The effect of vitamin D₃ supplementation on markers of glycaemia, lipidaemia and oxidative stress in Saudi women with poorly controlled type 2 diabetes mellitus

Alaa Hatim Qadhi
PhD 2016
The effect of vitamin D₃ supplementation on markers of glycaemia, lipidaemia and oxidative stress in Saudi women with poorly controlled type 2 diabetes mellitus

Alaa Hatim Qadhi

A thesis submitted in partial fulfilment of the requirements of the Manchester Metropolitan University for the degree of Doctor of Philosophy

Department of School of Healthcare Science
the Manchester Metropolitan University
2016
Abstract

Saudi Arabia has amongst the highest incidence of type 2 diabetes in the world, with nearly 20 percent of the adult population suffering from the condition. The prevalence of vitamin D deficiency in type 2 diabetics has been shown to be twice that of non-diabetic individuals. Recent studies have shown, over 95 percent of the Saudi adolescent population are vitamin D deficient. Pancreatic beta-cells express vitamin D receptors, as well as the vitamin-D₃-activating enzyme 1-alpha-hydroxylase. Vitamin D plays a key role in both insulin production and glucose homeostasis. Supplementation with vitamin D could help tackle morbidity in diabetes by decreasing insulin resistance and reducing levels of advanced glycation endproducts (AGEs) which contribute to the onset and progression of diabetic complications. Despite vitamin D deficiency and diabetes being highly prevalent amongst the female Saudi population, the effect of vitamin D₃ supplementation on improving diabetic outcomes is an area that is currently understudied.

This was a double-blind randomized control study of 156 overweight Saudi females with poorly controlled type 2 diabetes mellitus recruited from Al-Noor Hospital in Saudi Arabia. Each subject was randomly allocated to either a placebo, 2000 IU/day or 4000 IU/day vitamin D₃ intervention group for 16 weeks. Serum measurements were analysed using routine laboratory procedures. Oxidative stress biomarkers, including total antioxidant levels, were measured using a colorimetric method. AGEs were measured using an AGE reader at baseline and 16 weeks. Significant improvements in vitamin D status, HbA1c, LDL and total cholesterol were demonstrated after 16 weeks of supplementation (p < 0.001), (p = 0.001), (p = 0.028) and (p = 0.049) respectively. There were no statistically significant changes in HOMA-IR, AGE concentrations, fasting insulin, fasting glucose, HDL-cholesterol and triglyceride levels.

This study suggests vitamin D may have a role in improving outcomes for type 2 diabetics and slowing the natural progression of the disease. Further research however is needed to determine the optimum dose, duration and form of delivery of supplementation in order to achieve these effects.
Declaration

I declare that this thesis is all my own work and has not been copied from any other sources, or accepted for any other degree in any University. To the best of my knowledge this thesis contains no material written or distributed previously by any other parties, apart from where I have otherwise stated.

Signature: Alaa Qadhi

Date: 14/07/2016
Acknowledgements

In the Name of Allah, the most gracious and the most merciful, I would like to express my gratitude and thankfulness to Allah who has given me the opportunity, strength and blessing to complete this research, and my ‘selawat’ and ‘salam’ to His righteous Prophet Mohammad. Peace be upon Him.

I would like to express my sincere appreciation to the Director of Studies, Dr Nessar Ahmed, for his ongoing support, supervision, encouragement, guidance and suggestions, which were essential for the completion of this research. I am also truly thankful to my second supervisor, Dr Emma Derbyshire, for her quick feedback and helpful comments which were truly inspiring. I am also deeply grateful to Dr Khaled Taybe, the Head of the Diabetes Centre at Al Noor Specialist Hospital, for his generous support and encouragement during this research.

Words cannot express how grateful I am to my wonderful husband, Mohammad Mesawa, and my two lovely children, Ramah and Talal, for their patience and support during all the hard times that I faced. My deep and sincere gratitude to my excellent parents, Hatim Kadi and Wafaa Shafei, and kind parents-in-law, Talal Mesawa and Nawal Sunbul, for their constant encouragement and moral support. A special thanks goes to my brothers, Ahmed and Hassan, and my three marvellous sisters, Marzy, Kholod and Dina.

I am sincerely thankful to his Excellency Professor Abdulfattah Mashat, the President of the University of Jeddah, and Dr Adel Abuznadah, the Vice President of King Fahd Medical Research Centre at King Abdulaziz University, for their generous assistance and support in making this work a success.

I am very grateful to all the staff at the Diabetes and Endocrine Centre at Al Noor Specialist Hospital. I would like to give special thanks to the head nurse, Mrs Susan Mueen, and all her staff, especially Mrs Duaa Brinjee and Mr Maher Alsaedi, for their help and assistance throughout. My deepest appreciation to everyone at the Al Noor Specialist Hospital Laboratory, including Dr Hani Faidah, Director of the Laboratory, Mr Emran Alwakeel, Chief of Lab Technologist, Miss Mashaael Aladwani, Senior Supervisor of the Biochemistry Lab, and her staff and the Hormone Lab staff for their great support and technical assistance.
I also wish to express my gratitude to everyone from Umm Al Qura University for their involvement in this thesis. Finally, a special thanks to Dr Mohammed Basalamh, Vice Dean for Graduate Studies and Research, for his help and support.
Conference presentations

Alaa Qadhi, Khaled Tayeb, Emma Derbyshire, Nessar Ahmed (2016) Effect of Vitamin D supplementation on markers of glycaemia in Saudi Women with poorly-controlled Type 2 Diabetes Mellitus. The 9th Saudi Student Conference at The ICC, Birmingham (13-14 February 2016).

Alaa Qadhi, Khaled Tayeb, Emma Derbyshire, Nessar Ahmed (2016) Effect of Vitamin D supplementation on markers of dyslipidaemia in Saudi Women with poorly-controlled Type 2 Diabetes Mellitus. The 19th vitamin D workshop, Boston, MA (29-31 March 2016).
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<td>µU/mL</td>
<td>Microunits/millilitre</td>
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<tr>
<td>AGEs</td>
<td>Advanced glycation endproducts</td>
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<tr>
<td>Apo-A1</td>
<td>Apolipoprotein A-1</td>
</tr>
<tr>
<td>ApoB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>AR</td>
<td>Aldose reductase</td>
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<tr>
<td>BCG</td>
<td>Bromcresol green</td>
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<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>Calciferol</td>
<td>Vitamin D</td>
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<td>Calcitriol</td>
<td>1,25-dihydroxycholecalciferol</td>
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<tr>
<td>CAT</td>
<td>Catalase</td>
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<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
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<tr>
<td>Cholecalciferol</td>
<td>Vitamin D$_3$</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>Ergocalciferol</td>
<td>Vitamin D$_2$</td>
</tr>
<tr>
<td>FBS</td>
<td>Fasting blood sugar</td>
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<tr>
<td>FGIR</td>
<td>Fasting glucose/insulin ratio</td>
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<tr>
<td>GLM</td>
<td>General Linear Model</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GRed</td>
<td>Glutathione reductase</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<tr>
<td>HOMA-B</td>
<td>Homeostasis model assessment of β-cell function</td>
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<tr>
<td>HOMA-IR</td>
<td>Homeostasis model assessment for insulin resistance</td>
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<tr>
<td>HSL</td>
<td>Hormone-sensitive lipase</td>
</tr>
<tr>
<td>IM injection</td>
<td>Intramuscular injection</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin resistance</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
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<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
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<tr>
<td>mg/dl</td>
<td>Milligram/decilitre</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
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<td>NF-κB</td>
<td>Nuclear factor -kappaB</td>
</tr>
<tr>
<td>ng/ml</td>
<td>Nanogram/millilitre</td>
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<tr>
<td>NIDDM</td>
<td>Non-insulin-dependent diabetes</td>
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<tr>
<td>NM-BAPTA</td>
<td>5-nitro-5’-methyl-BAPTA</td>
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<tr>
<td>NPDR</td>
<td>Non-proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>Oxidised LDL</td>
</tr>
<tr>
<td>PCO</td>
<td>Pro-oxidants protein carbonyl</td>
</tr>
<tr>
<td>PDR</td>
<td>Proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>QUICKI</td>
<td>Quantitative insulin-sensitivity check index</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptors for advanced glycation endproducts</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized control trial</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>sdLDL</td>
<td>Small dense low density lipoprotein</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>TAC</td>
<td>Total antioxidant capacity</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>TC</td>
<td>Total cholesterol</td>
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<tr>
<td>TG</td>
<td>Triglycerides</td>
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<tr>
<td>TINIA</td>
<td>Turbidimetric inhibition immunoassay</td>
</tr>
<tr>
<td>UKPDS</td>
<td>UK Prospective Diabetes Study</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VDBP</td>
<td>Vitamin D binding protein</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D response element</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-low density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Interleukin-1 α</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>1,25-dihydroxycholecalciferol</td>
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Chapter 1. Introduction
1.1 General Introduction
Diabetes Mellitus is a group of metabolic disorders characterised by defects in insulin secretion and insulin sensitivity resulting in chronic hyperglycaemia (WHO 2016). According to World Health Organisation (2016) statistics, there are over 422 million people suffering from diabetes mellitus worldwide. It is the sixth leading cause of death globally and the mortality rates amongst diabetic patients are almost double that of healthy individuals of the same age (WHO 2014). Furthermore, the long-term health complications of diabetes are numerous and include neuropathy, retinopathy and nephropathy, as well as atherosclerosis and cardiovascular disease (Alberti and Zimmet 1998; Rother 2007).

1.2 Type 2 Diabetes Mellitus
Type 2 Diabetes Mellitus (T2DM), also known as non-insulin-dependent diabetes (NIDDM), accounts for approximately 90–95% of all diabetic cases (American Diabetes Association 2010). Modern sedentary lifestyles containing high calorie diets have contributed to a global epidemic of the disease, with global prevalence nearly doubling since 1980 (WHO 2016). Saudi Arabia has the fourth highest incidence of type 2 diabetes in the Middle East and the seventh highest in the world, with approximately 19% of adults aged between 20 and 79 suffering from the condition (International Diabetes Federation 2012). Mirroring the global trend, type 2 diabetes in Saudi Arabia is also on the rise and the prevalence amongst the population is predicted to increase by 39.5% by 2022 (Al-Quwaidhi et al., 2014).

1.3 Pathophysiology of Type 2 Diabetes
Type 2 diabetes results from a combination of insulin resistance and inadequate secretion (Cersosimo et al., 2014). The pathophysiology is complex, involving an interplay between genetic and environmental risk factors that contribute to beta-cell failure and the development of insulin resistance (DeFronzo 2009). The results are decreased peripheral glucose uptake, utilisation and storage, leading to a state of chronic hyperglycaemia (Kahn et al., 1993; Leahy 2005). The length of exposure to hyperglycaemia and its severity has been shown to be directly proportional to the development of vascular wall impairment and subsequent diabetic complications in patients with type 2 diabetes (Fuller et al., 1979; Balkau et al., 1998; UKPDS 1998).
Hyperglycaemia disturbs endothelial cell homeostasis, prompting modifications to the macro- and microvasculature, including increased generation of reactive oxygen species (ROS), enhanced oxidative stress and increased cell membrane permeability (Popov 2010).

1.4 Complications of Diabetes

The complications associated with diabetes are grouped into two subsets: microvascular disease (damage to small blood vessels) and macrovascular disease (damage to larger arteries) and are shown in Figure 1.1. Microvascular complications include nephropathy, retinopathy and neuropathy. Macrovascular complications include cardiovascular disease, resulting in strokes and myocardial infarction (Fuller et al., 1979; Balkau et al., 1998; UKPDS 1998).

![Figure 1.1: Diagram representing the long-term complications of Type 2 Diabetes](image)

1.4.1 Retinopathy

Diabetic retinopathy occurs in 90% of diabetic patients after approximately 20 to 30 years from onset of the condition. The most advanced form of the disease affects 60% of diabetic individuals (Fong et al., 2003) and it is the leading cause of new diagnoses of adult blindness worldwide (Aiello et al., 1998; Frank 2004). One study has found an incidence of 36.1% of diabetic retinopathy in Saudi Arabian patients with a mean...
duration of diabetes of 13 years (El-Bab et al., 2012). Diabetic retinopathy is a result of microvascular damage to the retina and can be classified in two stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) (Miura et al., 2004). NPDR is characterised by thickening of the basement membrane of the retina. Eventually this leads to a reduction in pericytes in the retinal blood vessels, resulting in haemorrhages and microaneurysms, as shown in Figure 1.2 (Al-Mesallamyy et al., 2011). Pericytes are contractile mesenchymal cells that wrap around the endothelial cells of capillaries. They regulate retinal blood flow by controlling vascular tone and the lumen size of retinal capillaries (Beltramo and Porta 2013; Wu et al., 2003; Kelley et al., 1987). A key histopathological change in diabetic retinopathy is the selective loss of pericytes. The exact mechanism by which this occurs is not fully understood, however it is thought that basement membrane thickening, systemic and local hypertension and the formation of advance glycation end-products contribute to pericyte apoptosis and the breaking of the tight structural connections between pericytes and endothelial cells (Beltramo and Porta 2013). It has been shown that in proliferative diabetic retinopathy, microaneurysms development following pericyte loss (Hirschi et al., 1998; Antonelli-Orlidge et al., 1989).

PDR is characterised by neovascularisation as blood vessels with reduced pericytes become more vulnerable to angiogenic factors (Motiejunaite and Kazlauskas 2008). It has been shown that a longer duration and increased degree of hyperglycaemia is associated with PDR (Park et al., 2008).
Diabetes is the most common cause of end-stage renal disease in the developed world and 40 to 50% of diabetics will develop nephropathy (Mauer and Chavers 1985). Diabetic nephropathy is characterised by glomerular hyper-filtration which leads to basement membrane thickening in the renal glomerulus and tubule. Initially, this is evidenced by microalbuminuria which advances to proteinuria, a reduced glomerular filtration rate and end-stage renal failure as the disease progresses, as shown in Figure 1.3 (Hong and Chia 1998; Wolf et al., 2003; Coughlan et al., 2005).

1.4.2 Nephropathy

Diabetes is the most common cause of end-stage renal disease in the developed world and 40 to 50% of diabetics will develop nephropathy (Mauer and Chavers 1985). Diabetic nephropathy is characterised by glomerular hyper-filtration which leads to basement membrane thickening in the renal glomerulus and tubule. Initially, this is evidenced by microalbuminuria which advances to proteinuria, a reduced glomerular filtration rate and end-stage renal failure as the disease progresses, as shown in Figure 1.3 (Hong and Chia 1998; Wolf et al., 2003; Coughlan et al., 2005).
Diabetic neuropathy is classified as damage to the nervous system and is a common complication of diabetes. More than one third of diabetics report experiencing neuropathic problems including diabetic ulcers, impotence, pain and incontinence (Young et al., 1993). A study of 375 Saudi Arabian diabetic patients with diabetes for nine years or more found symptoms of nerve dysfunction in 38% of the study population (Nielsen 1998b).

1.4.4 Macrovascular complications

Damage caused to the larger arteries in diabetes results in an increased risk of cardiovascular disease, peripheral vascular disease and cerebrovascular disease (Johansen and Birkeland 2003). The risk of developing cardiovascular disease is increased two- to four fold in diabetic patients (Poornima et al., 2006) and is the leading cause of diabetic morbidity and mortality (Bucala et al., 1994). Macrovascular disease is characterised by endothelial dysfunction and accelerated atherosclerosis which develops due to a multitude of factors, including hyperglycaemia, hyperlipidaemia, oxidative stress and insulin resistance (Laakso and Lehto 1998; Donnelly and Davis 2000; Candido et al., 2003).

1.5 Pathogenesis of Diabetic Complications

Several studies have demonstrated chronic hyperglycaemia to be the principal factor in the onset and progression of diabetic complications (Stratton et al., 2000; Reusch

Figure 1.3: Differences between the healthy and diabetic glomerulus

(Wright 2015)
Several biological pathways are initiated by the presence of elevated glucose levels and have been implicated in the development of macrovascular and microvascular diabetic complications (see Figure 1.4). These include:

- Polyol pathway
- Activation of protein kinase C (PKC)
- AGE formation pathway
- Oxidative stress

![Figure 1.4: Biological pathways resulting in diabetic complications](Adapted from Hadi and Suwaidi 2007)
1.5.1 Oxidative stress

Biological processes in the body create reactive oxygen species (ROS) that are damaging to cells and tissues (Skrha 2003). The production of ROS is limited by antioxidant defences comprising both endogenous and exogenous elements. Oxidative stress is defined as the imbalance between the formation of these reactive oxygen species and antioxidant defences (Betteridge 2000). In healthy individuals both the generation and inhibition of ROS are balanced, however oxidative stress may arise if either the formation of ROS increases or the body’s defensive mechanisms are not fully effective (West 2000). Both of these may exist in diabetes where higher ROS formation is accompanied by insufficient antioxidant defences (West 2000).

It has been suggested that diabetes contributes to the production of reactive oxygen species through different pathways, which will be discussed in greater detail in this chapter. High concentrations of intracellular glucose in endothelial cells have been shown to activate ROS production in mitochondria, accelerating glycation and cellular damage (Lorenzi 2007). A major target of oxidative stress is the vascular endothelium, which plays a vital role in the pathophysiology of diabetic complications (Hadi and Suwaidi 2007). Specifically, oxidative stress increases vascular endothelial permeability and promotes leucocyte adhesion, initiating the early stages of diabetic microangiopathy (Lum and Roebuck 2001).

ROS production in healthy individuals is usually balanced by a system of endogenous and exogenous scavenger enzymes and antioxidants. Exogenous sources of antioxidant scavengers, such as vitamins A, C and E, lipoic acid and carotenoids, can be derived from a healthy diet (Jakus 2000). Several potent endogenous antioxidants, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRed) have been studied both in vivo and in vitro in acute and chronic hyperglycaemic conditions (Skrha 2003). These enzymes have been shown to work either directly or indirectly to catalyse the removal of ROS, preventing further oxidative stress (Maritim et al., 2003). Numerous studies have demonstrated significantly reduced antioxidant activity in patients with type 2 diabetes (Illing et al., 1951; Sundaram et al., 1996; Maxwell et al., 1997; Opara et al., 1999). Furthermore, research has shown that in those patients with severely reduced antioxidant levels, the incidence of diabetic complications was far greater (Reunanen et al., 1998; Opara et al., 1999).
1.5.2 Polyol pathway

The polyol (or sorbitol) pathway explains how glucose is metabolised when intracellular levels are elevated (Gabbay et al., 1973; Oates 2002). In states of hyperglycaemia, glucose is reduced to sorbitol via aldose reductase (AR) and NADPH and then further metabolised to fructose by sorbitol dehydrogenase (Lorenzi 2007).

This has many implications for diabetic patients. Firstly, the fructose produced can be phosphorylated to fructose-3-phosphate which is broken down to 3-deoxyglucosone, both of which are powerful glycating agents involved in the formation of advanced glycation endproducts (AGEs) (Gonzalez et al., 1988; Szwergold et al., 1990).

Secondly, the use of NADPH by AR results in less co-enzyme availability for glutathione reductase. This enzyme is vital for the maintenance of the intracellular pool of reduced glutathione (GSH), a potent antioxidant, lessening the cells capability to balance ROS (Barnett et al., 1986). Chronic hyperglycaemia therefore exposes cells to oxidative stress, lessens antioxidant defences and increases AGE formation, resulting in amplified rates of cellular damage and advancing diabetic vascular complications.

1.5.3 Activation of protein kinase C

Many studies have shown activation of the DAG-PKC pathway to be linked to the development of diabetic complications (Koya and King 1998). The DAG-PKC pathway is one of the most studied of the cellular signalling pathways initiated in diabetes. Hyperglycaemia promotes diacylglycerol (DAG) accumulation within cells due to an increase in glycolytic intermediate dihydroxyacetone phosphate. This is reduced to glycerol-3-phosphate which subsequently increases synthesis of DAG. In diabetics, increased levels of DAG have been demonstrated in vascular tissues including the retina, aorta, heart and renal glomeruli (Craven et al., 1990; Shiba et al., 1993).

DAG is a physiological activator of protein kinase C (PKC), a family of enzymes responsible for regulating the function of other intracellular proteins (Inoguchi et al., 1992; Xia et al., 1994). PKC has been associated with vascular dysfunction, including increased permeability and contractility, synthesis of extracellular matrix, cell growth and apoptosis, angiogenesis, leucocyte adhesion and superoxide production (Geraldes and King 2010).
1.5.4 AGE formation pathways

The formation of advanced glycation endproducts (AGEs) is a major biochemical abnormality dominant in diabetes mellitus (Hadi and Suwaidi 2007). In healthy individuals, AGEs form at a continuous rate, beginning in early embryonic development. In diabetics, however, the increased availability of glucose means AGEs form and accumulate at an accelerated rate (Peppa et al., 2003). AGEs have been strongly linked to a number of diabetic complications due to their ability to form covalent crosslinks between proteins, such as the cellular matrix, basement membranes and vascular endothelium, altering their structure and function (Peppa et al., 2003). Examination of post-mortem aorta samples have, for example, demonstrated aortic stiffness to directly correlate with AGE accumulation in the tissue (Sims et al., 1996). Other pathogenic features of AGEs involve their interaction with a variety of cell-surface binding receptors for advanced glycation endproducts (RAGE), initiating cell degradation or activation and pro-oxidant, pro-inflammatory actions (Peppa et al., 2003).

1.5.4.1 Glycation

AGEs form through a succession of chemical reactions first described by Maillard (Litchfield et al., 1999) in which reducing sugars covalently bind to the free amino groups of proteins (Ahmed 2005). The Maillard reaction, as shown in Figure 1.5, begins with the nucleophilic addition of the carbonyl group of a reducing sugar to the amino group of a protein, producing a glycosylamine, known as the Schiff base. The Schiff base is a highly labile compound and therefore undergoes rearrangement to form the more stable Amadori product (a ketoamine), of which glycated haemoglobin (HbA1c) is the best known (Monnier et al., 1981; Watkins et al., 1985; Yaylayan and Huyghues-Despointes 1994; Boel et al., 1995). These initial reactions are concentration-dependent and in the presence of low glucose levels, the sugars will become detached from the amino groups. Conversely, in chronic hyperglycaemic states, the opposite is true and these glycated proteins will undergo further fragmentation, creating dicarbonyl intermediates, which eventually give rise to AGEs (Monnier 2003; Peppa et al., 2003; Ahmed 2005).
In addition to glycation, autoxidation of glucose also contributes to the production of AGEs, as well as free radicals (Wolff et al., 1989). In the presence of transition metals, glucose undergoes autoxidation to form an enediol radical (Ahmed 2005). This enediol radical then reduces oxygen to form a superoxide radical which itself becomes oxidised to a dicarbonyl ketoaldehyde (see Figure 1.6). In turn, the dicarbonyl ketoaldehyde can react with protein amino groups to form a ketoimine (a compound involved in AGE production), similar to an Amadori product but far more reactive (Wolff and Dean 1988). Superoxide radicals combine in a reaction catalysed by superoxide dismutase (SOD) to form hydrogen peroxide. When in contact with
transition metals, hydrogen peroxide produces the extremely toxic hydroxyl radical, inducing further oxidative damage (Baynes 1991). Whilst glucose autoxidation directly increases the formation of ROS, the generation of AGEs by autoxidative glycation is also accelerated by the presence of ROS (Stehouwer and Schaper 1996).

![Figure 1.6: Autoxidative glycation in the presence of transition metals, leading to the production of free radicals and AGEs](image)

(Adapted from Ahmed, 2005)

### 1.5.4.3 AGE receptors

Whilst there are many proven AGE receptors, the best characterised and most studied of the AGE receptors is the RAGE receptor (see Figure 1.7) (Mackic et al., 1998; Schmidt et al., 1999). In diabetes mellitus, the expression of RAGE is increased and the interaction between AGEs and their receptor is now considered fundamental in the pathogenesis of AGE-induced diabetic complications (Hudson et al., 2002; Ahmed 2005). These pathological consequences are driven through cellular activation and subsequent induction of oxidative stress and other broad signalling mechanisms when AGEs bind with RAGE. AGE-RAGE interaction in macrophages has been shown to induce the generation of intracellular free radicals and oxidative stress and subsequent
activation of the transcription factor NF-κB (Schmidt et al., 1994; Yan et al., 1994; Lander et al., 1997). NF-κB controls the gene transcription of endothelin-1, tissue factor and thrombomodulin and the generation of pro-inflammatory cytokines such as interleukin-1 α (IL-1α), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) (Neumann et al., 1999). In addition, there is the enhanced expression of adhesion molecules, which lead to increased vascular permeability amongst other effects (Singh et al., 2014). AGE interaction with RAGE also triggers the activation of NADPH oxidase leading to generation of reactive oxygen species (ROS) and further oxidative stress (Wautier et al., 2001). There is also evidence to show that RAGE may be directly involved in mediating inflammatory cell recruitment (Chavakis et al., 2003). Together, the effects of RAGE activation in endothelial cells, macrophages and smooth muscle cells contribute to the pathogenesis of diabetic macrovascular and microvascular complications (Ramasamy et al., 2011).

1.5.4.4 Role of AGEs in pathogenesis of diabetic complications
A growing body of evidence has demonstrated that AGEs are significant pathogenic mediators of nearly all micro- and macro-vascular diabetic complications (Peppa et al., 2003). The ability of AGEs to alter the structure and function of key proteins in the cellular matrix, basement membrane and vascular endothelium is key to their role in the pathogenesis of diabetic microangiopathies (Peppa et al., 2003). Furthermore, their interaction with RAGE receptors alters intracellular signalling, gene expression, release of pro-inflammatory molecules and free radicals that all contribute to the pathology of diabetic complications (Ahmed 2005). For example, AGEs have been found in the retinal blood vessel walls of diabetic patients and are believed to contribute to increased permeability of retinal endothelial cells and vascular occlusion, resulting in vascular leakage (Stitt 2003). Furthermore, the severity of retinopathy has been shown to directly correlate with serum AGE levels (Peppa et al., 2003).

In diabetic patients, levels of AGEs were also found to be increased in the axons and Schwann cells of peripheral neurones (Sugimoto et al., 1997). In vitro, Schwann cells and neuronal cells undergo apoptosis when incubated with AGEs (Takeuchi et al., 2000; Sekido et al., 2004). This may be due to AGE-induced hypoxia as a result of vasoconstriction. Whilst AGEs promote nitric oxide synthase expression leading to an increase in available nitric oxide, they have also been shown to directly react with and quench nitric oxide. As a result, vasoconstriction occurs and there is consequently
reduced blood flow to peripheral nerves (Amore et al., 1997; Bucala et al., 1991; Chakravarthy et al., 1995; Amore et al., 1997). AGEs also modify neurofilaments and tubulin, which are structures involved in nerve signalling along axons. This causes a disturbance in axonal transport, which contributes to the development of atrophy and degeneration of neurone fibres typical of diabetic neuropathies (Wada and Yagihashi 2005; Vlassara et al., 1981).

Several studies have demonstrated AGE levels in the kidney to be higher in diabetic patients compared to healthy subjects (Miyata et al., 1997; Monnier et al., 1999; Weiss et al., 2000; Miura et al., 2003; Monnier et al., 2005; Yoshida et al., 2005). The characteristic structural changes of diabetic nephropathy, including thickening of the glomerular basement membrane and mesangial expansion, can all be attributed to the action of AGEs in the nephron (Peppa et al., 2003). AGEs accumulate on collagen in the basement membrane, trapping plasma proteins and contributing to basement membrane thickening. This leads to altered filtration and ultimately loss of glomerular function (Monnier et al., 1992). Mesangial cells, which modulate glomerular filtration, express RAGE on their cell surface. In vitro, AGE-RAGE interaction can stimulate mesangial cells to produce matrix proteins (Dworkin et al., 1983; Schlondorff 1987; Skolnik et al., 1991). This results in expansion of the mesangial matrix and is thought to be one of the main factors in diabetic renal impairment due to the effect on glomerular filtration (Steffes et al., 1989).

AGEs have been identified in atheromatous plaques in the coronary arteries of diabetic subjects, implicating a role for AGEs in the formation of atherosclerosis and cardiovascular disease (Obayashi et al., 1996). Endothelial cells, smooth muscle cells and monocytes have been shown to express RAGE receptors. The interaction of AGE with RAGE receptors within these cells induce inflammatory response pathways, atherogenesis and vasoconstriction. Eventually, this leads to atherosclerosis, thrombosis and coronary dysfunction as demonstrated in the macrovascular complications of diabetes (Hartog et al., 2007).

1.5.5 Dyslipidaemia

Dyslipidaemia is one of the major causes of cardiovascular disease in diabetes mellitus (Mooradian 2009). Type 2 diabetes is associated with characteristically high plasma triglyceride concentrations, low high density lipoprotein (HDL) concentrations, and
increased small dense low density lipoprotein concentrations (sdLDL) (American Diabetes Association 2003). The exact mechanisms of diabetic dyslipidaemia are still poorly understood, however growing evidence suggests that insulin resistance has a crucial role in the pathogenesis of the condition (Mooradian 2009).

1.5.5.1 Pathogenesis of diabetic dyslipidaemia
The fundamental features of diabetic dyslipidaemia can be explained by increased free fatty-acid release from insulin-resistant adipose tissues (Taskinen 2003; Krauss and Siri 2004; Del Pilar Solano and Goldberg 2005; Chahil and Ginsberg 2006). In a healthy individual, when insulin binds to the insulin receptors on adipocytes, the activity of hormone-sensitive lipase (HSL) within the cell is suppressed (Frayn and Coppack 1992; Kershaw et al., 2006). In the presence of insulin resistance, HSL is not suppressed, leading to the hydrolysis of triglycerides to free fatty acids and glycerol (Frayn and Coppack 1992). Free fatty acids are released into the circulation and travel to the liver. In the state of insulin resistance, the increased flux of free fatty acids into the liver stimulates triglyceride production, which in turn promotes the secretion of apolipoprotein B (ApoB). Apolipoprotein B is a large protein which is the major component of very-low density lipoprotein (VLDL) cholesterol (Mooradian 2009). When the ability of insulin to inhibit free fatty-acid release is impaired, VLDL cholesterol production increases, leading to enhanced hepatic fat accumulation (Frayn 2001; Adiels et al., 2007).

The increased numbers of VLDL cholesterol particles and increased plasma triglyceride levels decrease the level of HDL cholesterol and increase the concentration of small dense LDL-cholesterol particles via several processes (see Figure 1.8). Firstly, cholesteryl ester transfer protein (CETP) acts on VLDL-transported triglyceride, substituting it for HDL-transported cholesteryl ester. This results in increased amounts of both atherogenic cholesterol-rich VLDL remnant particles and triglyceride-rich, cholesterol-depleted HDL particles. The triglyceride-enriched HDL is subsequently hydrolyzed by hepatic lipase in the liver and readily absorbed by hepatocytes, lowering circulating concentrations of HDL cholesterol in the blood (Mooradian et al., 2004; Mooradian et al., 2008).
The increased concentration of sdLDL cholesterol can also be explained by a similar lipid exchange process. CETP once again facilitates the transfer of triglyceride to LDL and cholesteryl ester to VLDL, resulting in increased levels of triglyceride-rich LDL. Triglyceride-rich LDL then undergoes hydrolysis by hepatic lipase in the liver, producing sdLDL. The sdLDL particles have a lower affinity for hepatocyte receptors and therefore there is reduced hepatocyte clearance and increased circulating levels of sdLDL in the blood (Mooradian 2009).

Figure 1.7: The role of insulin resistance in diabetic dyslipidaemia
LPL = lipoprotein lipase; TG = triglycerides; ApoB = apolipoprotein B; VLDL = very low density lipoprotein; CE = cholesteryl ester; CETP = cholesteryl ester transfer protein; HL = hepatic lipase; LDL = low density lipoprotein; SD LDL = small density low density lipoprotein; FFA = free fatty acids (Mooradian 2009)

1.5.5.2 Complications of diabetic dyslipidaemia
Atherosclerosis is the most severe consequence of diabetic dyslipidaemia and is the leading cause of death in diabetic patients (Ahmed 2005). It is characterised by formation of atherosclerotic plaques within the arterial endothelium, leading to occlusion of blood flow and hypertension, and eventual myocardial cell death. Raised concentrations of LDL leads to accelerated atherosclerosis through several mechanisms. In healthy individuals, circulating levels of LDL are recognised by receptors on hepatocytes in the liver and cleared from the bloodstream. However, in diabetic patients there is increased glycation and oxidation of LDL particles (Ahmed 2005). This impairs hepatic clearance as glycated and oxidised LDL (ox-LDL) particles are not recognised by the native LDL receptor (Skrha 2003). Instead, the
modified LDL molecules are bound by scavenger receptors on macrophages and their subsequent transformation into foam cells is accelerated (Skrha 2003). The deposition of these foam cells within the arterial endothelium provides the basis for the formation of atherosclerotic plaques and the initial manifestations of cardiovascular disease (Ahmed 2005).

1.6 Prevention of Diabetic Complications

Complications of diabetes are a significant public health burden leading to increased morbidity and a decrease in life expectancy (Gu et al., 1998). Diabetic patients with low fasting plasma glucose (FPG) have been found to have a significantly decreased risk of both microvascular and macrovascular complications when compared with those with a higher FPG (Colagiuri et al., 2002). In order to diagnose diabetes a FPG of 126 mg/dl or a 2-hour postprandial plasma glucose level of 200 mg/dl is required (WHO, 2006). In type 2 diabetes there is an increase in glycaemic levels as the disease progresses and studies have shown that patients who present with a lower HbA1c at diagnosis experience fewer diabetic complications (Colagiuri et al., 2002).

Consequently, intensive glycaemic control is vital in the prevention and delayed progression of diabetic retinopathy, neuropathy and nephropathy, as well as cardiovascular disease (Keen 1994; Molyneaux et al., 1998; Stratton et al., 2000). Whilst maintaining adequate glycaemic control will remain the mainstay of diabetic treatment, research has begun to consider developing alternative therapies for halting the progression of diabetic complications (Bonnefont-Rousselot 2001). One such therapy is the use of certain dietary antioxidants aimed at inhibiting AGE formation alongside reducing oxidative stress and dyslipidaemia (Golbidi et al., 2011).

1.6.1 Exogenous antioxidants

Many studies have established that oxidative stress, facilitated by hyperglycaemia-induced free radical production, worsens the progression of diabetes and its related complications. It has therefore been suggested that diabetic patients may benefit from supplementation with exogenous antioxidants to ameliorate free radical oxidation and the subsequent development of micro- and macrovascular complications (Aruoma 1998). Antioxidant supplementation has also been shown to improve hyperglycaemia and insulin efficiency, as well as limiting the oxidation of low-density lipoproteins vital to the atherogenesis process (Bonnefont-Rousselot 2001). Furthermore, some
antioxidants are able to fight against the harmful effects of AGEs either by preventing their cellular action or by inhibiting AGE formation altogether (Ahmed 2005). These findings have prompted several clinical trials using antioxidant supplements in diabetic patients to limit the long-term complications of the disease. To date the antioxidant properties of vitamins E and C, and more recently vitamin D, have been studied with thus far inconclusive results (Golbidi et al., 2011).

1.7 Vitamin D
Whilst the regulation of calcium homeostasis and bone metabolism are the most widely recognised functions of vitamin D, there is an increasing appreciation for its non-calcaemic properties; one of the least studied of which is its use as an antioxidant (Halicka et al., 2012). Numerous cross-sectional studies have demonstrated low serum concentrations of vitamin D to be associated with higher fasting blood glucose levels, increased insulin resistance and a greater risk of developing type 2 diabetes (Saedisomeolia et al., 2013). Initial interventional studies investigating the effect of vitamin D supplementation on diabetic outcomes have to date provided inconsistent results (Raghuramulu et al., 1992; Orwoll et al., 1994; Taylor and Wise 1998; De Boer et al., 2008; Bonakdaran and Afkhami Zadeh 2011; Nikooyeh et al., 2011; Al-Daghri et al., 2012; Tayebinejad et al., 2012; Nikooyeh et al., 2014; Tabesh et al., 2014; Mohamad et al., 2015).

1.7.1 Structure and function
Vitamin D (calciferol) is a group of steroids which are fat-soluble and regulate many biological functions including calcium homeostasis, cell proliferation and differentiation and immune function (Holick 2007; Tsiaras and Weinstock 2011). The two most important compounds of vitamin D are vitamin D$_2$ (ergocalciferol) and vitamin D$_3$ (cholecalciferol). The structure of these two compounds is very similar as demonstrated in Figure 1.9. Studies have shown vitamin D$_2$ to be less biologically active than vitamin D$_3$ (Lehmann et al., 2013).
There are two main sources of vitamin D: diet and sunlight. However, very few naturally occurring foods contain either vitamin D$_2$ or vitamin D$_3$ and therefore in many countries foods such as cereals, milk and butter are often fortified with an artificial form of the vitamin (Holick 2004; Holick 2007). The majority of vitamin D comes from ultraviolet B radiation from sunlight (Holick 1994).

Cholecalciferol (D$_3$) is synthesised in the skin layers from 7-dehydrocholesterol when exposed to ultraviolet (UV) light in the wavelength range 280-320nm (Neyestani 2013). This form of vitamin D is biologically inactive and cannot be utilised by cells until converted to 1,25-dihydroxycholecalciferol (1,25(OH)$_2$D$_3$) (see Figure 1.10) (Jones et al., 1998; Holick 2007; Holick 2009). This process of vitamin D metabolism occurs through two hydroxylation reactions in the liver and kidneys to produce 25(OH)D$_3$ and 1,25(OH)$_2$D respectively. Cytochrome P450 mixed-function oxidases (CYPs) are responsible for these reactions. CYP2R1, found in the endoplasmic reticulum of hepatocytes, is the key enzyme for 25-hydroxylation and produces 25(OH)D$_3$. 25(OH)D$_3$ is then transported to the proximal tubules of the kidneys where CYP27B1, the sole enzyme for 1α-hydroxylation, found in the mitochondria converts it to 1,25(OH)$_2$D. Another important CYP is CYP24A1, which is responsible for catabolizing both 25(OH)D$_3$ and 1,25(OH)$_2$D (Bikle 2013).

Active vitamin D is an unstable molecule and will rapidly degrade unless bound to the vitamin D binding protein (VDBP) in the bloodstream. It is the high affinity of active vitamin D for this binding protein that gives it many of its biological properties (Andress 2005). Once the active vitamin D-VDBP complex reaches a target cell, it must be released from the binding protein. Most tissues of the body have a vitamin D receptor (VDR) (located on chromosome 12q13.11) which uptakes the active vitamin
D once released in the target cell (Institute of Medicine Committee to Review Dietary Reference Intakes for Vitamin 2011; Lee et al., 2011). Once bound to the VDR, active vitamin D can interact with other transcriptional factors within the target cell nucleus, affecting gene transcription. One such factor is the retinoid X receptor which allows recognition of, and binding to, vitamin D responsive elements in the regulatory sequences of genes under the control of vitamin D (Haussler et al., 2013). This in turn alters gene expression and cellular activities within the target cell, including protein synthesis and secretion (Neyestani 2013).
1.7.2 Factors affecting vitamin D status

In 2008 it was estimated one billion people were diagnosed with vitamin D deficiency (James 2008). One recent study concluded that serum vitamin D concentrations were below satisfactory (30 ng/ml) in every region of the world, however severe deficiency (concentration below 10 ng/ml) was found to be most prevalent in South Asia and the
Lower concentrations of circulating 25(OH)D have been associated with increasing age, gender, dietary customs, latitude, exposure to sunlight and skin pigmentation (Holick 2004). In Saudi Arabia, the prevalence of vitamin D deficiency (defined as less than 20 ng/ml) in females has been estimated to be between 30% and 85% depending on the study size, age group, season and region studied, as shown in Table 1.1.

**Table 1.1: Prevalence of low vitamin D status amongst the Saudi population**

<table>
<thead>
<tr>
<th>Study paper</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Percentage of vitamin D deficiency (&lt;20 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlBuhairan <em>et al.</em>, (2015)</td>
<td>Saudi adolescents (n=12,575)</td>
<td>Cross-sectional</td>
<td>95.6%</td>
</tr>
<tr>
<td>Kanan <em>et al.</em>, (2013)</td>
<td>Saudi females (n=1556)</td>
<td>Retrospective</td>
<td>Premenopausal (80%-summer, 85%-winter)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Postmenopausal (60%-summer, 76% winter)</td>
</tr>
<tr>
<td>Ardawi <em>et al.</em>, (2011)</td>
<td>Saudi females =1172</td>
<td>Cross-sectional</td>
<td>80%</td>
</tr>
<tr>
<td>Al-Turki <em>et al.</em>, (2008)</td>
<td>Saudi Females N=100</td>
<td>Cross-sectional</td>
<td>25-35yrs (30%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;50 yrs (55%)</td>
</tr>
</tbody>
</table>

Low concentrations of vitamin D in the Saudi population have been noted since the 1980s (Sedrani *et al.*, 1983; Fonseca *et al.*, 1984), and this may in part be due to cultural attitudes, religious observances and dietary habits. Saudi Arabia is a Muslim country where women adopt religious dress such as the niqab (full-face veil) and hijab (head scarf) and clothing which only exposes the hands and occasionally face. One study has found no difference in the serum concentrations of vitamin D between veiled and non-veiled women; this is likely due to the face and hands contributing little to the cutaneous synthesis of vitamin D (Ardawi *et al.*, 2011).
Sun exposure in Saudi Arabia may be avoided in part due to the cultural attitude to favour pale skin colour, along with increased urbanisation (Kanan et al., 2013). In addition to this, melanin in the skin reduces vitamin D synthesis and therefore the darker the skin pigmentation, the lower the production of vitamin D for the equivalent sun exposure (Menon et al., 2013). Dietary intake of vitamin D is dictated by two main factors: dietary preference and concentration of vitamin D fortification in processed foods. One study comparing the levels of vitamin D fortification in certain foods such as cereals or orange juice in America and Saudi Arabia concluded that many of the Saudi Arabian versions were not fortified at all, and even those that were contained less fortified vitamin D than the recommended levels in the USA (Sadat-Ali et al., 2013).

### 1.7.3 Vitamin D and diabetes

The prevalence of vitamin D deficiency in type 2 diabetics has been found to be almost double that in non-diabetic subjects (Scragg et al., 1995; Isaia et al., 2001; Scragg et al., 2004; Al-Zahrani 2013). The risk factors for vitamin D deficiency and type 2 diabetes are often shared and include greater BMI, increasing age, and lack of physical activity (Brancati et al., 2000; Saintonge et al., 2009). Seasonal fluctuations in glucose and insulin concentrations have also been demonstrated (Desouza and Meier 1987). This may, at least in part, be due to variations in 25(OH)D concentrations resulting from shorter durations of sun exposure in cold seasons (Pittas et al., 2007b). Furthermore, research has demonstrated that when accompanied by vitamin D deficiency, type 2 diabetes is associated with a greater risk of cardiovascular mortality (Cigolini et al., 2006; Joergensen et al., 2010).

These observations have led to a need for randomised clinical trials to investigate the actual effects of vitamin D intake on the glycaemic status of type 2 diabetics. From such studies, several theories for the mechanisms of action of vitamin D in diabetes have been established. Firstly, vitamin D has been shown to improve markers of glycaemia via increased insulin secretion and reduced peripheral insulin resistance (Al-Daghri et al., 2012; Soric et al., 2012; Talaei et al., 2013; Labban 2014; Nasri et al., 2014; Tabesh et al., 2014; Yousef Rad et al., 2014; Mohamad et al., 2015). Secondly, an improvement in the lipid profiles of type 2 diabetics has been demonstrated when supplemented with dietary vitamin D (Raghuramulu et al., 1992; Bonakdaran and Afkhami Zadeh 2011; Al-Daghri et al., 2012; Tabesh et al., 2014; Mohamad et al.,
2015). Finally, vitamin D has been shown to upregulate endogenous antioxidants and therefore may have the potential to attenuate oxidative stress in diabetic individuals (Bao et al., 2008).

1.7.3.1 Vitamin D and glycaemia
Several mechanisms have been proposed to explain how vitamin D may improve markers of glycaemia in diabetic patients. The discovery of the vitamin D receptor on pancreatic beta cells, as well as the presence of a vitamin D response element (VDRE) within the promoter region of the insulin gene, have provided robust evidence for a causal relationship between the vitamin and insulin production (Johnson et al., 1994; Bland et al., 2004). Furthermore, 1,25(OH)$_2$D has been shown to directly activate transcription and regulate the expression of the insulin receptor, suggesting vitamin D has a role in insulin resistance as well as production (Maestro et al., 2000; Maestro et al., 2002).

Pancreatic beta cells are known to express the vitamin D receptor (VDR). One hypothesis is that circulating 1,25(OH)$_2$D binds to the VDR on pancreatic beta cells, initiating a direct cellular response from the vitamin D response element in the promoter region of the insulin gene. This in turn increases the transcription of the insulin gene, and therefore the secretion of insulin from the cell (Maestro et al., 2003), as shown in Figure 1.11 (Pittas et al., 2007b). Pancreatic beta cells have also been found to express the 25-hydroxyvitamin D-1α-hydroxylase enzyme which converts 25(OH)D to active 1,25(OH)$_2$D within the pancreatic beta cells (Bland et al., 2004).
The indirect role of vitamin D in type 2 diabetes is proposed to be through its role in calcium homeostasis. Vitamin D is known to regulate the concentration of extracellular calcium and its influx through pancreatic beta cell membranes (Pittas et al., 2007b). Insulin secretion is a calcium-dependent process and therefore any alteration in calcium concentrations within pancreatic beta cells caused by low circulating vitamin D concentrations may negatively influence insulin secretion (Milner and Hales 1967; Pittas et al., 2007b).

1,25(OH)₂D is known to directly activate transcription of the insulin receptor gene within target cells, increasing the number of peripheral insulin receptors (Maestro et al., 2002). In turn, this increases cellular insulin responsiveness and decreases overall insulin resistance (Maestro et al., 2000). The indirect role of vitamin D is likely once again to be mediated via calcium homeostasis. As previously mentioned, calcium is essential in certain tissues in order to effectively execute insulin-mediated cellular processes (Williams et al., 1990; Ojuka 2004). The optimum range of intracellular calcium that allows for these insulin-mediated cellular functions to work is very narrow. Therefore any small changes in the intracellular concentration of calcium may lead to peripheral insulin resistance (Draznin et al., 1987; Draznin et al., 1989). Studies have however indicated that the effect of vitamin D on insulin resistance is likely to be less than its effect on pancreatic beta cell function (Orwoll et al., 1994; Pittas et al., 2007a; Mitri et al., 2011).
1.7.3.2 Vitamin D and dyslipidaemia

Whilst vitamin D has been demonstrated to improve the lipid profile in diabetic patients, the exact mechanisms by which this occurs remain uncertain (Raghuramulu et al., 1992; Bonakdaran and Afkhami Zadeh 2011; Al-Daghri et al., 2012; Tabesh et al., 2014; Mohamad et al., 2015). Several hypotheses have however been proposed. Firstly, it has been suggested that vitamin D increases absorption of intestinal calcium which in turn reduces the amount of fat absorbed by the gut. Calcium binds to fatty acids and bile acids, forming calcium-fatty acid soaps which are excreted in the faeces, decreasing the absorption of fat from the gut (Boon et al., 2007). As a result serum total and LDL-cholesterol, as well as triglyceride concentrations are reduced (Saunders et al., 1988; Grundy and Denke 1990; Van der meer et al., 1990; Vaskonen et al., 2002; Vaskonen 2003; Cho et al., 2005; Zittermann et al., 2009). A second hypothesis is through the action of vitamin D on serum parathyroid hormone (PTH) concentrations. PTH has been shown to reduce lipolysis in vitro. As vitamin D suppresses PTH secretion in the body it may reduce serum lipids via increased peripheral removal (Zemel et al., 2000).

HDL cholesterol is comprised of 70% apolipoprotein A-1 (Apo-A1) and an increase in serum concentrations of this protein is known to lead to a direct increase in HDL particle formation and increased reverse cholesterol transport (Vacek et al., 2012; Teixeira et al., 2014). Apolipoprotein A1 has been demonstrated to persistently correlate with vitamin D concentrations (Satin 2000; Gagnon et al., 2012; Salum et al., 2012; Vacek et al., 2012). Furthermore, the expression of the apolipoprotein gene in hepatocytes (HepG2) and intestinal (CaCo-2) cells, responsible for production of the apolipoproteins A-1 and B, is known to be regulated by vitamin D receptor modulators (Wehmeier et al., 2008).

1.7.3.3 Vitamin D and oxidative stress

It is only in recent years that a possible relationship between vitamin D and oxidative stress has gained attention. Current animal studies have shown vitamin D deficiency to promote oxidative stress, whilst supplementation with the vitamin has been shown to upregulate the antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px) by up to 200% in diabetic rats (Kallay et al., 2002; Hamden et al., 2009; Argacha et al., 2011). However, the antioxidant properties of vitamin D in improving markers of diabetes are only recently coming to light and data
remains scarce (Nikooyeh et al., 2011; Tayebinejad et al., 2012; Nikooyeh et al., 2014). As previously demonstrated, chronic hyperglycaemia can induce oxidative stress via several mechanisms. In one recent cross sectional study on diabetic subjects a significant inverse relationship was demonstrated between vitamin D and markers of oxidative stress, including LDL susceptibility to oxidation and AGEs (Gradinaru et al., 2012).

There are multiple possible mechanisms by which vitamin D may influence oxidative stress levels. In vitro studies have shown calcitriol to have an inhibitory effect on RAGE expression and protein glycation, indicating that vitamin D may protect against AGE formation (Yamagishi et al., 2008). In vitamin D deficient subjects, PTH levels are known to be raised, leading to activation of 1a-hydroxylation of 25(OH)D in the kidneys to produce 1,25(OH)2D. This in turn increases intracellular calcium and the promotion of reactive oxygen species (Vidal et al., 2006; Sun and Zemel 2007). Furthermore, some experimental findings have demonstrated vitamin D to upregulate endogenous antioxidants, most notably glutathione (Neyestani 2013). However, other studies have found 25(OH)D to have no beneficial effect on antioxidant defenses (Noyan et al., 2005; Eftekhari et al., 2013; Yiu et al., 2013). The mechanisms by which vitamin D may play a role in limiting the progression of diabetic complications are summarised in Figure 1.12.
Previous related research

The exact mechanism by which vitamin D plays a role in the development and progression of type 2 diabetes remains controversial. The earliest studies appear to demonstrate no improvement in glycaemic control following supplementation with vitamin D (Orwoll et al., 1994). More recently however, many trials have found a positive association between vitamin D supplementation and improved markers of glycaemic control (Al-Daghri et al., 2012; Soric et al., 2012; Talaei et al., 2013; Labban 2014; Nasri et al., 2014; Tabesh et al., 2014; Yousefi Rad et al., 2014; Mohamad et al., 2015). Similarly, the existing literature has yielded inconsistent results on the role of vitamin D in improving the lipid profile in type 2 diabetics. The majority of studies have demonstrated no improvement in dyslipidaemia when vitamin D supplements are given (Jorde and Figenschau 2009; Patel et al., 2010; Witham et al., 2010; Breslavsky et al., 2013; Eftekhari et al., 2013; Talaei et al., 2013; Yiu et al., 2013; Al-Zahrani et al., 2014; Kampmann et al., 2014; Yousefi Rad et al., 2014; Al-
Shahwan et al., 2015; Al-Sofiani et al., 2015; Sadiya et al., 2015). However several studies exist which contradict this and have displayed significant improvements in the lipid profile following supplementation (Raghuramulu et al., 1992; Bonakdar and Afkhani Zadeh 2011; Al-Daghri et al., 2012; Tabesh et al., 2014; Mohamad et al., 2015).

Few studies exist which have analysed the effect of vitamin D on oxidative stress and AGEs in human subjects (Tayebinejad et al., 2012; Yiu et al., 2013; Nikooyeh et al., 2014; Krul-Poel et al., 2015; Shab-Bidar et al., 2015). However, of those one-third have demonstrated a significant reduction in AGEs following vitamin D supplementation (Tayebinejad et al., 2012; Nikooyeh et al., 2014). To the best of the researcher’s knowledge, only one study exists demonstrating a reduction in oxidative stress following vitamin D supplementation (Shab-Bidar et al., 2015). The findings of these studies are summarised in Table 1.2.
Table 1.2: Vitamin D intervention studies in diabetic and obese patients.

<table>
<thead>
<tr>
<th>Study paper</th>
<th>Study Population</th>
<th>Inclusion Criteria</th>
<th>Study design</th>
<th>Length of intervention</th>
<th>Type and Dose of Vitamin D</th>
<th>Outcome measures</th>
<th>Association (+ p value)</th>
<th>serum 25(OH)D, at baseline</th>
<th>serum 25(OH)D, at end of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Raghuramulu et al., 1992)</td>
<td>N=42 30-60 yrs</td>
<td>T2DM (India)</td>
<td>Intervention</td>
<td>1 month</td>
<td>25(OH)D$_3$ 300,000 IU IM injection</td>
<td>Cholesterol, Calcium, Fasting serum insulin</td>
<td>Yes (p &lt;0.02)</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>(Orwoll et al., 1994)</td>
<td>N=20 40-70 yrs</td>
<td>T2DM (USA)</td>
<td>Double-blind RCT</td>
<td>4 days</td>
<td>1, 25(OH)D$_2$ 200 IU/day, 1, 25-Dihydroxyvitamin D</td>
<td>FBS, Insulin secretion, Calcium</td>
<td>No significant change</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>(Taylor and Wise 1998)</td>
<td>N=3 Male=1 Female=2</td>
<td>T2DM (UK)</td>
<td>Case study</td>
<td>3 months</td>
<td>Ergocalciferol 300,000 IU stat IM injection</td>
<td>serum 25(OH)D, FBS, TG, fasting inulin, HbA1c</td>
<td>Increase in all, not significant</td>
<td>Patient 1= 5.2 ng/ml</td>
<td>Patient 1= 20.4 ng/ml</td>
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<td></td>
<td>Decreased significantly</td>
<td>Patient 2= 5.2 ng/ml</td>
<td>Patient 2= 18.8 ng/ml</td>
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<td>Patient 3= 3.2 ng/ml</td>
<td>Patient 3= 13.2 ng/ml</td>
</tr>
<tr>
<td>Study</td>
<td>N</td>
<td>Intervention</td>
<td>Duration</td>
<td>Intake</td>
<td>Outcome Measures</td>
<td>Results</td>
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<tr>
<td>(Borissova et al., 2003)</td>
<td>10</td>
<td>T2DM (Bulgaria)</td>
<td>1 month</td>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt; 1332 IU/day</td>
<td>serum 25(OH)D, HOMA-IR</td>
<td>Yes (p&lt;0.05) &amp; achieved sufficient level</td>
<td>14.12 ng/ml</td>
<td>N/A</td>
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<tr>
<td>(De Boer et al., 2008)</td>
<td>2291</td>
<td>T2DM (USA)</td>
<td>7 years</td>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt; 400 IU/day</td>
<td>serum 25(OH)D, HOMA-IR, insulin level, FBS, BMI</td>
<td>No significant change</td>
<td>&lt; 32 ng/ml for 89% of participants</td>
<td>N/A</td>
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<tr>
<td>(Sugden et al., 2008)</td>
<td>34</td>
<td>T2DM (UK)</td>
<td>2 months</td>
<td>25(OH)D&lt;sub&gt;2&lt;/sub&gt; 100,000 IU stat dose</td>
<td>serum 25(OH)D, FBS, HbA&lt;sub&gt;1c&lt;/sub&gt;, calcium, insulin</td>
<td>Yes (p &lt;0.02) but not did not achieve the sufficient level.</td>
<td>16 ng/ml</td>
<td>25.16 ng/ml</td>
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<tr>
<td>(Jorde and Figenschau 2009)</td>
<td>36</td>
<td>T2DM (Norway)</td>
<td>6 months</td>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt; 40,000 IU/week</td>
<td>serum 25(OH)D, FBS, serum insulin, HbA&lt;sub&gt;1c&lt;/sub&gt;, HOMA-B, HOMA-IR, calcium, lipid profile, BMI</td>
<td>Yes (p &lt;0.001) and did achieve the sufficient level.</td>
<td>24 ng/ml</td>
<td>47.32 ng/ml</td>
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<tr>
<td>Study</td>
<td>N</td>
<td>T2DM Location</td>
<td>Study Design</td>
<td>Duration</td>
<td>Intervention</td>
<td>Outcome Measures</td>
<td>Vitamin D Dose</td>
<td>Outcomes</td>
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<tr>
<td>(Witham et al., 2010)</td>
<td>61</td>
<td>(Scotland)</td>
<td>Double-blind RCT</td>
<td>4 months</td>
<td>25(OH)D$_3$</td>
<td>serum 25(OH)D, HbA1c, HOMA-IR, cholesterol</td>
<td>100,000 IU, 200,000 IU</td>
<td>Yes (p =0.003) only sufficient in the 200,000 IU group</td>
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<td></td>
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<td>stat oral dose</td>
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<td>25(OH)D$_3$</td>
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<td></td>
<td>No significant change</td>
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<td>100,000 IU group = 23.6 ng/ml</td>
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<td>200,000 IU group = 30.4 ng/ml</td>
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<tr>
<td>Patel, 2010</td>
<td>24</td>
<td>(Israel)</td>
<td>Pilot prospective randomized trial no control</td>
<td>4 months</td>
<td>25(OH)D$_3$</td>
<td>serum 25(OH)D, HbA1c, FBS, lipid profile, BMI, calcium</td>
<td>400 IU/day, 1200 IU/day</td>
<td>Yes (p&lt;0.001) but did not achieve the sufficient level at either dose</td>
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<td>400 IU/day group = 17.6 ng/ml</td>
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<td>1200 IU/day group = 25.5 ng/ml</td>
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<td>1200 IU/day group = 27.4 ng/ml</td>
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<tr>
<td>(Bonakdaran and Afkhami Zadeh 2011)</td>
<td>58</td>
<td>(Iran)</td>
<td>Intervention study</td>
<td>2 months</td>
<td>1,25(OH)$_2$D$_3$ (calcitriol) 0.5 microgram per day</td>
<td>FBS, HbA1c, cholesterol, TG, HOMA-IR, LDL</td>
<td>N/A</td>
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<tr>
<td>Study</td>
<td>N</td>
<td>Gender</td>
<td>Study Group</td>
<td>Duration</td>
<td>Treatment</td>
<td>Biomarker</td>
<td>p-Value</td>
<td>Results</td>
<td>Blood Level</td>
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<tr>
<td>(Al-Daghri et al., 2012)</td>
<td>92</td>
<td>Male: 34, Female: 58</td>
<td>T2DM (Saudi)</td>
<td>18 months</td>
<td>Intervention: 25(OH)D$_3$ 2000 IU/day</td>
<td>serum 25(OH)D</td>
<td>Yes (p &lt; 0.001)</td>
<td>but did not achieve the sufficient level.</td>
<td>12.88 ng/ml</td>
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<td>Corrected calcium</td>
<td>Yes (p = 0.003)</td>
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<td>LDL</td>
<td>Yes (p = 0.004)</td>
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<td>TC</td>
<td>Yes (p &lt; 0.001)</td>
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<td></td>
<td>HOMA-B</td>
<td>Yes (p = 0.002)</td>
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<td>HOMA-IR, Insulin</td>
<td>Yes (p &lt; 0.0001)</td>
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<tr>
<td><strong>Summary:</strong> improved lipid profile more significant in females</td>
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<td>Soric, 2012</td>
<td>37</td>
<td>Female: 22</td>
<td>T2DM (USA)</td>
<td>3 months</td>
<td>Single-blind RCT</td>
<td>25(OH)D$_3$ 2000 IU/day</td>
<td>HbA1c</td>
<td>Significant decrease (p &lt; 0.013) in patients with baseline HbA1c &gt;9%</td>
<td>N/A</td>
</tr>
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</table>

**Note:** N/A indicates not applicable or not available.
<table>
<thead>
<tr>
<th>Study</th>
<th>Participants</th>
<th>Intervention</th>
<th>Duration</th>
<th>Outcomes</th>
<th>Results</th>
<th>Serum 25(OH)D</th>
<th>HbA1c, HOMA-IR, BMI, FBS</th>
<th>25(OH)D</th>
<th>46.9 ng/ml</th>
<th>69.3 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heshmat, 2012</td>
<td>N= 42</td>
<td>T2DM (Iran)</td>
<td>3 months</td>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt; serum stat IM injection</td>
<td>Yes (p = 0.009) and did achieve the sufficient level</td>
<td>25(OH)D</td>
<td>serum 25(OH)D</td>
<td>25(OH)D</td>
<td>Yes (p = 0.001) and did achieve the sufficient level</td>
<td>Yes (p &lt; 0.001) and achieved the sufficient level.</td>
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<td>Male=15</td>
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<td>HbA1c, HOMA-IR, BMI, FBS</td>
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<td></td>
<td>Female=27</td>
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<td></td>
<td>46.9 ng/ml</td>
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<td></td>
<td>37-79 years</td>
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<td>69.3 ng/ml</td>
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<tr>
<td>Tayebinejad,</td>
<td>N= 60</td>
<td>T2DM (Iran)</td>
<td>3 months</td>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt; fortified yogurt drink 1000 IU/day vitamin D</td>
<td>Yes (p&lt;0.001) and achieved the sufficient level.</td>
<td>Serum of AGEs</td>
<td>Serum of AGEs</td>
<td>Serum of AGEs</td>
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<tr>
<td>2012</td>
<td>Males and</td>
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<td>Yes (p = 0.029)</td>
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<td></td>
<td>females</td>
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<td>30-60 years</td>
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<td>Yes (p = 0.001)</td>
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<td>Yes (p &lt; 0.001)</td>
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<td>Breslavsky,</td>
<td>N= 47</td>
<td>T2DM (Israel)</td>
<td>12 months</td>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt; 1000 IU/day</td>
<td>No significant change</td>
<td>serum 25(OH)D</td>
<td>Serum of AGEs</td>
<td>Serum of AGEs</td>
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<tr>
<td>2013</td>
<td>Male=22</td>
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<td>Yes (p = 0.001)</td>
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<td></td>
<td>Female=25</td>
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<td>Yes (p &lt; 0.001)</td>
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<td>11.8 ng/ml</td>
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<td>17.6 ng/ml</td>
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<tr>
<td>Yiu, 2013</td>
<td>N= 100</td>
<td>T2DM (Hong Kong)</td>
<td>Double-blind RCT</td>
<td>3 months</td>
<td>25(OH)D$_3$ 5000 IU/day</td>
<td>serum 25(OH)D</td>
<td>Yes (p &lt;0.001) and achieved the sufficient level</td>
<td>21.1 ng/ml</td>
<td>58.6 ng/ml</td>
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<td></td>
<td>Male = 50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Oxidative stress, FBS, HbA1c, lipid profile</td>
<td>No significant change</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Female = 50</td>
<td></td>
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</tr>
<tr>
<td>Talaei, 2013</td>
<td>N= 100</td>
<td>T2DM (Iran)</td>
<td>Single blind study</td>
<td>2 months</td>
<td>25(OH)D$_3$ 50,000 IU/week</td>
<td>serum 25(OH)D</td>
<td>Yes (p =0.02) but were already sufficient at baseline</td>
<td>43.03 ng/ml</td>
<td>60.12 ng/ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70% women</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>lipid profile</td>
<td>No significant change</td>
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<tr>
<td></td>
<td>30% men</td>
<td></td>
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<tr>
<td></td>
<td>30 to 70 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>serum insulin</td>
<td>Yes (p =0.02)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HOMA-IR</td>
<td>Yes (p =0.008)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FBS</td>
<td>Yes (p =0.02)</td>
<td></td>
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</tr>
<tr>
<td>Study</td>
<td>N=</td>
<td>Male</td>
<td>Female</td>
<td>Age Range</td>
<td>Type of Disease</td>
<td>Study Details</td>
<td>Primary Outcome</td>
<td>Secondary Outcomes</td>
<td>Follow-up</td>
<td>Serum 25(OH)D</td>
</tr>
<tr>
<td>---------------</td>
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</tr>
<tr>
<td>Alkharfy, 2013</td>
<td>499</td>
<td>267</td>
<td>232</td>
<td>21-60 yrs</td>
<td>T2DM (Saudi)</td>
<td>RCT 12 months</td>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt; 2000 IU/day</td>
<td>serum 25(OH)D BMI, FBS TG, LDL, Total cholesterol, HDL</td>
<td>Yes (p =0.04) but did not achieve sufficient levels</td>
<td>13.64 ng/ml</td>
</tr>
<tr>
<td>Eftekhari, 2013</td>
<td>70</td>
<td>35</td>
<td>35</td>
<td>30-75 yrs</td>
<td>T2DM (Iran)</td>
<td>Double-blind RCT 3 months</td>
<td>2 x capsules of calcitriol (0.25 µg 1,25dihydroxy cholecalciferol per capsule) per day</td>
<td>HDL, oxidative stress LDL, Total cholesterol, TG</td>
<td>No significant change</td>
<td>N/A</td>
</tr>
<tr>
<td>Ryu, 2014</td>
<td>62</td>
<td></td>
<td></td>
<td>47 to 63 yrs</td>
<td>T2DM (Korea)</td>
<td>RCT 6 months</td>
<td>2000 IU/day + calcium</td>
<td>25(OH)D</td>
<td>Yes (p &lt; 0.001) and achieve the sufficient level</td>
<td>12.3 ng/ml</td>
</tr>
<tr>
<td>Study</td>
<td>N=</td>
<td>Male=</td>
<td>Female=</td>
<td>Age Range</td>
<td>T2DM Location</td>
<td>Study Design</td>
<td>Duration</td>
<td>Intervention</td>
<td>Outcome Measures</td>
<td>Outcome</td>
</tr>
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<td>---------------</td>
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<td>----------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>Yousefi RAD, 2014</td>
<td>58</td>
<td>36</td>
<td>22</td>
<td>30-60</td>
<td>Iran</td>
<td>Double-blind RCT</td>
<td>2 months</td>
<td>25(OH)D3 4000 IU/day</td>
<td>serum 25(OH)D, HbA1c, insulin concentration, HDL, LDL, Total cholesterol, TG</td>
<td>Yes (p &lt; 0.001) but did not achieve sufficient level</td>
</tr>
<tr>
<td>Labban, 2014</td>
<td>722</td>
<td>360</td>
<td>362</td>
<td>36-61</td>
<td>Morocco</td>
<td>RCT</td>
<td>2 months</td>
<td>25(OH)D3 2000 IU/day</td>
<td>serum 25(OH)D, HbA1c</td>
<td>Increased significantly (p &lt; 0.001) but did not achieve the sufficient level</td>
</tr>
<tr>
<td>Study</td>
<td>N</td>
<td>Country</td>
<td>Design</td>
<td>Duration</td>
<td>Intervention</td>
<td>Outcome Measures</td>
<td>Outcome</td>
<td>Change in Vitamin D Level</td>
<td>p-Value</td>
<td>Change in HbA1c</td>
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</tr>
<tr>
<td>Alzahrani, 2014</td>
<td>248</td>
<td>Saudi Arabia</td>
<td>RCT</td>
<td>3 months</td>
<td>25(OH)D₃</td>
<td>serum 25(OH)D</td>
<td>Yes</td>
<td>No significant change</td>
<td>0.01</td>
<td>No significant change</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45,000 IU/day</td>
<td>HbA1c, FBS, lipid profile, BMI</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>for 2 months</td>
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<td>Stat dose 45,000 IU in the last month</td>
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<tr>
<td>Kampmann, 2014</td>
<td>16</td>
<td>Denmark</td>
<td>Double-blind RCT</td>
<td>3 months</td>
<td>25(OH)D₃</td>
<td>serum 25(OH)D</td>
<td>Yes</td>
<td>No significant change</td>
<td>0.02</td>
<td>No significant change</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11,200 IU/day</td>
<td>HbA1c, HOMA, BMI, lipid profile, calcium, FBS, serum insulin</td>
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<td>for 2 weeks</td>
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<td>5,600 IU/day</td>
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<td>for 10 weeks</td>
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38
<table>
<thead>
<tr>
<th>Study</th>
<th>N</th>
<th>Gender</th>
<th>Age</th>
<th>Type</th>
<th>Intervention</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tabesh, 2014</td>
<td>118</td>
<td>60</td>
<td>30 years+</td>
<td>T2DM (Iran)</td>
<td>Double-blind RCT 2 months 25(OH)D$_3$ fortified yogurt drink + vitamin D + 1,000 mg/day calcium</td>
<td>Serum 25(OH)D, serum insulin, HbA1c, HOMA-IR, LDL, Total cholesterol, HOMA-B, HDL BMI</td>
</tr>
<tr>
<td>Nikooyeh, 2014</td>
<td>90</td>
<td>Males and females</td>
<td>30-60 years</td>
<td>T2DM (Iran)</td>
<td>RCT 3 months 25(OH)D$_3$ fortified yogurt drink 1000IU/day vitamin D</td>
<td>Serum of AGEs HbA1c Markers of oxidative stress</td>
</tr>
</tbody>
</table>

<p>| 11.2 ng/ml | N/A  |
| 44.5 ng/ml | 74.6 ng/ml |</p>
<table>
<thead>
<tr>
<th>Study</th>
<th>Sample Size</th>
<th>Population</th>
<th>Intervention Duration</th>
<th>Intervention</th>
<th>Outcome</th>
<th>Study Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasri, 2014</td>
<td>N= 60</td>
<td>T2DM (Iran)</td>
<td>3 months</td>
<td>25(OH)D₃ 50,000 IU/week</td>
<td>serum 25(OH)D HbA1c FBS</td>
<td>Yes (p =0.001) but already had sufficient levels at baseline Significant reduction in males</td>
</tr>
<tr>
<td>Krul-poel, 2015</td>
<td>N= 245</td>
<td>T2DM (Netherlands)</td>
<td>6 months</td>
<td>25(OH)D₃ 50,000 IU/month</td>
<td>serum 25(OH)D HbA1c, AGEs</td>
<td>Yes (p &lt; 0.01) and achieved sufficient levels No significant change</td>
</tr>
<tr>
<td>Mohamad, 2015</td>
<td>N= 100</td>
<td>T2DM (Egypt)</td>
<td>2 months</td>
<td>25(OH)D₃ 4500 IU/day</td>
<td>serum 25(OH)D FBS, HbA1c, LDL, Total cholesterol HDL TG</td>
<td>Yes (p &lt; 0.05) and achieved sufficient level Decreased significantly Increased significantly Significantly decreased in females</td>
</tr>
<tr>
<td>Study</td>
<td>N</td>
<td>Gender</td>
<td>Age</td>
<td>Type</td>
<td>Intervention</td>
<td>Duration</td>
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<tr>
<td>Alsofani, 2015</td>
<td>20</td>
<td>Male=15</td>
<td>21 to 75y</td>
<td>T2DM (Saudi)</td>
<td>Double-blind RCT</td>
<td>3 months</td>
</tr>
<tr>
<td>Sadiya, 2015</td>
<td>87</td>
<td>All males</td>
<td>30-60y</td>
<td>T2DM (UAE)</td>
<td>Double-blind RCT</td>
<td>6 months (2 phase of 3 months each)</td>
</tr>
<tr>
<td>Al-shahwan, 2015</td>
<td>45</td>
<td>Male=21</td>
<td>21 years +</td>
<td>T2DM (Saudi)</td>
<td>Intervention study</td>
<td>1 year</td>
</tr>
<tr>
<td>Study</td>
<td>N=</td>
<td>Gender</td>
<td>Design Type</td>
<td>Intervention</td>
<td>Outcomes</td>
<td></td>
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</tr>
<tr>
<td>Shab Bidar, 2015</td>
<td>100</td>
<td>Male=43</td>
<td>RCT</td>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt; fortified yogurt drink 1000IU/day vitamin D</td>
<td>Yes (p&lt;0.001) but did not achieve the sufficient level</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female=57</td>
<td></td>
<td>25(OH)D</td>
<td>FBS</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>HbA1c</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Markers of oxidative stress including Total antioxidant</td>
<td>Yes (p&lt;0.001)</td>
<td></td>
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<td></td>
<td>15.4 ng/ml</td>
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<td></td>
<td></td>
<td></td>
<td>28.8 ng/ml</td>
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</tbody>
</table>
As shown in Table 1.2, evidence from recent experimental and observation studies has highlighted a potential role for vitamin D in improving glycaemic control, dyslipidaemia and markers of oxidative damage in diabetic patients. Vitamin D has been demonstrated to improve pancreatic beta cell function and decrease insulin resistance, reduce serum LDL-cholesterol and triglyceride levels and to upregulate potent endogenous antioxidants to combat oxidative stress. Despite these encouraging developments, the role of vitamin D supplementation in diabetes remains controversial and there are still many questions that need to be answered. In the Saudi population where both type 2 diabetes and vitamin D deficiency are so prevalent, the potential for vitamin D as an adjuvant to conventional diabetic therapies is all the more exciting.

1.8 Aims and objectives

The aim of this study was to understand glycaemia, dyslipidaemia and oxidative stress in Saudi Arabian women who were vitamin D deficient with poorly controlled type 2 diabetes.

The objectives were as follows:

- To assess the effect of vitamin D₃ supplementation on markers of glycaemia, including fasting serum glucose level, insulin resistance and secretion, and glycated haemoglobin in Saudi adult females
- To assess the effect of vitamin D₃ supplementation on diabetic dyslipidaemia, including serum low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides (TG) and total cholesterol in Saudi adult females
- To assess the effect of vitamin D₃ supplementation on oxidative stress, including total serum antioxidant levels and advanced glycation endproducts (AGEs) in Saudi adult females
- To establish whether different doses of vitamin D₃ elicit different responses in relation to markers of glycaemia, dyslipidaemia and oxidative stress
Chapter 2. Materials & Methods
2.1 Materials, equipment and software

2.1.1 Materials

❖ Microcrystalline cellulose placebo; 2000 IU vitamin D₃; 4000 IU vitamin D₃ (Metabolics Ltd, UK)

❖ 96-well microtiter plate format (Eppendorf company, German)

❖ Uric acid standard 100mg; reaction buffer 400ul; copper ion reagent 1.0mL; stop solution 1.5mL (Cell Biolabs, USA)

❖ 1N NaOH (Sigma Aldrich, USA)

❖ Albumin colorimetric assay kit (Cobas Albumin Gen.2, Roche Diagnostics, USA)

❖ Tina-quant Haemogblon A1c Gen.3 turbidimetric inhibition immunoassay kit (Cobas A1C-3, Roche Diagnostics, USA)

❖ Calcium Gen. 2 assay kit (Roche Diagnostics, USA)

❖ Glucose HK GLUC3 enzymatic assay kit (Roche Diagnostics, USA)

❖ Cholesterol Gen. 2 colorimetric assay kit (Roche Diagnostics, USA)

❖ Triglycerides enzymatic colorimetric assay kit (Roche Diagnostics, USA)

❖ HDL-Cholesterol plus 3rd generation enzymatic colorimetric assay kit (Roche Diagnostics, USA)

❖ LDL-Cholesterol plus 2nd generation enzymatic colorimetric assay kit (Roche Diagnostics, USA)

❖ Insulin immunoassay kit (Roche Diagnostics, USA)

❖ Vitamin D total 25-hydroxyvitamin D immunoassay kit (Roche Diagnostics, USA)

❖ Eppendorf tubes (Eppendorf company, German)

❖ Sterile Gloves (Ansell, Canada)
❖ Deionized water (Local store, Saudi Arabia)

2.1.2 Equipment
❖ Automated electrochemiluminescence assays (Roche Cobas E601, USA)
❖ Automated electrochemiluminescence assays (Roche Cobas E501, USA)
❖ OxiSelect TAC assay kit (Cell Biolabs, USA)
❖ Pipettes (Eppendorf company, German)
❖ Spectrophotometric microplate reader (BioTek, USA)
❖ AGE reader (Diagnoptics Technologies B.V., Groningen, the Netherlands)
❖ 50uL to 300uL adjustable single and multichannel micropipettes (Eppendorf company, German)
❖ Standard tape measure (Seca, Germany)
❖ Free-standing stadiometer (Seca, Germany)
❖ Weighing scales (Seca, Germany)
❖ 21G needle (BD, USA)
❖ EDTA, serum and lithium heparin tubes for blood collection (BD Vacutainer®, USA)
❖ Centrifuge 5702 (Eppendorf, Germany)
❖ Laboratory freezer minus 20 (Philip Kirsch, German)
❖ Laboratory freezer at minus 80 (Philip Kirsch, German)
❖ Laboratory fridge (Philip Kirsch, German)
❖ Orbital shaker (Lab tech, Korea)
❖ Informed consent form (see appendices)
❖ Pre-screening questionnaire (see appendices)
❖ Supplement recording calendar (see appendices)
❖ Debriefing questionnaire (see appendices)
Blood analysis flow chart (see appendices)

2.1.3 Software

- Microsoft office 2013 (Microsoft, USA)
- Statistics Package for Social Sciences (SPSS, version 20 for Windows) (SPSS Inc., Chicago, USA)
- Microsoft excel 2013 (Microsoft, USA)
- Powerful statistical software (Minitab 17) (Minitab Inc, USA)
- Microsoft Paint (Microsoft, USA)
- BMI calculator (NHS Tools, UK)

2.2 Ethical approval

Ethical approval was granted on the 8th August 2013 by the Manchester Metropolitan Ethics Committee. Authorisation from Al-Noor Specialist Hospital, Saudi Arabia, was received on the 9th December 2013, and submitted to the Manchester Metropolitan Ethics Committee. Ethical approval was also granted from the Ethics committee at Al-Noor Specialist Hospital, Saudi Arabia, following receipt of the research proposal.

Funding has been granted through the Saudi Arabian Government Scholarship scheme by the Saudi Arabian Culture Bureau.

2.3 Study overview

In this study, 156 poorly-controlled T2DM Saudi female patients were recruited from Al-Noor hospital (Saudi Arabia), between December 2013 and January 2014. Subjects who met the inclusion criteria (Figure 2.1) were randomly allocated to one of three test groups: placebo, 2000 IU vitamin D₃ daily and 4000 IU vitamin D₃ daily.

Participants were referred to the study by the consultant overseeing their care. If the participant met the inclusion criteria they were invited for an initial meeting between the participant and researcher where the objectives and expectations were outlined and informed consent obtained and documented clearly. Anthropometric measurements were taken, along with blood samples for analysis and a 16-week course of
supplements were allocated. A follow-up appointment was made at 16 weeks where repeat blood samples and anthropometric measurements were taken, a debriefing questionnaire completed and any remaining supplements collected. The general outline of the study is shown in Figure 2.1.
Figure 2.1: Study design and progression flow chart.

Inclusion Criteria
- age 18-60 years
- overweight (BMI > 25 kg/m²)
- type 2 diabetic
- no renal / gastrointestinal disease
- not taking vitamin supplements up to 3 months before or during study
- not pregnant

Screened for eligibility (n=3750)
- Screening questionnaire

Enrolment after meeting the inclusion criteria

Randomised allocation
n=156

Baseline - Meeting 1
- Communicate vit D regimen
- Informed written consent
- Blood samples and anthropometric measurements taken
- Supplements and pill calendar supplied

Week 16 – Follow-up
- Debriefing questionnaire and interview on compliance
- Repeat blood samples and anthropometric measurements taken
- Remainder of supplements collected and counted

Analysed (n=128)
Via SPSS

Lost to follow-up:
- Group A (n=4)
- Group B (n=3)
- Group C (n=2)

Excluded from analysis*
- Group A (n=6)
- Group B (n=6)
- Group C (n=7)

4000 IU vitamin D (group A)
n=52

Placebo group (group C)
n=52

2000 IU vitamin D (group B)
n=52

*see 3.3 Reasons for attrition
2.4 Interventions
Participants were randomly allocated one of three groups, 4000 IU per day (Group A), 2000 IU per day (Group B) or placebo (Group C). Both these supplement doses have been shown to be effective in raising serum vitamin D concentrations in a number of previous studies (Von Hurst et al., 2008; Mitri et al., 2011; Al-Daghri et al., 2012). The placebo contained only microcrystalline cellulose as used in previous similar studies (Pittas et al., 2007a) and was chosen as it cannot be readily absorbed by the body (Grossmann and Tangpricha 2010; Kaptchuk et al., 2010).

The random allocation of patients to one of the three study groups was also imperative to minimize bias and ensure a balanced distribution of important variables between groups (Practice Committee of American Society for Reproductive 2008). In this study, patients were randomly allocated one of the three groups depending on the order in which they presented for their scheduled follow-up appointment with the doctor. These appointments had already been allocated by an impartial third party prior to this study commencing. For example, the patient attending appointment one was allocated group A, patient attending appointment two was allocated group B and so on. This use of third party randomisation enabled the order of allocation to the groups to be concealed to the researcher, thus eliminating bias. Furthermore, the researcher and the patient were both blind to which group would be assigned which supplement dose or placebo. In order to maintain the blinding, all tablets and packaging looked the same. Blinding has been a key facet of trials analysing vitamin D₃ supplementation on markers of diabetic control in order to minimise bias (Pittas et al., 2007a; De Boer et al., 2008; Von Hurst et al., 2008; Nagpal et al., 2009; Mitri et al., 2011).

2.5 Safety margins
Vitamin D intoxication is an uncommon but avoidable issue, which would result in hypercalcaemia. Safety is paramount in research on human subjects and thus the upper limit of safe vitamin D supplementation had to be determined prior to study initiation. The current consensus is that 10,000 IU/day is the recommended highest daily intake to be sure of no adverse outcomes (Hathcock et al., 2007). As this study used 2000 IU and 4000 IU vitamin D₃ supplementation, it can be assured that all supplementation was within safe parameters.
In order to prevent hypercalcaemia as a result of vitamin D intervention, levels of corrected calcium were measured prior to inclusion in the study. Any patient found to have higher than acceptable levels of corrected calcium became ineligible for further participation in the study.

2.6 Recruitment and sittings

The target population was specifically Saudi females with poorly controlled type 2 diabetes between the ages of 18 and 60 years. Al-Noor hospital is the hub for outpatient diabetes clinics in the Makkah area. The researcher first determined eligible women for the study by reviewing around 125 files daily of Saudi diabetic females at the Diabetic Centre, looking for patients who met the inclusion criteria using the screening questionnaire for recruiting participants. A list of suitable patients was compiled for the attention of the specialist conducting the diabetic clinic to refer the patients on after their appointment. The women referred by the consultants were between 18 and 60 years of age and had poorly controlled T2DM. Participants who met the eligibility criteria were then invited to take part in the study via phone call. Participants were also sent flyers about the benefit of vitamin D and invited by What’sapp (if they use the app) to attend a meeting with the researcher after their next appointment with the specialist. During the appointment the study researcher explained the possible benefits of vitamin D in diabetic patients and what would be expected of them if they were to participate in the study. If they chose to participate it was also explained how to fill in a supplement recording calendar, the vitamin D$_3$ supplements were given out, and blood samples taken for baseline measurements and initial AGE measurements taken.

All patient appointments and biological tests were carried out in Saudi Arabia. Patient appointments were conducted at the Al-Noor hospital. Laboratory tests were carried out by trained laboratory staff at the Al-Noor clinical laboratory. Study coordination, analysis and subsequent work were all undertaken at the Manchester Metropolitan University, Manchester, UK.

2.7 Inclusion and exclusion criteria

Inclusion criteria for the study as shown in Figure 7 was as follows: Saudi Arabian women, whose parents were both Saudi Arabian, between the ages of 18 and 60 years
with poorly controlled type 2 diabetes (defined as glycated haemoglobin > 8%) (Kuo et al., 2006; Wolff-McDonagh et al., 2010) and BMI above 25 kg/m² with no prior history of renal, liver or gastrointestinal disease.

Patients with renal, liver or gastrointestinal disease, pregnancy or prior use of vitamin D supplements (three months prior to or during the study time) were excluded from the study as seen in Table 2.1. Vitamin D has a circulating half-life of 15 days and turnover in the body of two months (Jones 2008) and therefore excluding patients who had taken supplements within the past three months negated any confounding results from previous supplementation. Vitamin D₃ is absorbed by the gut and then activated in the liver and kidneys. Previous studies have shown that renal or liver disease and gastrointestinal disease limits the activation or absorption of vitamin D₃, thus rendering the intervention less effective (Ritz et al., 1979; Farraye et al., 2011; Vavricka and Rogler 2012; Stokes et al., 2013). Pregnancy has been linked to vitamin D deficiency and resulting clinical outcomes such as gestational diabetes (Barrett and McElduff 2010). Pregnancy can further complicate the measurement of diabetes as diabetic control is more difficult as glucose levels change according to hormonal factors (Rafat and Ahmad 2012). Due to the added complications that pregnancy could bring to diabetic control, pregnant women were excluded from the study.

**Table 2.1: Study Inclusion and exclusion criteria.**

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>✓ Females aged 18-60 years</td>
<td>➢ Renal, liver or gastrointestinal disease e.g. Crohn’s disease or liver failure</td>
</tr>
<tr>
<td>✓ BMI ≥ 25</td>
<td>➢ Those taking supplements that include vitamin D</td>
</tr>
<tr>
<td>✓ Glycated haemoglobin &gt; 8% (Poorly controlled type 2 diabetics)</td>
<td>➢ Pregnancy</td>
</tr>
<tr>
<td>✓ Participant and their parents are both Saudi Arabian</td>
<td>➢ Hypoglycaemic changes</td>
</tr>
</tbody>
</table>
2.8 Sample size calculation

ANCOVA was used to assess changes in outcomes after intervention, but the limitation of ANCOVA is that the statistical interpretations based on the F tests are sensitive to sample size. When the sample size is too small, there is inadequate power to calculate the F statistic and the hypothesis cannot be appropriately tested. The use of sample sizes which are inadequate can lead to misrepresentative results which may have profoundly negative clinical outcomes (Bailar et al., 1992).

Specific power calculations for this study were therefore undertaken using G*Power 3 (Faul et al., 2007) and the recommendations of a statistician to ensure that the sample size was sufficient to detect the effect of vitamin D₃ supplementation on biological outcomes. A power level of 80% was assumed, an effect size explaining 25% of the variance in vitamin D concentrations, with three groups to the study, a minimum sample size of N=128 was calculated to obtain significance at the p=0.05 level. Inevitably, some patients were lost to follow up. To compensate for this the number recruited was increased by 20%. If loss to follow up was greater than this, the analysis might be unsound and may not be publishable (Schulz and Grimes 2002). Consultation on the power calculations was taken with Dr M Sullivan, a Senior Lecturer at Manchester Metropolitan University. On consultation of similar previous interventional studies, it was noted that sample sizes ranging from between 37 and 92 participants had achieved significant validated results over various durations of one to 18 months (Raghuramulu et al., 1992; Sugden et al., 2008; Witham et al., 2010; Al-Daghri et al., 2012; Soric et al., 2012).

2.9 Informed consent form

Written information sheets contribute to patient understanding of a trial more than verbal discussion of the information (Langdon et al., 2002; Shukla et al., 2012). It has also been shown that such information sheets are often overly complicated in the language that they use, especially if the target audience is young or has low literacy levels (Franck and Winter 2004). The optimum design for an informed consent form has been shown to be brief and to the point (Epstein and Lasagna 1969). In this study, an informed consent sheet was given to the participants which explained what was expected of them during the study and what would happen at each stage. The language used in the form was simple and easy to understand. The participant was asked to
confirm in writing that they understood the information presented to them and to accept or decline the offer to take part in the study. Contact information for the researcher was included on the information sheet so that participants could raise any questions directly and at any time. The researcher also spent a one-to-one session with each participant, which has been shown to increase participant understanding in previous studies (Flory and Emanuel 2004).

2.10 Pre-study screening questionnaire

Potential participants attended a meeting with the researcher where their eligibility for the study was assessed. The researcher first checked that the potential participants met the inclusion criteria and did not breach the exclusion criteria. Those participants that met the inclusion criteria and agreed to participate in the study then went on to complete the pre-screening questionnaire which is discussed below.

In order to produce an effective questionnaire, the language used must be simple, understandable and precise. The language used should not be too formal or scientific in order to minimise confusion and misunderstanding (McLeod 2008; Gould 2011). The questionnaire for this study was developed in English and then translated to Arabic. The responses were then translated back in to English for analysis. The questions asked in this questionnaire fell in to two categories: personal information and medical information. These were arranged in a logical order, with the personal information questions first (Berdie et al., 1986; Hague 1993). The questions were closed where possible, with a minimum number of open ended questions as these are more difficult to analyse (Oppenheim 1992). For closed questions, a set number of responses were provided, and the subject prompted to circle the most appropriate answer.

The questionnaire was given out in a face-to-face meeting with the study researcher. This ensures that any misunderstanding is rectified and the errors or unanswered questions kept to a minimum and so response rates improved (Oppenheim 1992). A number of background questions were used to determine patients’ gender, age, marital status, education level, employment history, ethnic origin and smoking status. The questionnaire also indicated whether the participants were currently taking daily vitamin supplements, or whether they were taking vitamin D supplements three months prior to inclusion in the study. Specific medical conditions were checked, together with listing the prescribed medications that the participants were taking. This
section of the questionnaire aimed to identify factors that potentially confound the data.

2.11 Anthropometric measurements

Body mass index (BMI) is known to be a strong indicator of diabetes risk (Schulze et al., 2012). Participants’ height and weight were measured and recorded by the study researcher. Height (in centimetres) was recorded whilst the subject was standing erect and barefoot using a free-standing stadiometer. Weight (in kilograms) was measured using a hospital grade scale (Jack et al., 1994). BMI was calculated as weight (kg) / height (m)$^2$.

2.12 Blood collection

10 mL of blood was collected at baseline and at 16 weeks. It was drawn by a trained phlebotomist from the medial cubital vein using aseptic technique and a 21G needle. Blood was taken to fill a 4 mL vacutainer plain tube (to test vitamin D$_3$ and insulin), 2 mL vacutainer EDTA tube (to test HbA1c) and 4 mL vacutainer lithium heparin tube (to test lipids, fasting glucose, calcium, albumin and antioxidant). The patient’s information was recorded on the blood bottles as Name, Date of Birth and Hospital number (or equivalent) for identification. In order to test fasting glucose levels, participants were asked to fast (no food or beverages except water) for a minimum of ten hours prior to the phlebotomy appointment (Nagpal et al., 2009). This is due to the fact that test results could be affected by consuming food and beverages. This can lead to imprecise results as during fasting, glucagon is activated which raises plasma glucose levels (The global diabetes community 2013).

The withdrawn blood was taken to the hospital lab to be centrifuged and kept in the freezer at minus 20°C until the end of the recruitment period. The only exception to this was the sample of total antioxidant levels which had to be stored off-site at King Abulaziz Research Centre at a temperature of -80°C. Once all the data had been collated at baseline and following the 16-week trial, the researcher went to the lab to run the tests with the technicians.
2.13 Supplement compliance

In this study, the aim was to quantify compliance and employ methods which increase compliance to optimal levels. A calendar and pill counting were used to assess compliance by Talaei et al. (2013) and von Hurst et al. (2010). Subject interview can be particularly useful as it provides qualitative details on why the subject may not have complied, not solely whether they complied or not. In the case of von Hurst et al. (2010), subjects reported difficulty in taking four capsules per day for six months. This was taken into account in the present study to reduce the number of capsules required as this could negatively affect compliance.

Pill counting of the surplus supply by weight or number is a proven method of obtaining an objective figure for compliance during the study period (Al-Sofiani et al., 2015). This method has been employed in a number of studies (De Boer et al., 2008; Dong et al., 2010; Talaei et al., 2013).

Other aids for improving compliance involve calendar charts, which enable the subject to record days when the pill was taken and thus highlights to the researcher the days when it was missed. In a meta-analysis of the success of compliance methods in 153 studies showed that no particular intervention had a clear advantage over another (Roter et al., 1998). However, methods that encompassed cognitive and behavioural components improved compliance substantially.

Whilst there is no gold standard for compliance assessment, this study incorporated a calendar for recording pill usage, along with a debriefing questionnaire and interview on compliance issues during the study period and counting remaining supply at 16 weeks. In addition, the researcher contacted each participant daily via What’sapp asking them to reply that they had taken their supplement that day. This method of contacting participants via SMS has been shown to improve medication adherence by 17.8% (Strandbygaard et al., 2010).

2.14 Debriefing Questionnaire

At the end of the 16-week trial, an appointment was made for every participant to meet with the researcher. Again, participants were reminded via phone one week in advance to attend their appointment. At this final appointment, any leftover tablets were collected, along with the calendars and the left over pills counted. A debriefing
questionnaire was administered to ensure that no changes had occurred during the study that would inadvertently affect the results. These included changes to prescribed medicines, onset of new medical conditions and use of any additional vitamins. The baseline anthropometric measurements were re-recorded, along with the AGEs measured and blood samples taken for the previous parameters.

2.15 Blood analysis

All blood sampling methods are in accordance with the procedures in place at the Al-Noor clinical laboratory and information received from Roche (Roche 2013). Storage and deposition of blood samples were in line with the Human Tissue Act and Manchester Metropolitan University guidelines.

2.15.1 Measurement of the concentration of vitamin D

Analysis was carried out using the Roche Cobas E601 analyser. This is a competitive electrochemiluminescent protein binding assay designed to measure total 25-OH vitamin D in serum or plasma, using vitamin D binding protein (VDBP) as capture protein to bind vitamin D₃ and vitamin D₂. 16µl of serum was incubated with pretreatment reagent, releasing the vitamin D from its binding partner Vitamin D Binding Protein (VDBP).

The sample was then incubated with ruthenium labelled VDBP, forming vitamin D-ruthenium labelled VDBP complexes. Finally, streptavidin-coated micro-particles were added which occupy any free sites in the ruthenium labelled VDBP. The sample was then aspirated into the measuring cell, and the microparticles magnetically attach to the surface of the electrode. A voltage was applied to the electrode, inducing chemiluminescent emission, which was measured by a photomultiplier. Results were determined via a calibration curve that was generated by the analysis system. Calibration was performed once every 24 hours. The control interval was set with the lower limit value between 13.180-20.620 ng/ml and the upper limit value of 25.200-37.800 ng/ml using PreciControl Varia.

Previous studies have looked at this method of vitamin D measurement and compared to other methods of measurement and found high concordance (Abdel-Wareth et al., 2013). According to the classification of The Endocrine Society, vitamin D deficiency
is defined using the serum concentrations shown in Table 2.2 (Holick 2004; Holick and Chen 2008; Holick et al, 2011).

Table 2.2: Classification of vitamin D deficiency according to The Endocrine Society, using blood serum concentration of vitamin D.
(Holick 2004; Holick and Chen 2008; Holick et al, 2011)

<table>
<thead>
<tr>
<th>Classification of vitamin D deficiency</th>
<th>Concentration of vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficiency</td>
<td>&lt; 20 ng/ml (&lt; 50 nmol/L)</td>
</tr>
<tr>
<td>Insufficient</td>
<td>21-29 ng/ml (51-74 nmol/L)</td>
</tr>
<tr>
<td>Adequate or sufficient</td>
<td>&gt; 30 ng/ml (&gt; 75 nmol/L)</td>
</tr>
</tbody>
</table>

2.15.2 Measurement of insulin

Insulin levels were measured using the electrochemiluminescent immunoassay, as used in (Nagpal et al., 2009; Tarcin et al., 2009; Shab-Bidar et al., 2011). The Roche electrochemiluminescent immunoassay was used and analysed using the Cobas e601 analysis system. The assay was designed to measure insulin in 20µl of serum in the same method as above, using a biotinylated monoclonal insulin-specific antibody, and a monoclonal insulin-specific antibody labelled with ruthenium. Results were determined via a calibration curve, which was generated by the analysis system. Calibration was performed once every 24 hours. The control interval was set with the lower limit value between 18.980-26.22 µU/mL and the upper limit value of 59.140-81.660 µU/mL Using PreciControl Multimarker/Universal.

2.15.3 Measurement of the concentration of glycated haemoglobin

This study used the Roche turbidimetric inhibition immunoassay (TINIA). This is a two-step assay where the sample and an anti-HbA1c antibody are mixed, causing glycohaemoglobin (HbA1c) to form soluble antigen-antibody complexes. The addition of polyhaptens then react with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complex which can be quantified turbidimetrically using the Cobas e501 analyser. A blood volume of 2 µl was diluted with 180 µl Hemolyzing Reagent. The control interval was set with the lower limit value between 4.80-6.12% using PreciControl HbA1c norm (normal) and the upper limit value of 9.3-11.7% using PreciControl HbA1c path (Pathogenic).
A diagnosis of diabetes is made with HbA1c levels of 6.5% or greater (WHO 2011; American Diabetes Association 2012). This study was interested in patients with poorly controlled T2DM, defined as HbA1c levels of greater than 8%, as used in previous studies (Rotchford et al., 2002; Kuo et al., 2006; Wolff-McDonagh et al., 2010).

2.15.4 Measurement of the concentration of lipids
This study used an enzymatic method to measure high-density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TG) and total cholesterol (TC). HDL and LDL was measured using the Roche enzymatic colorimetric assay system. The concentration of HDL- and LDL- cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase. Cholesterol esters are broken down into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to 4-cholestenone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-amino-antipyrine and HSDA to form a purple-blue dye. The colour intensity of this dye is directly proportional to the cholesterol concentration and is measured photometrically using the Cobas analyser e501. A volume of 2.5µl of serum was used to measure HDL and 2µl of serum for LDL. The control intervals for HDL- cholesterol were set as 23.7-32.5 mg/dL for the lower limit using PreciControl ClinChem Multi 1 and 53.2-73.6 mg/dL for the upper limit using PreciControl ClinChem Multi 2. The control intervals for LDL- cholesterol were set 44.6-61.4 mg/dL for the lower limit and 80.3-110.7 mg/dL for the upper limit, again using PreciControl Multi 1 & 2 respectively.

Triglycerides (TG) were measured using a Roche assay that uses a lipoprotein lipase for the rapid and complete hydrolysis of triglycerides to glycerol followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff. The colour intensity of the red dyestuff formed is directly proportional to the triglyceride concentration and can be measured photometrically. A sample size of 2 µl was used and the control intervals set as 93-113 mg/dL for the lower limit using PreciControl ClinChem Multi 1 and 184-224 mg/dL for the upper limit using PreciControl ClinChem Multi 2.
Total cholesterol (TC) was measured using a Roche assay. Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids. Cholesterol oxidase then catalyzes the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide formed effects the oxidative coupling of phenol and 4-aminophenazone to form a red quinone-imine dye. The colour intensity of the dye formed is directly proportional to the cholesterol concentration. It is determined by measuring the increase in absorbance using the Cobas e501 analyser. A volume of 2µl of serum was sampled and the control intervals set as 74.1-90.5 mg/dL for the lower limit using PreciControl ClinChem Multi 1 and 155-191 mg/dL for the upper limit using PreciControl ClinChem Multi 2.

Table 2.3 shows the desirable, low, borderline high, high and very high cut off points for lipid measurements, as defined by the American Heart Association (The American Heart Association 2013). These cut offs were used to compare the lipid levels pre-intervention and post-intervention to assess whether vitamin D₃ has significantly altered lipid values.

The American Diabetes Association published guidelines in 2012 on desirable lipid levels for diabetic patients, whilst adding a limit for a low HDL cut-off (American Diabetes Association 2012). These are in agreement with the optimal levels shown in Table 2.3.
Table 2.3: Defining the cut offs for lipid measurements.
(Adapted from The American Heart Association 2013).

<table>
<thead>
<tr>
<th></th>
<th>Desirable</th>
<th>Low</th>
<th>Border line high</th>
<th>High</th>
<th>Very high</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL</td>
<td>≥40 mg/dL for men&lt;br&gt;≥50 mg/dL for women</td>
<td>&lt; 35 mg/dL</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>LDL</td>
<td>&lt;100 mg/dL</td>
<td>N/A</td>
<td>130 to 159 mg/dL</td>
<td>160 to 189 mg/dL</td>
<td>≥190 mg/dL</td>
</tr>
<tr>
<td>TG</td>
<td>&lt;150 mg/dL</td>
<td>N/A</td>
<td>150–199 mg/dL</td>
<td>200–499 mg/dL</td>
<td>≥500 mg/dL</td>
</tr>
<tr>
<td>TC</td>
<td>&lt;200 mg/dL</td>
<td>N/A</td>
<td>200 to 239 mg/dL</td>
<td>≥240 mg/dL</td>
<td>N/A</td>
</tr>
</tbody>
</table>

2.15.5 Measurement of the concentration of fasting Glucose

Fasting glucose levels were determined using the Roche enzymatic reference method combining 2µl of serum with hexokinase. Hexokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate by ATP. Glucose-6-phosphate dehydrogenase oxidizes glucose-6-phosphate in the presence of NADP to gluconate-6-phosphate. No other carbohydrate is oxidized. The rate of NADPH formation during the reaction is directly proportional to the glucose concentration and is measured photometrically using the Cobas e501 analyser. The control intervals were set as 92-112 mg/dL for the lower limit using PreciControl ClinChem Multi 1 and 212-260 mg/dL for the upper limit using PreciControl ClinChem Multi 2.

2.15.6 Measurement of the concentration of corrected calcium

The most popular method of calcium analysis in the previous studies is chemiluminescence (Orwoll et al., 1994; Mitri et al., 2011; Shab-Bidar et al., 2011; Talaei et al., 2013). Calcium ions in 3 µl of serum react with 5-nitro-5’methyl-BAPTA (NM-BAPTA) under alkaline conditions, forming a complex. This complex reacts in the second step with EDTA to form a calcium EDTA complex + NM-BAPTA. The
change in absorbance is directly proportional to the calcium concentration and is measured photometrically. The control intervals were set as 8.00-9.40 mg/dL for the lower limit using PreciControl ClinChem Multi 1 and 12.6-15.0 mg/dL for the upper limit using PreciControl ClinChem Multi 2.

About 45% of calcium in the blood is normally transported partly bound to plasma proteins, notably albumin. When albumin concentrations fluctuate substantially, total calcium levels may vary (Goltzman 2000). Such discrepancies can be ‘corrected’ using the formula:

Corrected calcium (mM) = measured total Ca (mM) + \[40 - \text{serum albumin (g/L)}\] \times 0.02

Albumin was measured using the Roche cobas c 501 colorimetric assay. A buffer solution is added to 3µl of serum to give a pH value of 4.1. At this concentration, albumin is able to bind with bromcresol green (BCG), an anionic dye, to form a blue-green complex. The colour intensity of the blue-green colour is directly proportional to the albumin concentration in the sample and is measured photometrically. The control intervals were set as 2.83-3.59 gm/dL for the lower limit using PreciControl ClinChem Multi 1 and 4.45-5.65 gm/dL for the upper limit using and PreciControl ClinChem Multi 2.

2.15.7 Measurement of Oxidative Stress (Total Antioxidant Capacity)

Total antioxidant capacity (TAC) has been demonstrated to be a reliable method for measuring oxidative stress, with a lower TAC representing higher levels of oxidative stress (Ceriello et al., 1997). TAC was assessed using a colorimetric method with the commercially available Cell Biolabs’ OXiSelect TAC assay kit (Antibodies online 2013). This method utilises a standard against which to measure the total antioxidant capacity (ascorbic acid; bovine serum albumin [BSA]; glutathione [GSH]; vitamin E; uric acid; butylated hydroxytoulene [BHT]). 20µl of plasma was collected with heparin and then centrifuged at 4°C for 10 minutes. To measure total antioxidant capacity in the samples, the samples were compared to a known concentration of uric acid standard within a 96-well microtiter plate format. Samples and standards were diluted with a reaction buffer and a reading obtained using a standard 96-well spectrophotometric microplate reader at 490nm. Upon addition of the copper ion reagent into each well the reaction proceeded for a few minutes. The reaction was then
stopped and once again read with the spectrophotometric microplate reader at 490nm. Quality control solutions of a fixed concentration of total antioxidants were ran in duplicate and the absorbance of the control sample measured as with the plasma samples.

2.16 Homeostasis Model Assessment

Insulin resistance and progressive pancreatic β-cell dysfunction have been shown to be paramount in the pathogenesis of type 2 diabetes (Song et al., 2007). There have been various methods employed to assess these factors, including fasting insulin level, fasting glucose/insulin ratio (FGIR), homeostasis model assessment for insulin resistance (HOMA-IR) and quantitative insulin-sensitivity check index (QUICKI) (Matthews et al., 1985; Bergman 1989; Katz et al., 2000; Cutfield et al., 2003; Kurtoglu et al., 2010). HOMA-IR, however has been found to be far superior to FGIR and QUICKI in accurately determining insulin resistance (Keskin et al., 2005). Therefore, HOMA-IR is a frequently used marker in clinical research studies (Wallace and Matthews 2002; Cutfield et al., 2003). The Homeostasis Model Assessment (HOMA) estimates both beta cell function (%B) insulin resistance (%IR), as percentages of a normal reference population. It is derived from calculating the balance between hepatic glucose secretion and insulin secretion from fasting levels of glucose and insulin using the following formulas: (Matthews et al., 1985; Wallace et al., 2004).

\[
\text{HOMA-B} = \frac{\text{Fasting insulin level}}{\text{Fasting glucose concentrations}} - 3.5 \quad \text{(Wallace et al., 2004)}. \\
\text{HOMA-IR} = \frac{\text{Fasting insulin (μU/mL)} \times \text{Fasting glucose (mg/dL)}}{405} \quad \text{(Matthews et al., 1985)}. \\
\]

As the HOMA model uses only a single measurement of fasting insulin and glucose it is seen to be an easy, cheap and reliable alternative to large-scale epidemiologic studies.

2.17 Advanced Glycation Endproducts (AGE) measurement

An AGE reader was used. This can assess AGE levels within the skin in less than 30 seconds and is a non-invasive procedure. Some AGEs have auto-fluorescence capabilities. The AGE reader has a UV light source which penetrates the skin to a
depth of 1 mm. The AGEs become excited, and emit auto-fluorescence at particular wavelengths. The detector within the AGE reader can assess the wavelengths of auto-fluorescence produced by the AGEs, and its intensity, which correlate to the quantity of AGEs present within the skin tissue (Diagnotics 2012).

2.18 Data protection
The researcher obtained an MRC data protection certificate prior to commencing the study and the study followed data protection 1998 guidelines to ensure that only those involved in the study had access to data and that all computers that store this data are encrypted with a password (Smith and Harding 2013). The study was anonymised and all subjects given a unique study number. A key linking patient names and their study number was held by the study supervisor.

2.19 Data handling and cleaning
All data was entered into and maintained in Microsoft Excel. All entries were double checked to minimise data entry errors. Before statistical analysis, data was cleaned and checked for mistakes. If during the course of the study, changes had occurred that would inadvertently affect the results, these subjects were excluded from the study. This included non-compliance with taking vitamin, changes to prescribed medicines, onset of new medical conditions, use of any additional vitamins and not returning after 16 weeks. Adherence to medication was calculated by taking the worst outcome from either the pill calendar, debriefing questionnaire or returned supplements. This value for the number of pills not taken during the study period was used to calculate percentage adherence (number of pills not taken/total number prescribed) x100. Where adherence was found to be less than 80%, participants were excluded from further analyses. This method of adherence calculation has been shown to be an acceptable level of adherence (Winkler et al., 2002; Osterberg and Blaschke 2005; Mitri et al., 2011).

2.20 Statistical analyses
Microsoft Excel was used to handle and maintain the data. All statistical analyses was performed using Statistics Package for Social Sciences (SPSS, version 20).
The population was characterised using descriptive statistics including mean, median, mode and range summary statistics for age, BMI and biological test results. Pill adherence was calculated as described in the previous section and compared between test groups by means of a one-way ANOVA. The Kolmogorov-Smirnov test was used to test the assumption that the clinical outcome measures are normally distributed. If necessary, appropriate transformations such as logarithms were used to normalise the data.

To compare pre- and post-treatment variables, a multivariate approach is needed (Young 2007). An ANCOVA model was used to adjust the mean values of the 16-week outcome measures by controlling for the within-subjects differences in the baseline measures, leading to precise estimates of the between-subjects effects of the treatments, taking into account the interactions between covariates and treatment groups (Albert 1999; Raab et al., 2000; Pocock et al., 2002). The ANCOVA was based upon the General Linear Model (GLM) procedure, with statistical significance $\alpha<0.05$. The GLM assumption of homogeneity of regression slopes (i.e. that the slopes of the lines of the outcome measures versus the baseline measures are equal for each test group) was tested by determining the significance of the interaction between covariates and the three treatment groups. A non-significant interaction at $\alpha=0.05$ implies homogeneity of regression slopes (Rutherford 2001). The effect sizes were interpreted according to the criteria of Ferguson (2009) where $\eta^2 < .04 = \text{negligible}; .041 \text{ to } .249 = \text{low}; .250 \text{ to } .639 = \text{moderate}; .640 \text{ to } 1.00 = \text{high}$.

Mixed ANOVA considers the number of times the dependent variable is measured per participant (Robert 2012). Two-way interaction is used to examine whether the effect of one factor depends on the level of another factor and vice versa (Robert 2012). In the present study, the effect of two different doses of vitamin D supplementation were compared against a placebo over time (pre-test and post-test). Mixed ANOVA was used to examine if there was an interaction within subject groups (pre-test and post-test) and between subject groups (A, B and C) on the dependent variable.
Chapter 3. Patient Demographics
3.1 Introduction

As with many diseases, certain traits and lifestyle factors can influence a person’s susceptibility to developing diabetes. There are many factors which confer a small amount of risk such as certain genetic polymorphisms, and others which confer a much larger risk such as obesity. Many studies have found that non-modifiable risk factors such as gender, age at onset of disease, duration of diabetes and the current age of the patient directly affect HbA1c (Verma et al., 2006).

It is well documented that middle-aged and older adults are at highest risk of developing type 2 diabetes (CDC 2014). A wider age range of 18-60 years was selected in this study to enable comparisons with other similar studies (Mitri et al., 2011; Al-Daghri et al., 2012). Several studies have shown a robust inverse relationship, especially in women, between T2DM prevalence and education level. The relationship between education level and health is, however, a complex one and is likely influenced by many factors, including lifestyle, access to health services and awareness of health promotion (Sacerdote et al., 2012). Lifestyle factors such as diet and smoking are considered to be strong risk factors for the development of diabetes. It is widely acknowledged that appropriate use of diet and exercise can increase insulin sensitivity, improve glycaemic control and reduce the need for intervention with oral medication or insulin (Ainsworth et al., 1993; Nielsen 1998a).

It is well documented that having a family history of diabetes increases the risk of developing the disease, however the contribution of genetics to diabetes is not a simple topic (Valdez et al., 2007). There is considerable variation in the heredity of diabetes among different populations and in different environments. Studies have also shown that an increased duration of diabetes correlates with higher HbA1c levels (Verma et al., 2006).

Obesity is one of the main non-genetic risk factors for diabetes (Hamman 1992). Weight gain has been shown to cause a decrease in insulin sensitivity, possibly as a mechanism to halt further weight gain (Hamman 1992). Increases in BMI amongst type 2 diabetics have been shown to be a strong indicator of changes in HbA1c as documented in longitudinal cohort studies. Similarly, patients who lose weight are far more likely to reach their target HbA1c than those whose weight remains stable or increases (Wilding 2014).
The risk factors that are linked to worse prognostic outcomes may confound results if they are unevenly distributed between groups (Practice Committee of American Society for Reproductive 2008). The aim of this study was to identify the relevant modifiable and non-modifiable risk factors and to control for their effects. As such, the following parameters were measured across the two groups (Group A: 4000 IU of vitamin D per day, and Group B: 2000 IU per day) and compared against the age-matched control group:

- Age, education level, smoking status, BMI and diet
- Family history and duration of type 2 diabetes

3.2 Materials & methods

Ethical approval was granted by the Manchester Metropolitan University Ethics Committee as stated in section 2.2. This was a double-blind, randomized control trial of 156 female type 2 diabetics with HbA1c levels greater than 8.0 percent. All participants were recruited from Al-Noor hospital in Saudi Arabia and informed consent was obtained as detailed in sections 2.6 and 2.9. Subjects who met the inclusion criteria were randomly allocated to one of three test groups. These were as follows: 2000 IU vitamin D per day; 4000 IU vitamin D per day; or a placebo, as described in section 2.3. Participants were also asked to attend a meeting and complete a pre-study screening questionnaire at baseline, and a debriefing questionnaire at 16 weeks as per sections 2.10 and 2.14.

3.3 Results

One hundred and fifty-six subjects were initially recruited, however, due to attrition between the baseline measurements and the final measurements (collected 16 weeks after the baseline), the total number of participants was n = 128: 42 in group A, 43 in group B and 43 in group C. The reasons for the attrition of 10 individuals from Group A and nine individuals each from Groups B and C (based on the responses to the debriefing questionnaire) are listed in Table 3.1.
Table 3.1: Reasons for attrition

<table>
<thead>
<tr>
<th>Reason</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-compliance with taking vitamin D supplements</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Contracted gastrointestinal malabsorption during the intervention</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Changed diabetic medication during the intervention</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Did not return after 16 weeks</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Took additional vitamin D</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

The demographic characteristics of the 128 participants, classified into groups A, B, and C, as reported in the Pre-Questionnaire, are summarized in Table 3.2. All of the participants were Saudi women. The most frequent age-group, representing about half of the women in Group A, B, and C was 45 to 54 years. The next most frequent age-group, representing about one third of the women in Group A, B, and C was 55 to 60 years. The most frequent levels of education of over half the women in each group were no schooling or primary school. About 14 percent of the women in each group were employed, and over 70 percent in each group were unemployed. Consequently, in terms of their demographic characteristics, the women in groups A, B, and C appeared to be approximately equivalent.
Table 3.2: Demographic characteristics of participants at baseline (N = 128)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Category</th>
<th>Group A</th>
<th></th>
<th>Group B</th>
<th></th>
<th>Group C</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Nationality</td>
<td>Saudi</td>
<td>42</td>
<td>100.0</td>
<td>43</td>
<td>100.0</td>
<td>43</td>
<td>100.0</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>42</td>
<td>100.0</td>
<td>43</td>
<td>100.0</td>
<td>43</td>
<td>100.0</td>
</tr>
<tr>
<td>Age</td>
<td>25-34</td>
<td>1</td>
<td>2.4</td>
<td>1</td>
<td>2.3</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>35-44</td>
<td>3</td>
<td>7.1</td>
<td>7</td>
<td>16.3</td>
<td>5</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>45-54</td>
<td>24</td>
<td>57.1</td>
<td>21</td>
<td>48.8</td>
<td>21</td>
<td>48.8</td>
</tr>
<tr>
<td></td>
<td>55-60</td>
<td>14</td>
<td>33.3</td>
<td>14</td>
<td>32.6</td>
<td>17</td>
<td>39.5</td>
</tr>
<tr>
<td>Education</td>
<td>No schooling</td>
<td>13</td>
<td>31.0</td>
<td>8</td>
<td>18.6</td>
<td>16</td>
<td>37.2</td>
</tr>
<tr>
<td></td>
<td>Primary school</td>
<td>10</td>
<td>23.8</td>
<td>13</td>
<td>30.2</td>
<td>11</td>
<td>25.6</td>
</tr>
<tr>
<td></td>
<td>Secondary school</td>
<td>11</td>
<td>26.2</td>
<td>7</td>
<td>16.3</td>
<td>5</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>High school</td>
<td>1</td>
<td>2.4</td>
<td>4</td>
<td>9.3</td>
<td>4</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>Diploma</td>
<td>3</td>
<td>7.1</td>
<td>2</td>
<td>4.7</td>
<td>2</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Bachelor’s degree</td>
<td>4</td>
<td>9.5</td>
<td>8</td>
<td>18.6</td>
<td>4</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>Postgraduate degree</td>
<td>0</td>
<td>0.0</td>
<td>1</td>
<td>2.3</td>
<td>1</td>
<td>2.3</td>
</tr>
<tr>
<td>Employment</td>
<td>Employed</td>
<td>6</td>
<td>14.3</td>
<td>6</td>
<td>14.0</td>
<td>6</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>Retired</td>
<td>2</td>
<td>4.8</td>
<td>6</td>
<td>14.0</td>
<td>3</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Unemployed</td>
<td>34</td>
<td>81.0</td>
<td>31</td>
<td>72.1</td>
<td>34</td>
<td>79.1</td>
</tr>
</tbody>
</table>

The health-related characteristics of the 128 participants, classified into groups A, B, and C, as reported in the Pre-Questionnaire, are summarized in Table 3.3. Supplement compliance was very high (over 96%) and those who had taken less than 80% of the tablets were excluded from the study. The majority of the participants (81.0 % in group A, 86.0 % in group B, and 69.8 % in group C) were classified as obese (BMI > 30 kg/m²). None of the women were pregnant. Only three or fewer women in each group were smokers or had a special diet. Over half of the participants in each group took vitamin supplements that did not contain vitamin D, before the study. The duration of diabetes of about two thirds or more of the participants in each group was over eight years. The family history of diabetes was very high in group A (95.2 %) and group B.
(93.0 %) but relatively low in group C (18.6 %). All of the participants reported complications, of which the most frequent were neuropathy (about 80 % or more in groups A, B, and C); and cardiovascular disease (over 80 % in groups A, B, and C). Hypothyroidism was present in about one fifth of the patients in each group. Consequently, in terms of their health related characteristics, groups A, B, and C appeared to be approximately equivalent (with the exception of family history of diabetes).

Table 3.3: Health-related characteristics of participants at baseline (N = 128)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Category</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Overweight (BMI = 25.1 to 30)</td>
<td></td>
<td>8</td>
<td>19.0</td>
<td>6</td>
</tr>
<tr>
<td>Obese (BMI &gt; 30)</td>
<td></td>
<td>34</td>
<td>81.0</td>
<td>37</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>Not pregnant</td>
<td>42</td>
<td>100.0</td>
<td>43</td>
</tr>
<tr>
<td>Smoking</td>
<td>Smoker</td>
<td>2</td>
<td>5.8</td>
<td>3</td>
</tr>
<tr>
<td>Diet</td>
<td>Special diet</td>
<td>3</td>
<td>7.1</td>
<td>3</td>
</tr>
<tr>
<td>Supplements (not containing vitamin D)</td>
<td>Yes</td>
<td>25</td>
<td>59.5</td>
<td>27</td>
</tr>
<tr>
<td>Duration of diabetes</td>
<td>1-2 years</td>
<td>7</td>
<td>16.7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3-5 years</td>
<td>2</td>
<td>4.8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>6-8 years</td>
<td>2</td>
<td>4.8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt; 8 years</td>
<td>31</td>
<td>73.8</td>
<td>28</td>
</tr>
<tr>
<td>Family history of DM</td>
<td>Yes</td>
<td>40</td>
<td>95.2</td>
<td>40</td>
</tr>
<tr>
<td>Complications</td>
<td>Yes</td>
<td>42</td>
<td>100.0</td>
<td>43</td>
</tr>
<tr>
<td>Condition</td>
<td>Count</td>
<td>Percentage</td>
<td>Median</td>
<td>Mean</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-------</td>
<td>------------</td>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>Nephropathy</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>37</td>
<td>88.1%</td>
<td>35</td>
<td>81.4</td>
</tr>
<tr>
<td>Diabetic Cataract</td>
<td>8</td>
<td>19.0%</td>
<td>6</td>
<td>14.0</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>12</td>
<td>28.6%</td>
<td>5</td>
<td>11.6</td>
</tr>
<tr>
<td>Dyslipidaemia</td>
<td>37</td>
<td>88.1%</td>
<td>39</td>
<td>90.7</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>12</td>
<td>28.6%</td>
<td>18</td>
<td>41.9</td>
</tr>
<tr>
<td>Comorbidities</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>10</td>
<td>23.8%</td>
<td>8</td>
<td>18.6</td>
</tr>
</tbody>
</table>

3.4 Discussion

In this chapter the modifiable and non-modifiable risk factors and their effect on diabetes will be discussed.

3.4.1 Age and duration of diabetes

The mean age group across all study groups was 45-54 years. This is unsurprising given that older age is a well-documented risk factor for the development of type 2 diabetes and the peak age of onset of type 2 diabetes is usually later than type 1 (CDC 2014). In the 25-34 age group there were only 1.5 percent (n=2) of participants. Orwoll et al. (1994) has previously suggested that young patients with type 2 diabetes mellitus may confound outcomes due to the perceived lowered insulin secretion in diabetics of shorter duration. Therefore, despite low numbers of type 2 diabetics in Orwoll’s 1994 study, it was important to take into account the duration of diabetes before analysis of the results.

The average duration of diabetes was over eight years in two-thirds of the study population. Studies have shown that an increased duration of diabetes correlates with higher HbA1c levels (Verma et al., 2006). With increased age and duration of diabetes...
the tendency is for the blood sugar level to increase, followed by an increase in the HbA1c and the fasting insulin level (Shera et al., 2004).

3.4.2 Education level
The mean education level of study participants was no schooling or primary school education (55.4 percent, n= 71). This supports what previous studies have found that lower levels of education are related to higher prevalence of type 2 diabetes amongst women (Robbins et al., 2001; Agardh et al., 2011; Sacerdote et al., 2012). Education level is not thought to directly influence the biology of type 2 diabetes; however its effect on the disease is mediated through other risk factors associated with low levels of education (smoking status, diet, exercise, BMI). Conversely, a higher level of education is thought to relate to positive health impacts (Winkleby et al., 1992; Berkman and Macintyre 1997). For example, those who are more educated are likely to be more amenable to health education and to have better access to healthcare, enabling them to exhibit more positive health practices. It is therefore perhaps unsurprising that the majority of the participants in this study had had little education.

3.4.3 Smoking
The incidence of smoking amongst participants in all groups was low (5.5 percent, n=7). This was surprising given that smoking is a well-documented risk factor for the development of type 2 diabetes (Eliasson 2003; Haire-Joshu et al., 2004). A systematic review by Willi et al., found that 24 of the 25 selected studies for their review showed a positive association between smoking and diabetes (Willi et al., 2007). A pooled analysis of these studies gave a relative risk for developing diabetes of 1.44 per cent for smokers compared to non-smokers. However, although there is a wealth of data to show that smoking is associated with diabetes, a causal relationship has yet to be established. Smoking is often associated with other risk factors such as lack of physical activity and poor diet, and so mechanisms for direct causality or indirect association must still be investigated. Similarly, the low prevalence of female smokers in this study may be explained by cultural factors. Studies have found gender disparities amongst young Arab smokers, with 65 percent of adolescent males claiming to be smokers, compared with just 11.1 percent of adolescent females (Abdalla et al., 2007). This is perhaps unsurprising given that Arab males are actively encouraged to spend time with friends and are not supervised as strictly as females. Arab females, however,
may consider smoking to affect their reputation and feminine Islamic image, consequently affecting their marriage prospects (Islam and Johnson 2003).

3.4.4 Obesity

Obesity has been shown to be a robust indicator for the development of type 2 diabetes with one study conducted in a cohort of over 110,000 women finding that for those with a BMI in the range of 23 to 23.9 kg/m² the relative risk for development of type 2 diabetes was 3.6 compared to women with a BMI of 22 kg/m² or lower (Colditz et al., 1990). The mean BMI across all study groups was above 30 kg/m², highlighting that the majority of the study population were not just overweight (21 %, n= 27) but fell into the category of obese (79 %, n= 101) as per the WHO classification for BMI (WHO expert consultation 2004). Previous studies in Saudi Arabia have drawn similar conclusions. Al-Nozha et al. (2005) found 72.5 percent of his study population to be obese or overweight. Prevalence was also higher in females than males, with 44 percent of Saudi females being obese compared to 26.4 percent of males. The reasons for this are plentiful. The Saudi population is experiencing a nutritional transition where traditional food is being substituted for high calorie, high fat, fast-food (Al-Nozha et al., 1991). Alongside this, the lifestyle is predominantly sedentary, especially for females, who spend the majority of their time indoors. Physical activity such as sports is practiced by a very small percent of the population and socialising is instead predominantly around meals that are high in carbohydrates and fats, for example meals composed of rice with whole lamb. The hot climate discourages individuals from participating in physical activity and vehicles are used for even moderate distances of travel (Al-Othaimeen et al., 2007).

3.4.5 Family history

The family history of diabetes was very high in group A (95.2 %) and group B (93.0 %) but relatively low in group C (18.6 %). It has been well-documented that first-degree relatives of diabetic parents have an increased risk of developing type 2 diabetes themselves (Midhet et al., 2010). There is strong evidence for hereditary aspects of diabetes, most notably because concordance rates are higher in monozygotic twins compared to dizygotic twins and sibling recurrence rates are higher than for the general population (O'Rahilly et al., 2005). However, roughly half of the risk of T2DM can be attributed to lifestyle, and half to genetics (Midhet et al., 2010). Whilst family
history is a good indicator of future development of type 2 diabetes, it is unlikely that having a positive or negative family history would affect the results of vitamin D supplementation (Krul-Poel et al., 2015).

4.4.6 Summary
All of the above measured modifiable and non-modifiable risk factors were roughly equal across all study groups. The only exception to this was the prevalence of a strong family history for diabetes amongst the subjects.
Chapter 4. Vitamin D$_3$ Status
4.1 Introduction

The Endocrine Society Clinical Practice Guidelines state that an optimal concentration of serum vitamin D is above 30 ng/ml (Holick et al., 2011). Vitamin D deficiency is defined as serum concentrations below 20 ng/ml, whilst insufficiency is defined as between 21 and 29 ng/ml (Bischoff-Ferrari et al., 2006; Lee et al., 2008; Holick et al., 2011). A minimum daily dose of 400 IU of vitamin D is expected to raise serum concentrations by 2.8 ng/ml in deficient subjects and this dose has been also recommended by Public Health England to be taken daily in autumn and winter (Heaney et al., 2003; Public Health England, 2016). However, the rise in serum vitamin D is related to the baseline concentration, and a severely deficient patient is likely to require an even higher dose to normalise their status (Heaney et al., 2003). Multiple studies have found improvements in markers of diabetes in those involving vitamin D deficient subjects, whose vitamin D status is then normalised through supplementation (Norman et al., 1980; Cade and Norman 1986). Furthermore, it has been shown that where sufficient serum vitamin D concentrations were not achieved, despite similar doses of supplementation, the effect on diabetic markers is far less (Sugden et al., 2008). It is therefore important that this study measured serum vitamin D concentrations before and after supplementation.

It has been proposed that high doses of vitamin D supplementation above 10,000 IU/day may lead to toxic effects due to hypercalcaemia (Yiu al., 2013; Holick et al., 2011). This can present with symptoms of anorexia, nausea, constipation and depression and can be life-threatening (Joshi et al., 2010). It was therefore important in this study to measure baseline and 16-week corrected calcium levels to ensure subject safety throughout the study. Vitamin D supplementation below 10,000 IU/day has not been found to have any adverse effects (Hathcock et al., 2007).

Obesity has long been associated with an increased risk of developing type 2 diabetes (Kahn et al., 2006). The current literature highlights an inverse relationship between vitamin D and obesity (Dalgard et al., 2011; Salekzamani et al., 2011). Serum concentrations of 25(OH) D have been shown to be lower in obese subjects and obese subjects appear to have a lower vitamin D intake than those of a normal weight (Kamycheva et al., 2003; Jorde et al., 2010). One hypothesis is that vitamin D deficiency increases parathyroid hormone (PTH) which in turn increases the cytosolic
calcium level, preventing lipolysis and promoting expression of fatty acid synthase (Ni et al., 1994; Zemel et al., 2000; Xue et al., 2001). However, to date, the research has failed to demonstrate that supplementation with vitamin D in obese subjects aids weight loss (Sneve et al., 2008; Zittermann et al., 2009).

This study aims to test the hypothesis that 16 weeks of vitamin D supplementation would lead to an improvement in serum vitamin D concentrations and BMI, with no adverse effect on the serum corrected calcium concentration.

4.2 Methodology
Blood samples were collected from all 128 participants at baseline and 16 weeks using the aseptic technique described in section 2.12. Vitamin D status was measured using the Roche electrochemiluminescent protein binding assay Cobas E601 as per section 2.15.1. Serum corrected calcium levels were analysed using chemiluminescence as indicated in section 2.15.6. Anthropometric measurements were taken at baseline and 16 weeks, and calculated using the BMI formula as described in section 2.11.

4.3 Results

4.3.1 Vitamin D Status
The changes in the mean serum vitamin D of the participants (post-test minus pre-test) in the 4000 IU group (A), the 2000 IU/day group (B), and the placebo group (C) are compared in Table 4.1. The difference in mean serum vitamin D concentrations between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 4.1.
Table 4.1: Post-hoc comparison of vitamin D status (ng/ml) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10.52 ±7.04</td>
<td>32.92 ±7.81</td>
<td>22.40 ±7.37*</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>B</td>
<td>12.54 ±7.90</td>
<td>28.93 ±7.61</td>
<td>16.39 ±7.95*</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>C</td>
<td>11.55 ±9</td>
<td>10.87 ±7.25</td>
<td>-0.68 ±4.45</td>
<td>0.510</td>
</tr>
</tbody>
</table>

<sup>a</sup>indicates a significant difference between pre-test and post-test as per p < 0.05

<sup>b</sup> indicates adjustment for multiple comparisons

The results of ANCOVA indicated that the mean serum vitamin D varied significantly between the three groups, (F (2, 124) 174.54, p <0.001) with a large effect size (eta squared = 0.738). Mixed ANOVA was used to test whether there was a statistically significant interaction between time (pre-test and post-test) and the three
supplementation groups (A, B and C). The results of mixed ANOVA showed that there was a significant interaction between time and the three groups (F (2, 125) = 133.87, p < 0.001). One-way ANOVA was used to examine any significant difference between A, B and C, for pre-test and post-test. The results of one-way ANOVA indicated that for pre-test the mean serum vitamin D did not vary significantly between the three groups (F (2, 125) = 0.645, p = 0.527). In contrast, for post-test the mean serum vitamin D varied significantly between the three groups (F (2, 125) = 103.23, p < 0.001).

Using post-hoc test for pairwise comparison at post-test, mean serum vitamin D for group A was significantly higher than groups B (mean change = 3.99 ng/ml, p = 0.016), and C (mean change = 22.05, p < 0.001), see Table 4.2. Mean serum vitamin D concentration for group B was significantly higher than C (mean change = 18.05 ng/ml, p < 0.001).

### Table 4.2: Post-hoc test comparisons of vitamin D status (ng/ml) between the three groups for post-test

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean change</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 weeks</td>
<td>A</td>
<td>B</td>
<td>3.996&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>22.050&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>18.054&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup>indicates a significant difference between two groups as per p < 0.05

<sup>b</sup>indicates adjustment for multiple comparisons

Comparing pre- and post-test using post-hoc test, there was a significant difference in serum vitamin D concentration between the pre-test and post-test for group A (mean change = 22.40 ng/ml, p < 0.001), and B (mean change = 16.39 ng/ml, p < 0.001), where vitamin D status increased in the post-test, see Table 4.1. No significant difference was found for group C (p = 0.510).
The frequencies of serum vitamin D concentrations of the three groups of participants (A, B, and C), as classified by The Endocrine Society (Holick et al, 2011), are presented in Figures 4.2 and 4.3.

![Figure 4.2: Vitamin D status of participants at baseline (%)](image1)

![Figure 4.3: Vitamin D status of participants at 16 weeks (%)](image2)

### 4.3.2 Corrected calcium (mg/dl)

The changes in the mean corrected calcium of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 4.3. The difference in mean corrected calcium between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 4.4.

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>9.66 ±0.33</td>
<td>9.77 ±0.35</td>
<td>0.11 ±0.40</td>
<td>0.060</td>
</tr>
<tr>
<td>B</td>
<td>9.52 ±0.37</td>
<td>9.58 ±0.37</td>
<td>0.06 ±0.38</td>
<td>0.302</td>
</tr>
<tr>
<td>C</td>
<td>9.79 ±0.25</td>
<td>9.76 ±0.27</td>
<td>-0.03 ±0.37</td>
<td>0.583</td>
</tr>
</tbody>
</table>

\(^{a}\)indicates a significant difference between pre-test and post-test as per \(p < 0.05\)

\(^{b}\) indicates adjustment for multiple comparisons
The results of ANCOVA indicated that the mean corrected calcium did not vary significantly between the three groups ($F (2, 124) = 2.137, p = 0.122$). In none of the three test groups did corrected calcium exceed the safety margin of 10.5 mg/dl. Mixed ANOVA testing revealed no significant interaction between time and the three groups ($F (2,125) = 1.555, p = 0.215$). Post-hoc test showed no significant difference in corrected calcium between pre-test and post for each group, see Table 4.3.

### 4.3.3 BMI

The changes in the mean BMI of the participants in groups A, B, and C (post-test minus pre-test) are compared in Table 4.4. The difference in mean BMI between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 4.5.
Table 4.4: Post-hoc comparisons of BMI (kg/m²) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>34.79 ±5.17</td>
<td>34.80 ±5.30</td>
<td>0.01 ±1.34</td>
<td>0.970</td>
</tr>
<tr>
<td>B</td>
<td>38.40 ±11.25</td>
<td>38.70 ±11.27</td>
<td>0.30 ±0.95</td>
<td>0.050</td>
</tr>
<tr>
<td>C</td>
<td>34.94 ±6.77</td>
<td>35.06 ±6.99</td>
<td>0.12 ±0.81</td>
<td>0.459</td>
</tr>
</tbody>
</table>

^aindicates a significant difference between pre-test and post-test as per p < 0.05

^b indicates adjustment for multiple comparisons

Figure 4.5: Mean BMI (kg/m²) across the three groups over time (pre-test and post-test)

The results of ANCOVA indicated that the mean BMI did not vary significantly between the three groups (F (2, 124) = 0.823, p = 0.442). Mixed ANOVA testing revealed no significant interaction between time and the three groups (F (2,125) = 83
Post-hoc test showed no significant difference in BMI between pre- and post test for each group, see Table 4.4.

4.4 Discussion

4.4.1 Vitamin D status

This study found a significant improvement in vitamin D status in both of the supplemented groups. The doses used (4000 IU/day and 2000 IU/day) were found to be safe, as the results did not demonstrate an adverse effect on the serum corrected calcium concentration.

The absence of any supporting evidence for a positive relationship between vitamin D and diabetes has often been accredited to suboptimal dosing and short study duration. Studies have found that normal serum concentrations of vitamin D can be achieved using supplementation doses of at least 1332 IU/day for one month to 50,000 IU/month for six months in vitamin D deficient subjects (Borissova et al., 2003; Yiu et al., 2013; Al-Zahrani et al., 2014; Kampmann et al., 2014; Nasri et al., 2014; Al-Sofiani et al., 2015; Krul-Poel et al., 2015; Mohamad et al., 2015; Sadiya et al., 2015). However, when doses of less than 1332 IU/day were used, such as de Boer’s (2008) study of 2291 type 2 diabetic females using 400 IU/day, sufficient serum concentrations of vitamin D were not achieved even with a study duration of seven years. Similarly, a study using 1000 IU vitamin D per day for 12 months was not adequate to raise serum vitamin D concentrations into the sufficient range (Breslavsky et al., 2013). Moreover, certain studies have highlighted how serum concentrations of vitamin D may be more important in improving markers of diabetes than supplementation alone. A systematic review of several studies found no improvement in diabetic outcomes in participants with already sufficient serum vitamin D concentrations (Pittas et al., 2007b). Whilst clear improvements have been demonstrated in the serum concentrations of vitamin D in both the supplemented groups in this study, neither dose was enough to ensure all participants had sufficient concentrations post intervention. Further work investigating the dose-response effect of vitamin D on serum status is warranted.
4.4.2 Corrected calcium
In this study mean corrected calcium levels did not increase significantly in either of
the supplemented groups. None of the participants exceeded the upper limit for normal
safe calcium levels and no patient developed hypercalcaemia. This supports what other
studies have concluded that using a safe dose of vitamin D supplements does not cause
serum corrected calcium levels to increase beyond the normal range (Sugden et al.,
2008; Jorde and Figenschau 2009; Al-Sofiani et al., 2015). Although some studies
found a significant increase in corrected calcium levels, which was attributed to the
role of vitamin D in calcium homeostasis, this never exceeded the normal upper limit
(Raghuramulu et al., 1992; Al-Daghri et al., 2012).

4.4.3 BMI
This study showed no significant change in BMI following 16 weeks of
supplementation with either dose of vitamin D. This supports several other studies
demonstrating vitamin D to have no significant impact on BMI in obese subjects with
type 2 diabetes (Sugden et al., 2008; Jorde and Figenschau 2009; Al-Daghri et al.,
2012; Heshmat et al., 2012; Alkharfy et al., 2013; Breslavsky et al., 2013; Al-Zahrani
et al., 2014; Kampmann et al., 2014; Ryu et al., 2014; Tabesh et al., 2014; Al-Sofiani
et al., 2015).

4.4.4 Summary
In conclusion, this study demonstrates a significant improvement in serum vitamin D
concentrations (p < 0.001) in both the 4000 IU/day and 2000 IU/day groups. The level
of corrected calcium remained within normal parameters in all subjects and no side
effects of vitamin D supplementation were demonstrated in any of the trial
participants. Vitamin D supplementation of either 2000 IU/day or 4000 IU/day did not
have an effect on BMI.
Chapter 5. Vitamin D₃ and Markers of Glycaemia
5.1 Introduction
HbA1c is the principle measure of glycaemia. One study reported that a fall in HbA1c of just one percent was associated with a 21 per cent decrease in diabetic complications and a 37 percent decrease in microvascular disease (Stratton et al., 2000). Given that Asian populations develop type 2 diabetes at an earlier age than their Caucasian counterparts (Barnett et al., 2006), controlling glycaemia through the monitoring of HbA1c is even more pertinent.

It has been established that high incidence of diabetes in the Middle East is strongly related to low vitamin D status (Zhang 2016). Several cross-sectional studies exist which demonstrate a relationship between low serum vitamin D concentrations and higher fasting blood glucose levels and insulin resistance (Saedisomeolia et al., 2013). Recent interventional studies exploring the effect of vitamin D supplementation on markers of glycaemia have, however, yielded inconsistent results and a definitive relationship between vitamin D and glucose metabolism is yet to be confidently established (Alvarez and Ashraf 2010). It has been proposed that vitamin D may affect glycaemia by binding to the vitamin D receptor (VDR) on pancreatic beta cells, resulting in increased transcription of the insulin gene and increased peripheral glucose uptake (Krul-Poel et al., 2014). Vitamin D may also facilitate glucose transport across target cells indirectly through the regulation of extra- and intra-cellular calcium (Krul-Poel et al., 2014).

This study aims to test the hypothesis that vitamin D supplementation improves insulin resistance and beta cell function, as reflected by a reduction in glycated haemoglobin and fasting blood glucose levels.

5.2 Methodology
Blood samples were taken from all 128 participants at baseline and 16 weeks using the aseptic technique described in section 2.12. Fasting insulin was measured using the Roche electrochemiluminescent immunoassay Cobas e601 as described in section 2.15.2. Fasting glucose levels were determined using the Roche enzymatic reference method described in section 2.15.5. Beta cell function and insulin resistance were calculated according to the Homeostasis Assessment Model as described in section
2.16. Glycated haemoglobin levels were determined using the Roche turbidimetric inhibition immunoassay as per section 2.15.3.

5.3 Results

5.3.1 Fasting blood glucose
The changes in the mean fasting blood glucose of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 5.1. The difference in mean fasting blood glucose between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 5.1.

Table 5.1: Post-hoc test comparisons of fasting blood glucose (mg/dl) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>215.21 ±70.67</td>
<td>175.14 ±73.31</td>
<td>-40.07 ±63.48*</td>
<td>0.001</td>
</tr>
<tr>
<td>B</td>
<td>192.13 ±76.80</td>
<td>183.16 ±73.55</td>
<td>-8.97 ±72.54</td>
<td>0.435</td>
</tr>
<tr>
<td>C</td>
<td>181.65 ±63.42</td>
<td>180.65 ±72.02</td>
<td>-1.00 ±87.09</td>
<td>0.931</td>
</tr>
</tbody>
</table>

*indicates a significant difference between pre-test and post-test as per p < 0.05

b indicates adjustment for multiple comparisons
The results of ANCOVA indicated that the mean fasting blood glucose did not vary significantly between the three groups ($F(2, 124) = 1.291, p = 0.279$). Mixed ANOVA testing revealed a significant interaction between time and the three groups ($F(2, 125) = 13.87, p = 0.044$). The results of one-way ANOVA for pre-test and post test indicated that the mean fasting blood glucose did not vary significantly between the three groups. Post-hoc test showed only a significant difference in fasting blood glucose between pre and post test for group A (mean change = -40.07 p-value < 0.001), see Table 5.1.

5.3.2 Fasting blood insulin

The changes in the mean insulin of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 5.2. The difference in mean fasting blood insulin between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 5.2.
Table 5.2: Post-hoc test comparisons of fasting insulin (μU/mL) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>15.34 ±10.40</td>
<td>17.80 ±12.64</td>
<td>2.46 ±10.05</td>
<td>0.213</td>
</tr>
<tr>
<td>B</td>
<td>16.03 ±10.79</td>
<td>15.91 ±10.05</td>
<td>-0.11 ±12.20</td>
<td>0.954</td>
</tr>
<tr>
<td>C</td>
<td>20.56 ±20.81</td>
<td>18.16 ±20.55</td>
<td>-2.40 ±15.28</td>
<td>0.218</td>
</tr>
</tbody>
</table>

<sup>a</sup>indicates a significant difference between pre-test and post-test as per p < 0.05

<sup>b</sup> indicates adjustment for multiple comparisons

Figure 5.2: Mean fasting insulin (μU/mL) across the three groups over time (pre-test and post-test)

The results of ANCOVA indicated that the mean fasting insulin did not vary significantly between the three groups, (F (2, 124) 0.776, p = 0.463). Mixed ANOVA testing revealed no significant interaction between time and the three groups (F (2,125)
Post-hoc test showed no significant difference in fasting blood insulin between pre and post test for each group, see Table 5.2.

5.3.3 Insulin resistance (HOMA-IR)
The changes in the mean HOMA-IR of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 5.3. The difference in mean HOMA-IR between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 5.3.

Table 5.3: Post-hoc test comparisons of HOMA-IR (%) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>8.69 ±8.43</td>
<td>8.05 ±7.50</td>
<td>-0.64 ±6.27</td>
<td>0.576</td>
</tr>
<tr>
<td>B</td>
<td>7.62 ±5.27</td>
<td>7.15 ±5.20</td>
<td>-0.47 ±6.44</td>
<td>0.672</td>
</tr>
<tr>
<td>C</td>
<td>11.32 ±20.13</td>
<td>9.08 ±14.46</td>
<td>-2.24 ±9.00</td>
<td>0.050</td>
</tr>
</tbody>
</table>

<sup>a</sup>indicates a significant difference between pre-test and post-test as per p < 0.05

<sup>b</sup> indicates adjustment for multiple comparisons

Figure 5.3: Mean HOMA-IR (%) across the three groups over time (pre-test and post-test)
The results of ANCOVA indicated that the mean HOMA-IR did not vary significantly between the three groups (F (2, 124) = 0.136, p = 0.873). Mixed ANOVA testing revealed no significant interaction between time and the three groups (F (2,125) = 0.753, p = 0.473). Post-hoc test showed no significant difference in HOMA-IR between pre and post test for each group, see Table 5.3.

5.3.4 Beta cell function (HOMA-B)

The changes in the mean HOMA-B of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 5.4. The difference in mean HOMA-B between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 5.4.

Table 5.4: Post-hoc test comparisons of HOMA-B (%) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td></td>
<td>Mean ±SD</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>42.45 ±26.53</td>
<td>82.57 ±76.49</td>
<td>40.12 ±73.92*</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>B</td>
<td>55.11 ±39.57</td>
<td>71.42 ±65.77</td>
<td>16.30 ±60.81</td>
<td>0.131</td>
</tr>
<tr>
<td>C</td>
<td>65.00 ±73.40</td>
<td>72.95 ±73.55</td>
<td>7.95 ±75.27</td>
<td>0.460</td>
</tr>
</tbody>
</table>

*indicates a significant difference between pre-test and post-test as per p < 0.05

^b indicates adjustment for multiple comparisons
The results of ANCOVA indicated that the mean HOMA-B did not vary significantly between the three groups (F (2, 124) = 1.271, p = 0.284). Mixed ANOVA testing revealed no significant interaction between time and the three groups (F (2,125) = 2.390, p = 0.096). Post-hoc test showed only a significant difference in HOMA-B between pre and post test for group A (mean change = 40.12%, p < 0.001), see Table 5.4.

5.3.5 Comparing the changes in HOMA-IR and HOMA-B with final vitamin D concentration

To investigate the relationship between HOMA-IR and HOMA-B with changes in the serum vitamin D concentration, final serum vitamin D concentration was subdivided into three groups: < 30 ng/ml, 30-40 ng/ml, and > 40 ng/ml. The results are shown in Table 5.5. The baseline vitamin D concentration for the majority of participants across all three sample groups was less than 30 ng/ml. After 16 weeks, HOMA-IR for participants in group A decreased once a serum vitamin D concentration of more than 30 ng/ml was achieved. Similar changes were noted for participants in group B, however the change in HOMA-IR was more pronounced once serum vitamin D concentrations were greater than 40 ng/ml.
Table 5.5: Changes in HOMA-IR at different serum vitamin D concentrations across the three groups (n = group size)

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Vitamin D concentration (ng/ml)</th>
<th>HOMA-IR Baseline</th>
<th>HOMA-IR After 16 week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n Mean ±SD</td>
<td>n Mean ±SD</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>&lt; 30</td>
<td>42 8.68 ±8.43</td>
<td>16 9.71 ±7.93</td>
</tr>
<tr>
<td></td>
<td>30 - 40</td>
<td>-</td>
<td>16 6.54 ±5.25</td>
</tr>
<tr>
<td></td>
<td>&gt; 40</td>
<td>-</td>
<td>10 7.81 ±9.84</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>42 8.68 ±8.43</td>
<td>42 8.05 ±7.50</td>
</tr>
<tr>
<td>B</td>
<td>&lt; 30</td>
<td>41 7.83 ±5.31</td>
<td>23 8.27 ±5.82</td>
</tr>
<tr>
<td></td>
<td>30 - 40</td>
<td>1 2.74</td>
<td>16 6.76 ±4.19</td>
</tr>
<tr>
<td></td>
<td>&gt; 40</td>
<td>1 3.94</td>
<td>4 2.23 ±0.39</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>43 7.62 ±5.27</td>
<td>43 7.14 ±5.20</td>
</tr>
<tr>
<td>C</td>
<td>&lt; 30</td>
<td>40 11.69 ±20.81</td>
<td>41.00 8.81 ±14.47</td>
</tr>
<tr>
<td></td>
<td>30 - 40</td>
<td>2 7.84 ±5.87</td>
<td>2.00 14.80 ±18.30</td>
</tr>
<tr>
<td></td>
<td>&gt; 40</td>
<td>1 3.20</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>43 11.32 ±20.13</td>
<td>43.00 9.08 ±14.46</td>
</tr>
</tbody>
</table>

Similarly, HOMA-B appeared to increase in participants in group A when serum vitamin D concentrations reached above 40 ng/ml as shown in Table 5.6. However, for participants in group B, HOMA-B appear to improve at serum vitamin D concentrations of between 30 and 40 ng/ml and less so at the higher concentration of greater than 40 ng/ml.
Table 5.6: Changes in HOMA-B at different serum vitamin D concentrations across the three groups (n = group size)

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Vitamin D concentration (ng/ml)</th>
<th>HOMA-B Baseline</th>
<th>HOMA-B After 16 week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ±SD</td>
<td>n</td>
</tr>
<tr>
<td>A</td>
<td>&lt; 30</td>
<td>42</td>
<td>42.45 ±26.54</td>
</tr>
<tr>
<td></td>
<td>30 - 40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>&gt; 40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>42</td>
<td>42.45 ±26.54</td>
</tr>
<tr>
<td>B</td>
<td>&lt; 30</td>
<td>41</td>
<td>56.85 ±39.57</td>
</tr>
<tr>
<td></td>
<td>30 - 40</td>
<td>1</td>
<td>34.92</td>
</tr>
<tr>
<td></td>
<td>&gt; 40</td>
<td>1</td>
<td>4.19</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>43</td>
<td>55.12 ±39.57</td>
</tr>
<tr>
<td>C</td>
<td>&lt; 30</td>
<td>40</td>
<td>67.46 ±75.44</td>
</tr>
<tr>
<td></td>
<td>30 - 40</td>
<td>2</td>
<td>34.37 ±29.75</td>
</tr>
<tr>
<td></td>
<td>&gt; 40</td>
<td>1</td>
<td>27.74</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>43</td>
<td>65.00 ±73.41</td>
</tr>
</tbody>
</table>

5.3.6 Glycated haemoglobin (HbA1c)

The changes in the mean HbA1c of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 5.7. The difference in mean HbA1c between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 5.5.
Table 5.7: Post-hoc test comparisons of HbA1c (%) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>9.91 ±1.19</td>
<td>9.50 ±1.72</td>
<td>-0.41 ±1.08&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.027</td>
</tr>
<tr>
<td>B</td>
<td>9.57 ±1.15</td>
<td>9.27 ±1.44</td>
<td>-0.30 ±1.29</td>
<td>0.106</td>
</tr>
<tr>
<td>C</td>
<td>9.70 ±1.29</td>
<td>10.19 ±1.39</td>
<td>0.48 ±1.18&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.009</td>
</tr>
</tbody>
</table>

<sup>*</sup>indicates a significant difference between pre-test and post-test as per p < 0.05

<sup>b</sup> indicates adjustment for multiple comparisons

Figure 5.5: Mean HbA1c (%) across the three groups over time (pre-test and post-test)

The results of ANCOVA indicated that the mean HbA1c varied significantly between the three groups, (F (2, 124) = 7.135, p = 0.001) although the effect size was relatively
low (eta squared = 0.103). The results from mixed ANOVA showed that there was a significant interaction between time and the three groups (F (2,125) = 125, p = 0.001). The results of one-way ANOVA indicated that the mean HbA1c for pre-test did not vary significantly between the three groups, (F (2, 125) = 0.905, p = 0.407). In contrast, for post-test, the mean HbA1c varied significantly between the three groups (F (2, 125) = 4.169, p = 0.018).

Post-hoc test for pairwise comparison at post-test indicated that the mean HbA1c for group A was significantly less than group C (mean change = -0.679 ng/ml, p = 0.042), see Table 5.8. In addition, group B was significantly less than group C (mean change = -0.914 ng/ml, p = 0.006) see Table 5.8.

Table 5.8: Post-hoc test comparisons of HbA1 (%) between the three groups for post-test

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean change</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-test</td>
<td>A</td>
<td>B</td>
<td>0.235</td>
<td>0.478</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>-0.679*</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>-0.914*</td>
<td>0.006</td>
</tr>
</tbody>
</table>

<sup>*indicates a significant difference between two groups as per p < 0.05</sup>

<sup>b</sup> indicates adjustment for multiple comparisons

Comparing pre- and post-test using post-hoc test showed a significant difference in HbA1c for group A (mean change = -0.41 %, p = 0.027), where HbA1c decreased at post-test, see Table 5.7. There was also a significant difference for group C (mean change = 0.48 %, p = 0.009), where HbA1c increased at post-test, see Table 5.7.

5.4 Discussion

5.4.1 Fasting blood glucose level
This study has found no significant change in fasting blood glucose level as a result of vitamin D supplementation of either 4000 IU or 2000 IU per day. These findings support previous studies that have found no relationship between vitamin D supplementation and improved fasting blood glucose levels (Patel et al., 2010; Al-Zahrani 2013; Kampmann et al., 2014; Nasri et al., 2014; Ryu et al., 2014; Tabesh et al., 2014; Sadiya et al., 2015). Orwoll et al. (1994) recruited 20 type 2 diabetic subjects
in a double-blind, placebo-controlled trial with 200 IU vitamin D supplemented daily for four days. The study concluded that vitamin D supplementation had no effect on fasting blood glucose levels in type 2 diabetics, supporting the results of this study. A similar randomised, placebo-controlled study of 47 type 2 diabetics given a daily dose of 1000 IU vitamin D for a longer period of 12 months, still showed no improvement in fasting blood glucose levels (Breslavsky et al., 2013). Even at higher doses and where sufficient serum concentrations have been achieved, vitamin D supplementation has not been found to improve fasting blood glucose levels. Jorde and Figenschau (2009) conducted a randomised control trial on 36 subjects with type 2 diabetes, supplemented with 40,000 IU per week of vitamin D. At six months, the fasting blood glucose levels were not significantly different from the baseline values. Furthermore, there was no difference in fasting blood glucose levels between the vitamin D and the placebo group. This is consistent with the findings from a larger 2012 study involving 100 type 2 diabetics randomised to either 5000 IU per day of vitamin D or a placebo for 12 weeks (Yiu et al., 2013). The study concluded that vitamin D supplementation had no effect on fasting blood glucose levels even at a higher dose. The results of this study suggest that vitamin D supplementation has no effect on fasting blood glucose levels.

A 2014 systematic review of 25 randomised control trials evaluating the effect of vitamin D supplementation on subjects without diabetes, with pre-diabetes and with type 2 diabetes drew somewhat different conclusions (Seida et al., 2014). Whilst the participants with type 2 diabetes showed no improvement in fasting blood glucose levels following supplementation, those with pre-diabetes had a significantly lower increase in fasting blood glucose when compared to those randomised to a placebo. A similar observation was made by Pittas et al. (2007a) in a randomised control study of 314 subjects given vitamin D for the treatment of osteoporosis (Pittas et al., 2007a). Of those studied, 92 exhibited an impaired fasting blood glucose level. After three years, those subjects allocated vitamin D supplements had a significantly lower increase in their fasting blood glucose in comparison to those randomised to placebo (0.4 mg/dl compared to 6.12 mg/dl). Similarly, Boucher et al. (1995) highlighted improvements in glucose tolerance after vitamin D supplementation in vitamin D deficient subjects with impaired glucose tolerance.
This study appears to support the existing evidence that vitamin D supplementation has no effect on fasting blood glucose levels in those with type 2 diabetes. Interestingly, a review of the literature does, however, suggest a potential role for vitamin D in stabilising fasting blood glucose levels in those with pre-diabetes.

5.4.2 Fasting insulin level, insulin resistance (HOMA-IR) and beta cell function (HOMA-B)

This study found no change in the fasting insulin levels amongst subjects in any of the three groups. Previous studies have similarly found no significant improvement in insulin level following vitamin D supplementation (Raghuramulu et al., 1992; De Boer et al., 2008; Jorde and Figenschau 2009; Harris et al., 2012; Heshmat et al., 2012; Kampmann et al., 2014; Al-Sofiani et al., 2015). Conversely, a randomised control trial by von Hurst, (2010) concluded that there was a significant decrease in fasting insulin level amongst 81 pre-diabetic subjects, following supplementation of 4000 IU/day for six months. Al-Daghri, (2012), however, found a significant increase in fasting insulin level, as well as an improvement in both insulin resistance and insulin secretion. Changes in fasting insulin levels can best be expressed as a measure of insulin resistance (HOMA-IR) and insulin secretion i.e. beta cell function (HOMA-B) (Laakso 1993).

This study found no significant decrease in insulin resistance (HOMA-IR) at supplementation doses of either 4000 IU/day or 2000 IU/day vitamin D compared to placebo. These findings support those of a large 2008 randomised control trial of 2291 type 2 diabetic females, supplemented with 400 IU per day vitamin D followed up for seven years (De Boer et al., 2008). A randomised control trial of shorter duration (four months) but with an increased supplementation of 100,000 IU and 200,000 IU vitamin D as a single stat dose in 61 type 2 diabetics concluded that there was still no improvement in HOMA-IR (Witham et al., 2010). These findings have been supported by several smaller studies using various supplement dosages and durations that also demonstrated vitamin D to have no effect on insulin resistance (Bonakdaran and Afkhami Zadeh 2011; Kampmann et al., 2014; Ryu et al., 2014; Yousefi Rad et al., 2014; Al-Sofiani et al., 2015). Pittas (2007a) demonstrated that whilst insulin resistance and fasting blood glucose remained unchanged after three years of supplementation with 700 IU vitamin D per day, in the placebo group both markers had significantly increased. This could suggest that whilst vitamin D may not actually
improve insulin resistance and fasting blood glucose, it may slow the natural progression of diabetes.

Other studies conducted in the Middle East have found vitamin D supplementation to have a significant effect on insulin resistance (Al-Daghri et al., 2012; Talaei et al., 2013; Al-Shahwan et al., 2015). A 2015 randomised control trial of 45 Saudi type 2 diabetics demonstrated a significant improvement in insulin resistance after one year of supplementation of 2000 IU vitamin D per day (Al-Shahwan et al., 2015). Von Hurst et al. (2010) not only highlighted how insulin resistance had significantly improved after six months of supplementation at 4000 IU/day, but that the improvement was most marked when serum 25(OH)D concentrations reached above 32 ng/ml. This suggests that achieving a certain serum vitamin D concentration may be of greater importance than the dose of supplement given. Similarly, Talaei (2013) reported improvements in insulin resistance only when serum vitamin D concentrations reached 40-60 ng/ml and found no effect when lower serum vitamin D concentrations were achieved. This corroborates what the present study found with regards to HOMA-IR and vitamin D concentration. Whilst these changes were descriptive and not statistically significant, the findings do suggest that the serum vitamin D concentration is more important than the dose of supplementation given. This suggests there is scope for further research on the role of vitamin D in insulin resistance when higher serum vitamin D concentrations have been achieved.

This study demonstrates an improvement in pancreatic beta cell function (HOMA-B) as a result of vitamin D supplementation, however this was not found to be significant. A growing body of evidence suggests that vitamin D plays a role in improving beta cell function (Borissova et al., 2003; Harris et al., 2012; Al-Sofiani et al., 2015), however this study did not support these findings. An 18 month prospective interventional study of 34 males and 58 females with type 2 diabetes in Saudi Arabia, supplemented with 2000 IU/day vitamin D, further showed that this improvement was more marked in females than in males (Al-Daghri et al., 2012). Harris et al. (2012) in a randomized control trial of 89 pre-diabetics and type 2 diabetics supplemented with 4000 IU/day vitamin D saw a significant improvement in beta cell function after 12 weeks.
Other studies, however, have found vitamin D to play no role in improving pancreatic beta cell function (Orwoll et al., 1994; Jorde and Figenschau 2009; von Hurst et al., 2010). A cross-sectional analyses of the National Health & Nutrition Examination Survey 1989-1994 (NHANES III) found serum 25(OH)D concentrations to be inversely related to diabetes risk and insulin resistance. However, no relationship was found between serum 25(OH)D concentrations and beta-cell function (Scragg et al., 2004). This discrepancy in the evidence may be related to insufficient doses of supplementation used, the duration of supplementation given and the validity of the study. Orwoll et al. (1994) found no relationship between vitamin D and improved beta cell secretion, however as previously mentioned the very small sample size used (n=20), small supplement dosage (400 IU/day) and short duration of trial (four days) made drawing any valid conclusions difficult. Sufficient serum vitamin D concentrations were also not achieved following intervention. Similarly, it is hard to draw reliable conclusions from the cross-sectional analyses of the NHANES III as it was an observational rather than an interventional study and therefore it is difficult to demonstrate causality. In contrast to this, Jorde and Figenschau (2009) and von Hurst et al. (2010) both used supplementation doses of 40,000 IU/ week and 4000 IU/day respectively and both for a duration of six months, yet still found no relationship between vitamin D and beta cell function.

In conclusion, this study has demonstrated no significant improvements in either beta cell function or insulin resistance following four months of supplementation with vitamin D. This is likely due to not achieving a serum vitamin D concentration above 40 ng/ml as previous studies have demonstrated. Furthermore, vitamin D has been shown to delay the progression of insulin resistance and beta cell dysfunction in those with impaired fasting glucose. As a result it may be the case that whilst vitamin D does not necessarily improve these two markers, it has a role in delaying the progression of diabetic complications.

5.4.3 Glycated haemoglobin
This study found a significant improvement in glycated haemoglobin in those supplemented with vitamin D. After 16 weeks of vitamin D supplementation, 62% of patients had a lower HbA1c level compared to baseline. Of those patients that received 4000 IU vitamin D per day this figure was even higher at 72%, compared to 51% of those that received the lower dose of 2000 IU per day. In comparison, 67% of those
patients given the placebo had an increase in their HbA1c from baseline after 16 weeks. These findings are consistent with several cross-sectional analyses also finding vitamin D to be inversely associated with HbA1c (Schwalfenberg 2008; Sabherwal et al., 2010; Dalgard et al., 2011; Zoppini et al., 2013; Sebekova et al., 2015). A 2012 cross-sectional analysis of 158 vitamin D deficient, type 2 diabetics demonstrated that as serum vitamin D concentrations improved, so too did glycated haemoglobin (Dalgard et al., 2011). Similarly, a 2013 cross-sectional study of 715 type 2 diabetics with known serum 25(OH)D concentrations concluded that serum vitamin D concentration was inversely associated with HbA1c (Zoppini et al., 2013). Furthermore, Sabherwal et al. (2010) found the greater the increase in serum vitamin D concentrations following supplementation, the greater the decrease in HbA1c in a retrospective study of 52 South Asians with both type 2 diabetes and vitamin D deficiency.

Discrepancies continue to exist within the current literature as to whether vitamin D supplementation improves HbA1c levels. Several interventional studies from the Middle East and North Africa have demonstrated a causal link between improved serum vitamin D concentrations and a decrease in HbA1c levels (Labban 2014; Nasri et al., 2014; Yousefi Rad et al., 2014; Mohamad et al., 2015). A 2014 randomised control trial of 722 Moroccan type 2 diabetics supplemented with 2000 IU/day vitamin D demonstrated a 21.0% improvement in HbA1c levels after three months (Labban 2014). Similarly, Nasri (2014) demonstrated a significant improvement in the HbA1c levels of 60 Iranian type 2 diabetic participants following supplementation with 50,000 IU vitamin D per week for three months. A 2008 case report of two female type 2 diabetics receiving 3000 IU/day and 2000 IU/day vitamin D respectively, found a substantial decrease in HbA1c level of 13.5 percent and 9.0 percent respectively after nine months (Schwalfenberg 2008).

Conversely, several large-scale interventional studies have demonstrated no causal relationship between vitamin D and HbA1c (Sugden et al., 2008; Jorde and Figenschau 2009; Patel et al., 2010; Witham et al., 2010; Heshmat et al., 2012; Al-Zahrani et al., 2014; Kampmann et al., 2014; Ryu et al., 2014; Al-Sofiani et al., 2015; Krul-Poel et al., 2015). A 2012 randomised double-blind control trial on 42 Iranian type 2 diabetic patients supplemented with a single intramuscular injection of 300,000 IU vitamin D found there to have been no change in HbA1c levels after three months (Heshmat et
The researcher concluded that in this case the sample size was likely too small to see significant improvements. Similarly, Sugden et al. (2008), Jorde and Figenschau (2009), Patel et al. (2010), Kampmann et al. (2014) and Al-Sofiani et al. (2015) all used sample sizes likely too small to demonstrate a significant effect of vitamin D therapy on glucose metabolism. Furthermore, a 2010 pilot prospective randomised trial of 24 type 2 diabetics with serum concentrations of vitamin D below 25 ng/ml, were supplemented with either 400 IU/day or 1200 IU/day of vitamin D, well below the 2000 IU required to achieve significant results (Patel et al., 2010). Therefore, this discrepancy in the evidence may be related to the different doses of supplementation used, the sample size and the ethnic background of the study population.

This study appears to support the existing evidence that vitamin D supplementation has a significant effect on glycated haemoglobin in participants from the Middle East and North Africa with type 2 diabetes. Interestingly, improvements were seen when vitamin D supplements of 2000 IU/day or higher were used and sample sizes ranged from between 58 to 722 participants.

5.4.4 Summary

In conclusion, this study demonstrates no significant relationship between vitamin D supplementation of either 2000 IU/day or 4000 IU/day on fasting insulin and glucose levels, insulin resistance and beta cell function. However, a significant improvement in glycated haemoglobin (p = 0.001) was demonstrated in both groups supplemented with vitamin D, with the higher dose proving to be the more effective of the two. This suggests vitamin D may play a positive role in diabetic control and prevention of diabetic complications, particularly in Middle Eastern countries. Further human studies at higher doses of vitamin D supplementation, achieving serum concentrations of 40-60 ng/ml, will be useful in cementing the role of vitamin D in the long-term management of type 2 diabetes.
Chapter 6. Vitamin D₃ and Lipidaemia
6.1 Introduction
A review of the literature indicates that the relationship between vitamin D and dyslipidaemia remains controversial. Observational studies have, however, shown low serum concentrations of vitamin D are associated with unfavourable lipid profiles and it has been suggested that vitamin D may affect serum lipid levels in a number of ways (Kendrick et al., 2009; Wang et al., 2012). Vitamin D increases absorption of intestinal calcium, which in turn affects the amount of fat absorbed in the gut. The calcium binds to fatty acids and bile acids, forming calcium-fatty acid soaps, which are excreted in the faeces (Boon et al., 2007). This decreases the absorption of fat from the gut, reducing serum total and LDL-cholesterol, as well as triglyceride concentrations (Saunders et al., 1988; Grundy and Denke 1990; Van der meer et al., 1990; Vaskonen et al., 2002; Vaskonen 2003; Cho et al., 2005; Zittermann et al., 2009). In addition, the action of vitamin D on serum parathyroid hormone (PTH) concentrations may inhibit dyslipidaemia (Jorde and Grimnes 2011). Raised serum PTH levels are associated with an increased risk of obesity and have been shown to reduce lipolysis in vitro (Kamycheva et al., 2004a; Kamycheva et al., 2004b; Jorde et al., 2005; Hagstrom et al., 2009). It has therefore been proposed that vitamin D may lower serum lipid concentrations through the suppression of PTH secretion from the parathyroid glands (Zemel et al., 2000).

Apolipoprotein A1 has been shown to persistently correlate with vitamin D concentrations (Satin 2000; Gagnon et al., 2012; Salum et al., 2012; Vacek et al., 2012). Elevations in serum apolipoprotein A1 concentrations lead to a direct increase in HDL particle formation and increase reverse cholesterol transport (Vacek et al., 2012). To date, the effects of vitamin D on apolipoprotein gene expression and cholesterol levels remains controversial and further research is required.

The aim of this study was to test the hypothesis that vitamin D supplementation improved markers of dyslipidaemia in poorly controlled type 2 diabetic females within the Saudi Arabian population.

6.2 Methodology
Blood samples were taken from all 128 participants to determine the lipid profile, including high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides
(TG) and total cholesterol (TC) using the Roche enzymatic colorimetric assay system as described in section 2.15.4.

6.3 Results

6.3.1 High density lipoprotein (HDL)

The changes in the mean HDL of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 6.1. The difference in mean HDL between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 6.1.

Table 6.1: Post-hoc test comparisons of HDL cholesterol (mg/dl) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>48.31 ±12.04</td>
<td>48.12 ±12.49</td>
<td>0.22 ±8.67</td>
<td>0.850</td>
</tr>
<tr>
<td>B</td>
<td>49.21 ±9.64</td>
<td>47.70 ±10.94</td>
<td>-1.51 ±6.61</td>
<td>0.184</td>
</tr>
<tr>
<td>C</td>
<td>46.74 ±9.66</td>
<td>46.18 ±10.46</td>
<td>-0.56 ±6.88</td>
<td>0.623</td>
</tr>
</tbody>
</table>

*indicates a significant difference between pre-test and post-test as per p < 0.05

b indicates adjustment for multiple comparisons

Figure 6.1: Mean HDL cholesterol (mg/dl) across the three groups over time (pre-test and post-test)
The results of ANCOVA indicated, however, that the HDL did not vary significantly between the three groups (F (2, 123) = 0.468, p = 0.628). Mixed ANOVA testing revealed no significant interaction between time and the three groups (F (2,125) = 573, p = 0.565). Post-hoc test showed no significant difference in HDL between pre and post test for each group, see Table 6.1.

6.3.2 Low density lipoprotein (LDL)

The changes in the mean LDL of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 6.2. The difference in mean LDL between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 6.2.

Table 6.2: Post-hoc comparisons of LDL cholesterol (mg/dl) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>105.45 ±36.25</td>
<td>103.71 ±39.84</td>
<td>-1.74 ±37.16</td>
<td>0.949</td>
</tr>
<tr>
<td>B</td>
<td>105.91 ±34.21</td>
<td>100.44 ±25.41</td>
<td>-5.47 ±32.06</td>
<td>0.282</td>
</tr>
<tr>
<td>C</td>
<td>102.79 ±32.53</td>
<td>115.30 ±36.37</td>
<td>12.51 ±30.18*</td>
<td>0.015</td>
</tr>
</tbody>
</table>

*indicates a significant difference between pre-test and post-test as per p < 0.05

b indicates adjustment for multiple comparisons
Figure 6.2: Mean LDL cholesterol (mg/dl) across the three groups over time (pre-test and post-test)

The results of ANCOVA indicated that the mean LDL varied significantly between the three groups (F (2, 123) = 3.684, p = 0.028) although the effect size was relatively low (eta squared = 0.057). The results of mixed ANOVA showed that there was a significant interaction between time and the three groups (F (2,125) = 3.342, p = 0.039). The results of one-way ANOVA indicated that at both pre and post test the mean LDL did not vary significantly between the three groups.

Using post-hoc test for pairwise comparison at post-test, mean LDL cholesterol in group B was significantly lower than group C (mean change = -14.86, p = 0.043), see Table 6.3. Comparing pre and post test using post-hoc test, the only significant difference in LDL was for group C (mean change = 12.51, p = 0.015), see Table 6.2.
Table 6.3: Post-hoc test comparisons of LDL cholesterol (mg/dl) between the three groups for post-test

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean change</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-test</td>
<td>A</td>
<td>B</td>
<td>4.86</td>
<td>0.526</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>-10.18</td>
<td>0.169</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>-14.86*</td>
<td>0.043</td>
</tr>
</tbody>
</table>

<sup>a</sup>indicates a significant difference between two groups as per p < 0.05
<sup>b</sup>indicates adjustment for multiple comparisons

6.3.3 Triglycerides

The changes in the mean triglycerides of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 6.4. The difference in mean triglycerides between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 6.3.

Table 6.4: Post-hoc comparisons of triglycerides levels (mg/dl) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>146.52 ±85.29</td>
<td>155.97 ±96.19</td>
<td>9.46 ±69.36</td>
<td>0.350</td>
</tr>
<tr>
<td>B</td>
<td>126.77 ±80.34</td>
<td>123.09 ±53.88</td>
<td>-3.67 ±54.42</td>
<td>0.713</td>
</tr>
<tr>
<td>C</td>
<td>142.91 ±64.54</td>
<td>151.22 ±96.28</td>
<td>8.32 ±70.96</td>
<td>0.405</td>
</tr>
</tbody>
</table>

<sup>a</sup>indicates a significant difference between pre-test and post-test as per p < 0.05
<sup>b</sup>indicates adjustment for multiple comparisons
The results of ANCOVA indicated, however, that the mean triglycerides did not vary significantly between the three groups (F (2, 124) 1.068, p = 0.347). Mixed ANOVA testing revealed no significant interaction between time and the three groups (F (2, 124) = 0.531, p = 0.589). Post-hoc test showed no significant difference in triglycerides between pre and post test for each group, see Table 6.4.

6.3.4 Total cholesterol
The changes in the mean total cholesterol of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 6.5. The difference in mean total cholesterol between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 6.4.
### Table 6.5: Post-hoc comparisons of total cholesterol levels (mg/dl) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>177.91 ±39.39</td>
<td>170.52 ±44.94</td>
<td>-7.38 ±38.50</td>
<td>0.209</td>
</tr>
<tr>
<td>B</td>
<td>176.00 ±41.47</td>
<td>160.84 ±31.50</td>
<td>-15.16 ±40.44*</td>
<td>0.010</td>
</tr>
<tr>
<td>C</td>
<td>176.70 ±34.82</td>
<td>178.07 ±41.44</td>
<td>1.37 ±34.56</td>
<td>0.813</td>
</tr>
</tbody>
</table>

<sup>a</sup>indicates a significant difference between pre-test and post-test as per p < 0.05

<sup>b</sup> indicates adjustment for multiple comparisons

![Graph showing mean total cholesterol levels across three groups over time](image)

**Figure 6.4: Mean total cholesterol levels (mg/dl) across the three groups over time (pre-test and post-test)**

The results of ANCOVA indicated that the mean total cholesterol varied significantly between the three groups, (F (2, 124) = 2.685, p = 0.049) although the effect size was low (eta squared = 0.042). The results of mixed ANOVA showed that there was a significant interaction between time and the three groups (F (2,125) = 4.43, p = 0.037).
The results of one-way ANOVA indicated that for pre and post test the mean total cholesterol did not vary significantly between the three groups.

Using post-hoc test for pairwise comparison at post-test, there was a significant difference in total cholesterol levels between groups B and C (mean change = -14.86, p = 0.046), with group B having a lower mean total cholesterol than group C, see table 6.6. Comparing pre and post test using post-hoc test, the mean total cholesterol was significantly lower (mean change = -15.16, p = 0.010) in group B, see Table 6.5.

Table 6.6: Post- hoc test comparisons of total cholesterol levels (mg/dl) between the three groups for post-test

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean change between two groups</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-test</td>
<td>A</td>
<td>B</td>
<td>9.69</td>
<td>0.263</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>-7.54</td>
<td>0.382</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>-14.86*</td>
<td>0.046</td>
</tr>
</tbody>
</table>

<sup>a</sup>indicates a significant difference between two groups as per p < 0.05

<sup>b</sup> indicates adjustment for multiple comparisons

6.4. Discussion

6.4.1 HDL cholesterol

This study found no significant relationship between vitamin D supplementation and changes in serum HDL cholesterol concentrations. This supports the existing literature base that vitamin D supplementation does not improve HDL cholesterol significantly (Jorde and Figenschau 2009; Patel et al., 2010; Al-Daghri et al., 2012; Breslavsky et al., 2013; Eftekhari et al., 2013; Talaei et al., 2013; Yiu et al., 2013; Al-Daghri et al., 2014; Kampmann et al., 2014; Ryu et al., 2014; Al-Sofiani et al., 2015; Sadiya et al., 2015). A 2015 randomised double blind control trial of 87 vitamin D-deficient obese, type 2 diabetics supplemented with 6000 IU/day vitamin D for three months, and 3000 IU/day for a further three months showed no effect on serum HDL concentrations (Sadiya et al., 2015). Similarly, a 2015 Saudi study of 20 diabetic subjects found no significant change in HDL cholesterol concentration following supplementation of 5000 IU/day for three months (Al-Sofiani et al., 2015). Even when a larger sample size and greater dose was used, such as in Talaei’s (2013) study of 100 type 2 diabetics
supplemented with 50,000 IU/ week for two months, no significant improvements were demonstrated in serum HDL cholesterol concentrations.

To the best of the researcher’s knowledge only two studies from the Middle East exist that demonstrate a significant improvement in HDL cholesterol following vitamin D supplementation (Yousefi Rad et al., 2014; Mohamad et al., 2015). Yousefi Rad (2014) conducted a randomised control trial of 58 type 2 diabetics, the majority of which were female, supplemented with 4000 IU/day for two months (Yousefi Rad et al., 2014). Despite the small sample size and short duration of the study, a significant improvement in HDL cholesterol was demonstrated following vitamin D supplementation. The reasons behind this are unclear. Interestingly, a 2015 study of 100 type 2 diabetics given 4500 IU/ day of vitamin D for two months demonstrated significantly higher HDL cholesterol levels in those subjects with double the sufficient concentration of serum vitamin D (> 61 ng/ml) post supplementation (Mohamad et al., 2015). This suggests further work on the dose response of vitamin D is warranted and achieving serum vitamin D concentrations greater than 61 ng/ml may show significant improvements in HDL cholesterol.

### 6.4.2 LDL cholesterol

This study demonstrates a significant decrease in LDL cholesterol in the vitamin D supplemented groups. Interestingly, LDL cholesterol levels increased in the placebo group after 16 weeks. These findings have been supported by similar Middle Eastern studies by Al-Daghri et al., (2012), Eftekhari (2013), Bonakdaran and Afkhami Zadeh (2011) and Mohamad (2015) who also demonstrated a significant improvement in LDL cholesterol following vitamin D supplementation of varying doses and durations. Despite the improvements in LDL cholesterol demonstrated by Eftekhari (2013) and Bonakdaran and Afkhami Zadeh (2011), it is hard to draw definite conclusions from these two studies. Both used lower doses (< 2000 IU/day) of vitamin D than the two other studies as well as the present study and did not measure serum concentrations of 25(OH)D. Al-Daghri’s 2012 study of 92 Saudi type 2 diabetics supplemented with 2000 IU/day for 18 months demonstrated a significant decrease in LDL cholesterol, more marked in females than males, when serum vitamin D concentrations also increased significantly. Similarly, Mohamad (2015) demonstrated a significant decrease in LDL cholesterol in subjects with greater than double the sufficient serum level of vitamin D (> 61 ng/ml) when compared to those with lower serum levels post
supplementation. This suggests that higher serum 25(OH)D concentrations may be required in order to achieve favourable effects on the lipid profile. This supports the findings of the present study; 61.9 percent of participants in the 4000 IU/day group achieved sufficient serum vitamin D concentrations post supplementation compared to just 41.9 percent of participants in the 2000 IU/day group.

Conversely, the bulk of literature conducted from the rest of the world to date appears to suggest vitamin D plays no significant role in improving LDL cholesterol (Patel et al., 2010; Breslavsky et al., 2013; Talaei et al., 2013; Yiu et al., 2013; Kampmann et al., 2014; Ryu et al., 2014; Yousefi Rad et al., 2014; Al-Sofiani et al., 2015; Sadiya et al., 2015). This may be explained partly by the sample sizes of the studies, all of which used a much smaller number of participants than the present study. To some extent, short study duration may also explain the inconsistency in the findings; only three of the cited trials had study durations equal to or greater than the present study (Breslavsky et al., 2013; Ryu et al., 2014; Sadiya et al., 2015). The majority of the studies that did not find any correlation between vitamin D supplementation and improved LDL cholesterol levels did not achieve sufficient post-supplementation serum vitamin D concentrations (> 30 ng/ml). Furthermore, only two studies (Talaei et al., 2013; Yiu et al., 2013) achieved close to the 61 ng/ml demonstrated in Mohamad’s (2015) study to be the optimal serum level for reduced LDL-cholesterol levels. The reliability of Talaei’s (2013) study may be questionable given that it was only a single blinded study. Similarly, Yiu et al., 2013 study was not conducted in the Middle East and therefore the reproducibility of the results in the Saudi population is dubious.

The evidence base suggests that significant improvements in LDL cholesterol levels have been achieved in Middle Eastern countries where a dose of at least 2000 IU/day vitamin D is used. Those studies that achieved serum concentrations of vitamin D above 61 ng/ml appeared to produce the most significant decrease on LDL cholesterol.

6.4.3 Triglycerides
This study found no significant relationship between serum triglycerides and improved vitamin D status. These findings are supported by the current body of literature which suggests there is no relationship between vitamin D supplementation and triglyceride levels (Jorde and Figenschau 2009; Talaei et al., 2013; Ryu et al., 2014; Yousefi Rad
et al., 2014; Al-Sofiani et al., 2015; Sadiya et al., 2015; (Bonakdaran and Afkhami Zadeh 2011; (Patel et al., 2010).

To the best of the researcher’s knowledge, only three studies conducted to date, all of which have been in the Middle East, have demonstrated a significant reduction in triglyceride levels following supplementation with vitamin D (Alkharfy et al., 2013; Eftekhari et al., 2013; Mohamad et al., 2015). Of note, is Alkharfy’s (2013) study of 499 Saudi participants supplemented with 2000 IU/day for 12 months. It is likely the large sample size and long study duration contributed to the finding of a significant relationship between vitamin D and improved triglyceride levels. In contrast to the current findings, a 1998 case study of three vitamin D–deficient subjects with type 2 diabetes receiving a single intramuscular dose of 300,000 IU of vitamin D, demonstrated a significant increase in serum triglycerides after three months. This suggests that too high a dose of vitamin D supplementation may in fact have negative effects on triglycerides (Taylor and Wise 1998). Once again, more research is warranted to assess the most appropriate dose of vitamin D supplementation in reducing triglyceride concentrations in 12 months.

6.4.4 Total cholesterol

This study demonstrates a significant decrease in total cholesterol in the vitamin D supplemented groups. In contrast, the placebo had a significant increase in total cholesterol after 16 weeks. To a certain extent, these findings can be explained by the parallel decrease in LDL cholesterol as total cholesterol is a sum of serum LDL-, HDL- cholesterol and triglyceride levels. These findings have echoed similar studies conducted in the Middle East that demonstrated significant decreases in serum total cholesterol following supplementation with vitamin D (Al-Daghri et al., 2012; Alkharfy et al., 2013; Eftekhari et al., 2013). However, the effect of vitamin D on total cholesterol in the current literature remains controversial and many studies have demonstrated no relationship between the two variables (Jorde and Figenschau 2009; Patel et al., 2010; Witham et al., 2010; Breslavsky et al., 2013; Talaei et al., 2013; Yiu et al., 2013; Kampmann et al., 2014; Ryu et al., 2014; Yousefi Rad et al., 2014; Al-Sofiani et al., 2015; Sadiya et al., 2015). This may be explained by ethnic background, sample size and short study duration of less than three months. It has been suggested that significant improvements in total cholesterol can be achieved in Middle Eastern
countries where studies include at least 70 participants and use a study duration of four months of greater (Alkharfy et al., 2013).

6.4.5 Summary
In conclusion, this study demonstrates no significant relationship between vitamin D supplementation of either 2000 IU/day or 4000 IU/day on serum HDL cholesterol and triglyceride levels. A significant improvement in serum LDL cholesterol (p = 0.028) and total cholesterol levels (p = 0.049) was demonstrated in both groups supplemented with vitamin D. Further interventional studies are clearly required using higher doses of vitamin D supplementation in order to achieve serum concentrations of greater than 61 ng/ml and to replicate these positive findings on the lipid profile of type 2 diabetics.

It is worth mentioning that the majority of the participants in the present study were already taking statins (87.5%, n = 112) prior to enrolment and thus a large percentage had a lipid profile within normal parameters at baseline. Statins have a considerable anti-inflammatory effect (Pickup 2004), that may have masked the anti-inflammatory effect of vitamin D. Alternatively, the extent of inflammation may already be so established in type 2 diabetics that four months of vitamin D supplementation had no effect. To the best of the researcher’s knowledge, no study to date has analysed the effect of vitamin D supplementation on the lipid profile of type 2 diabetic patients who are not already taking statins. This indicates there is scope for future work to assess the effect of vitamin D in deficient, type 2 diabetics with uncontrolled lipid profile.
Chapter 7. Vitamin D₃ and Oxidative Stress
7.1 Introduction

Oxidative stress is considered to be a vital component in the pathogenesis and development of diabetic vascular complications (Ceriello et al., 2002). Sustained hyperglycaemia is associated with heightened production of reactive oxygen species (ROS) as well as a reduction in the intrinsic antioxidant defence mechanisms, rendering diabetic individuals more susceptible to oxidative damage (Tsai et al., 1994; Kakkar et al., 1998). Type 2 diabetics have consistently been shown to have far lower total antioxidant capacities (TAC) than their healthy counterparts (Illing et al., 1951; Sundaram et al., 1996; Maxwell et al., 1997; Opara et al., 1999; Serafini and Del Rio 2004). This imbalance between oxidant and antioxidant levels leads to both functional and morphological damage to blood vessels and results in the development of micro- and macrovascular complications (Baynes 1991). Oxidative reactions also increase the formation of advanced glycation endproducts (AGEs) and their accumulation in cardiovascular tissues (Smith and Thornalley 1992).

Advanced glycation endproducts are a group of compounds formed non-enzymatically by glycation and oxidation of proteins. In type 2 diabetics, the formation of AGEs is increased as a result of hyperglycaemia and an abundance of free radicals (Singh et al., 2001). The accumulation of AGEs in the skin has been shown to be a strong indicator for the development of both micro- and macrovascular complications (Gerrits et al., 2008a; Meerwaldt et al., 2008). AGEs bind to the RAGE receptor in endothelial cells, resulting in further oxidative stress, superoxide radical generation and cell apoptosis (Vincent et al., 2007).

Vitamin D has been shown to enhance antioxidant defences and reduce the damage caused by both oxidative stress and AGEs, and may be of benefit in the treatment of diabetic vascular complications (Codoner-Franch et al., 2012; Gradinaru et al., 2012; Jain et al., 2013). Recent animal studies have shown vitamin D to upregulate the activity of certain antioxidant enzymes, such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) in diabetic rats (Noyan et al., 2005). Furthermore, vitamin D may improve vascular endothelium function through the modulation of radical generation and RAGE expression, averting the overproduction of
ROS and AGE formation (Dong et al., 2012). Despite these positive findings, research on the antioxidant properties of vitamin D for use in type 2 diabetes remains scarce.

The aims of this study were to test the hypothesis that vitamin D supplementation improved markers of oxidative stress, including advanced glycation endproducts (AGEs), in poorly controlled type 2 diabetic females within the Saudi Arabian population.

7.2 Methodology

Blood samples were taken from all 128 participants at baseline and 16 weeks using the aseptic technique described in section 2.12. Total antioxidant capacity was measured using the colorimetric method with the commercially available Cell Biolabs’ OXiSelect TAC assay kit as described in section 2.15.7. Skin AGE levels were assessed using a skin autofluorescence AGE reader as described in section 2.17.

7.3 Results

7.3.1 Total antioxidant capacity

The changes in the mean total antioxidant capacity of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 7.1. The difference in mean total antioxidant capacity between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 7.1.

Table 7.1: Post-hoc comparisons of total antioxidant capacity (mM) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.62 ±0.32</td>
<td>0.42 ±0.10</td>
<td>-0.20 ±0.31&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>B</td>
<td>0.80 ±0.27</td>
<td>0.40 ±0.09</td>
<td>-0.40 ±0.29&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>C</td>
<td>0.47 ±0.13</td>
<td>0.37 ±0.07</td>
<td>-0.10 ±0.13&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.013</td>
</tr>
</tbody>
</table>

<sup>*</sup>indicates a significant difference between pre-test and post-test as per p < 0.05

<sup>b</sup> indicates adjustment for multiple comparisons
The results of ANCOVA indicated, however, that the mean total antioxidant did not vary significantly between the three groups (F (2, 124) = 1.981, p = 0.142). Mixed ANOVA testing revealed no significant interaction between time and the three groups (F (2,125) = 2.83, p = 0.063). Post-hoc test showed a significant decrease in total antioxidant capacity between pre and post test for each group, for group A (p < 0.001), group B (p < 0.001), and group C (p = 0.013), see Table 7.1.

7.3.2 Skin Advanced glycation endproducts (AGEs)
The changes in the mean skin AGEs of the participants (post-test minus pre-test) in groups A, B, and C are compared in Table 7.2. The difference in mean skin AGEs between the post-test and the pre-test, as well as the differences across the three groups is shown in Figure 7.2.
Table 7.2: Post-hoc comparisons of skin AGEs (arbitrary units) between baseline (pre-test) and after 16 weeks (post-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (pre-test)</th>
<th>After 16 weeks (post-test)</th>
<th>Mean change (post-test minus pre-test)</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.13 ±0.87</td>
<td>3.12 ±0.81</td>
<td>-0.01 ±0.59</td>
<td>0.971</td>
</tr>
<tr>
<td>&lt;sup&gt;B&lt;/sup&gt;</td>
<td>2.98 ±0.65</td>
<td>2.86 ±0.57</td>
<td>-0.13 ±0.64</td>
<td>0.524</td>
</tr>
<tr>
<td>&lt;sup&gt;C&lt;/sup&gt;</td>
<td>3.17 ±2.82</td>
<td>2.94 ±1.18</td>
<td>-0.23 ±2.05</td>
<td>0.234</td>
</tr>
</tbody>
</table>

<sup>a</sup>indicates a significant difference between pre-test and post-test as per p < 0.05

<sup>b</sup> indicates adjustment for multiple comparisons

Figure 7.2: Mean skin AGEs (arbitrary units) across the three groups over time (pre-test and post-test)

The results of ANCOVA indicated that the mean AGEs did not vary significantly between the three groups (F (2, 124) = 1.478, p = 0.232). Mixed ANOVA testing revealed no significant interaction between time and the three groups (F (2,125) =
Post-hoc test showed no significant difference in AGEs between pre and post test for each group, see Table 7.2.

7.4 Discussion

7.4.1 Total Antioxidant Capacity

This study found no significant improvement in TAC following supplementation with either 2000 IU/day or 4000 IU/day vitamin D. To the best of the researcher’s knowledge this is only the second interventional study to assess the role of vitamin D on TAC in type 2 diabetics. The first, a 2015 study of 100 type 2 diabetics supplemented with a fortified yoghurt drink containing 1000 IU vitamin D, demonstrated a significant improvement in TAC after three months (Shab-Bidar et al., 2015). A 2013 cross-sectional analyses of 100 type 2 diabetics also demonstrated a positive correlation between serum vitamin D concentrations and TAC when compared to healthy individuals, although given the nature of the study it was difficult to establish causality (Saedisomeolia et al., 2013). Two further interventional studies have also shown vitamin D supplementation to significantly increase TAC in non-diabetic subjects (Asemi et al., 2013; de Medeiros Cavalcante et al., 2015). Whilst the present study has shown no relationship between vitamin D and TAC this appears to be in contrast with the, albeit limited, existing evidence base.

There appears to be some discrepancy in the literature concerning the most appropriate parameter by which to measure an individual’s oxidative stress (Argüelles et al., 2004; Serafini and Del Rio 2004). Other studies have used alternative markers to determine the effect of vitamin D supplementation on oxidative stress. Nikooyeh et al. (2014) demonstrated a significant improvement in the antioxidant superoxide dismutase (SOD) and a reduction in the pro-oxidants protein carbonyl (PCO) and myeloperoxidase (MPO) in 90 type 2 diabetics supplemented with a 1000 IU fortified vitamin D yoghurt drink daily for four months. Conversely, Yiu et al. (2013) demonstrated no significant change in SOD levels following administration of 5000 IU/day vitamin D for three months. Both Eftekhar et al. (2013) and Tayebinejad et al. (2012) failed to demonstrate any improvements in oxidised LDL (ox-LDL) or malondialdehyde (MDA) following supplementation with vitamin D for three months. Once again, these studies had shorter durations than the present study which may explain the discrepancy in findings. Regardless of the marker used, the existing
literature base provides inconsistent evidence for the role of vitamin D in ameliorating oxidative stress in diabetic individuals. It is suggested that intervention studies on a larger scale are warranted. These should use greater doses of vitamin D supplementation and have a study duration longer than the present study, using a variety of different markers to measure oxidative stress.

7.4.2 Skin Advanced Glycation Endproducts

This study found no significant relationship between vitamin D supplementation and reduced levels of advanced glycation end products. These findings are supported by a 2015 randomised control trial of 245 type 2 diabetics given 50,000 IU/month vitamin D for six months (Krul-Poel et al., 2015). Whilst skin autofluorescence (as a measure of AGE accumulation) was found to be significantly higher in those with a serum 25(OH)D concentration of less than 20 ng/ml, compared to patients with serum concentrations above 30 ng/ml, there was no reduction in AGE levels after six months of intervention. This is likely due to the fact that skin AGEs have a mean half life of 10 to 15 years (Gerrits et al., 2008b) and therefore the length of the intervention may be too short to demonstrate any perceivable outcomes. Furthermore, using an AGE-reader to measure the accumulated AGEs in the skin may not be sufficient to record all AGE types, for example circulating AGEs and AGEs without fluorescent properties (Krul-Poel et al., 2015). Similarly, a 2015 cross-sectional study of 233 type 2 diabetics, concluded that vitamin D deficiency did not enhance the skin autofluorescence or plasma AGE levels (Sebekova et al., 2015).

In contrast, studies performed on diabetic rats have shown vitamin D supplementation to reduce AGE deposition within the vasculature and to downregulate the effects of the RAGE receptor (Salum et al., 2013; Lee et al., 2014). AGEs have been shown to bind to the RAGE receptor in endothelial cells, resulting in further oxidative stress, superoxide radical generation and cell inflammation (Vincent et al., 2007). Two recent in vitro studies demonstrated a reduction in endothelial inflammation following supplementation with vitamin D. It has been suggested that vitamin D may act as a vascular protective agent, offsetting the harmful effects of AGEs on endothelial cells (Talmor et al., 2008; Salum et al., 2013; Krul-Poel et al., 2015). Similar findings have also been demonstrated in human studies. Two recent randomised control trials of 60 and 90 type 2 diabetics respectively found significant decreases in serum AGEs
following supplementation with two 500 IU fortified yoghurt drinks a day (Tayebinejad et al., 2012; Nikooyeh et al., 2014). It is worth noting that this improvement was in serum AGEs, corresponding with our observations in Chapter 5. Whilst this study has demonstrated no relationship between vitamin D and skin AGE accumulation, the positive findings from both in vitro and in vivo studies suggests further work within this field is warranted with longer study durations for both serum and skin AGE concentrations.

7.4.3 Summary
In conclusion, this study has demonstrated no significant relationship between markers of oxidative stress and vitamin D in type 2 diabetic Saudi females. Current evidence, whilst limited, is inconsistent and more research within this field is required to assess the role for vitamin D, if any, in ameliorating oxidative stress. These studies should use longer durations and supplement doses, as well as assessing AGE response in both skin and serum.
Chapter 8. Discussion
8.1 General Discussion

Diabetes Mellitus represents a condition that is characterised by chronic hyperglycaemia, resulting from deficits in insulin secretion and sensitivity (WHO 2016). Saudi Arabia has amongst the highest incidence of type 2 diabetes in the world, with nearly 20 percent of adults suffering from the condition (International Diabetes Federation 2012). Mortality rates amongst diabetics are nearly twice that of healthy individuals and are largely due to microvascular and macrovascular complications that arise from the disease (Roglic et al., 2005).

Biological processes within the body produce reactive oxygen species (ROS) that harm tissues and cells (Skrha 2003). In healthy individuals this process is limited by exogenous and endogenous antioxidant defences (Betteridge 2000). In type 2 diabetes high concentrations of intracellular glucose increase the production of ROS (Lorenzi 2007). This is compounded by significantly reduced antioxidant levels and accelerated formation of advanced glycation endproducts (AGEs). This imbalance between oxidant and antioxidant defences results in functional and morphological damage to the vasculature and the initiation of diabetic angiopathy (Baynes 1991). AGEs are a group of compounds formed non-enzymatically via the glycation and oxidation of proteins. Their formation is accelerated in type 2 diabetics as a result of hyperglycaemia and an increased number of free radicals (Singh et al., 2001). The accumulation of AGEs in the skin has been shown to cause the development of diabetic vascular complications (Gerrits et al., 2008a; Meerwaldt et al., 2008).

Vitamin D has been shown to enhance antioxidant defences and limit the harm caused by both oxidative stress and AGEs and therefore may be of benefit in the treatment of diabetic complications (Codoner-Franch et al., 2012; Gradinaru et al., 2012; Jain et al., 2013). The prevalence of vitamin D deficiency in type 2 diabetics has repeatedly been shown to be nearly twice that of non-diabetic individuals; within the Saudi Arabian population it is estimated that somewhere between 30 and 85% of females are vitamin D deficient (Scragg et al., 1995; Isaia et al., 2001; Scragg et al., 2004; Al-Zahrani 2013). Despite a number of recent interventional studies on vitamin D and diabetes in Saudi Arabia, none to date have considered the effect of vitamin D supplementation on markers of oxidative stress. Whilst being the first study of this kind, unfortunately the present study has demonstrated no significant effect on total antioxidant capacity or
skin autofluroscence (AGEs) in the type 2 diabetic Saudi population. There have been few previous studies on vitamin D, diabetes and oxidative stress in any population and all have provided conflicting results. This study concludes that vitamin D has limited, if any effect, antioxidant properties.

Administration of vitamin D supplementation has differed between studies, with daily doses proving to be more effective in raising serum concentrations than weekly or monthly doses (Von Hurst et al., 2010). This is likely to be due to more consistent absorption in the gut (Chel et al., 2008). Recent meta-analysis of 51 randomised control trials using doses of vitamin equivalent to 1000 IU per day has failed to demonstrate any beneficial effects of vitamin D on diabetic outcomes (Elamin et al., 2011). However, for studies using higher doses of vitamin D the results have proved more inconclusive.

In the present study, the therapeutic effect of daily oral supplementation of vitamin D in 128 female patients with type 2 diabetes mellitus and hypovitaminosis D was investigated. The results demonstrate that daily oral supplementation with doses of 2000 IU and 4000 IU was both safe and effective in significantly (p < 0.001) improving vitamin D status in patients with type 2 diabetes. The majority of Saudi studies have used a supplement dose of 2000 IU for varying durations, yet this is the first to consider the effect of a higher dose of 4000 IU on diabetic parameters. Serum vitamin D concentrations increased by 212% in the 4000 IU group and 131% in the 2000 IU group (compared to a 5% decrease in the placebo group). However, only 61.9% of the 4000 IU group and 41.9% of the 2000 IU group had achieved sufficient (>30 ng/ml) vitamin D concentrations post supplementation. No patients developed either clinically or biochemically significant hypercalcaemia.

These findings confirm that greater doses of vitamin D supplementation are likely to be required in order to achieve sufficient serum concentrations amongst female type 2 diabetics. This may in part be due to the baseline BMIs of participants; one of the inclusion criteria was for subjects to have a BMI greater than 25. Wortsman et al. (2003) reported that obese patients were able to raise their serum vitamin D concentrations by no more than 50 percent when compared to their non-obese counterparts and hence this may be accountable for the suboptimal increase in many of the participants. However, this study demonstrated no relationship between BMI and
vitamin D. Other similar studies have demonstrated that using 6000 IU vitamin D per day for three months was effective in safely increasing serum concentrations from deficient to sufficient (Sadiya et al., 2015; Al-Zahrani et al., 2014). However, it was noted that this dose may still be inadequate to achieve optimum vitamin D function.

Whilst the effects of vitamin D on oxidative stress have been somewhat disappointing, the present study has demonstrated significant improvements ($p = 0.001$) in the glycaemic profile of participants. After 16 weeks, subjects who had taken vitamin D supplements had a significantly lower HbA1c level than at baseline. HbA1c is a marker of average serum glucose concentrations over the previous three months and therefore is indicative of glucose homeostasis (Wuerle et al., 2004). The UK Prospective Diabetes Study (UKPDS) demonstrated a one percent reduction in HbA1c to be associated with a 21 percent decrease in diabetic complications and a further 37 percent reduction in microvascular disease (Stratton et al., 2000). The findings of this study suggest that vitamin D supplementation may therefore produce a significant reduction in diabetes-related morbidity and mortality, and may be used as an adjuvant to regular anti-hyperglycaemic agents.

It has been further suggested that improvements in HbA1c are more likely to be seen in vitamin D-deficient female type 2 diabetics than in their insufficient and sufficient counterparts. Sabherwal’s 2010 study demonstrated a 0.70% improvement in HbA1c levels amongst vitamin D-deficient type 2 diabetics compared to a 0.21% improvement in those who were vitamin D insufficient (Sabherwal et al., 2010). Similarly, Jorde and Figenschau (2009) and Krul-Poel et al. (2015) found that vitamin D replacement therapy had no effect on HbA1c in type 2 diabetics with sufficient serum vitamin D concentrations. Due to the similar numbers of deficient and insufficient subjects across all of the intervention groups, it is impossible to either prove or disprove this theory in the present study. It was found that giving a higher dose of 4000 IU vitamin D per day improved HbA1c levels more than a dose of 2000 IU per day. Of the 37 female subjects found to be vitamin D deficient at baseline who received the higher dose, 80% of them elevated their vitamin D status after 16 weeks. This was in comparison to 76% of those in the lower supplementation group and therefore suggests a higher dose may not be that beneficial.
After 16 weeks of supplementation with vitamin D, no improvements were found in pancreatic beta cell function and insulin resistance. Fasting blood glucose did not show an improvement at all. A recent study similarly demonstrated that after three years of supplementation with 700 IU vitamin D per day, insulin resistance and fasting blood glucose remained unchanged (Pittas et al., 2007a). Interestingly however, in the placebo group both markers had significantly increased after three years. This suggests that whilst vitamin D may not actually improve insulin resistance and fasting blood glucose, it may slow the natural progression of the disease. The majority of vitamin D interventional studies to date all have durations between one and six months and few have considered the long-term effect of supplementation on diabetic outcomes.

Dyslipidaemia is one of the leading causes of cardiovascular disease in type 2 diabetics (Mooradian 2009). Diabetes mellitus is associated with increased small-dense low-density lipoprotein (sdLDL) and triglyceride concentrations, alongside low concentrations of cardio-protective high-density lipoprotein (HDL) (American Diabetes Association 2003). The exact mechanism behind the pathogenesis of diabetic dyslipidaemia remains uncertain, however evidence has shown insulin resistance to be critical in the development of the condition (Mooradian 2009).

Many observational studies have demonstrated high serum vitamin D concentrations to be associated with more favourable lipid profiles (Jorde and Grimnes 2011). Unfortunately, interventional studies to date have provided inconsistent results. These studies have been limited by comparatively small sample sizes, short study durations and supplement doses not sufficient to achieve optimal serum 25(OH)D concentrations, as well as study locations outside the Middle East (Ponda et al., 2012). In the present study, significant reductions in LDL- and total cholesterol (p = 0.028 and p = 0.049 respectively) have been demonstrated, following 16 weeks of supplementation with vitamin D. Furthermore, both LDL- and total-cholesterol increased in the placebo group after 16 weeks. No significant changes were noted in HDL cholesterol and triglyceride concentrations.

Mohamad et al. (2015) demonstrated that diabetic subjects with high serum vitamin D concentrations (> 61 ng/ml) had significantly lower levels of total and LDL cholesterol, compared to those with sufficient or insufficient serum concentrations. As such, higher serum concentrations than what are currently considered to be sufficient
may be required in order to produce auspicious gains on the lipid profile. None of the
participants in the present study achieved final serum vitamin D concentrations above
47.28 ng/ml. Supplementation doses were selected on the basis of recommendations
from the Endocrine Society Clinical Guidelines (Holick et al, 2011), however these
have been derived from interventional studies centred on skeletal outcomes. To date,
there has been little consideration as to the effective supplementation doses required
for the non-skeletal properties of vitamin D and further research in this area is clearly
warranted (Jorde and Grimnes 2011).

It is presumable that if vitamin D supplementation were to effect serum lipid levels,
this would be most evident in individuals with both hypovitaminosis D and
dyslipidaemia. Unfortunately, the majority of participants in the present study were all
taking statins prior to enrolment and therefore very few had unfavourable lipid profiles
at baseline (14% in group A, 14% group B, 4% in group C). As it is a recommendation
(Grundy, 2006) that all newly diagnosed type 2 diabetics be commenced on a statin, it
was impossible to find a population in which the lipid profile had not already been
controlled for. However, until such studies are performed the effect of vitamin D
supplementation on the serum lipid profile will remain controversial.

8.2 Limitations

The results of this study may have been affected by a number of limitations:

- Not all participants in both supplemented groups achieved sufficient (> 30
  ng/ml) serum concentrations of vitamin D following intervention
- All subjects were taking statins at the time of enrolment and therefore very few
  had unfavourable lipid profiles at baseline
- Insulin resistance and beta cell function were calculated using the HOMA
  model which is less invasive and time-consuming but not as accurate as the
  glucose clamp method
- The study duration of 16 weeks was relatively short and it is likely that to elicit
  any tangible effects on insulin resistance, beta cell function and oxidative stress
  warrants longer durations of supplementation
8.3 Scope for future work
To date, recommendations for vitamin D supplementation doses have been based solely on its calcaemic properties. Further research is currently warranted into the dose of vitamin D that is not only adequate for bone health but for its potential non-calcaemic actions. It is recommended that these studies use doses starting at 6000 IU per day aiming to achieve serum concentrations of greater than 60 ng/ml. Longer study durations of 18 months or greater are warranted to assess the long-term effect of vitamin D supplementation on insulin resistance, beta cell function and using different markers to measure oxidative stress. Furthermore, further work is warranted to assess the different accumulation of AGEs in serum and skin for longer study durations as well to demonstrate which marker is the most effective measurement of oxidative stress. This study has highlighted the need for further investigation in newly diagnosed female type 2 diabetics who have not yet been commenced on statins in order to accurately assess the effect of vitamin D on the lipid profile.

8.4 Overall conclusion
In conclusion, the results from the present study indicate that HbA1c levels significantly decreased following 16 weeks of supplementation with vitamin D. These findings were most evident at the greater dose of 4000 IU day (p < 0.001), however this was still not adequate to achieve sufficient serum vitamin D concentrations in all participants. Similar improvements were noted in LDL- and total cholesterol. No significant improvements in oxidative stress were demonstrated. This study adds to a growing body of evidence that vitamin D supplementation may be beneficial not only in bone-related conditions, but as an adjuvant therapy in female type 2 diabetes management in the Middle Eastern diabetic population.
References


Antibodies online. (2013) Total Antioxidant Capacity (T-AOC).


Gerrits EG, Lutgers HL, Kleefstra N, Groenier KH, Smit AJ, Gans RO (2008b) Skin advanced glycation end product accumulation is poorly reflected by glycemic control


Tsai EC, Hirsch IB, Brunzell JD, Chait A (1994) Reduced plasma peroxyl radical trapping capacity and increased susceptibility of LDL to oxidation in poorly controlled IDDM. *Diabetes* 43: 1010-1014.


Screening Questionnaire for recruiting participants

Name:………………………………………………… Today’s date: ____/_____/_____

Address:……………………………………………………………………………………………...

Telephone:………………………………………………………………………………………

Patient’s file number…………………………………………

Patient’s next appointment ……………………………..

1- Age?
   (1) Less than 18  (2) 18 - 24  (3) 25 - 34  (4) 35 - 44  (5) 45 - 54  (6) 55-60
   (7) 61 and over
   Date of birth: ------/-/-/-

2- Does the patient have type 2 diabetes?
   (1) Yes  (2) No
   • Current HbA1c _____________%
   • Calculated BMI _____________m²
   • Participant’s Height? _____________cm
   • Participant’s Weight? _____________kg
   • Calcium level _____________mmol/l

3- Does the patient have any of the following medical conditions?
   (1) Renal failure
   (2) Gastrointestinal Malabsorption (e.g. Crohn’s disease, Colitis)
   (3) Thyroid and parathyroid disease
   (4) Hypercalcemia
   (5) Liver disease

4- Does the patient take vitamin supplements?
   (1) Yes  (2) No
   If yes, please specify type and brand
   ……………………………………………………………………………………………………..

5- Is the patient taking vitamin D?
   (1) No  (2) Yes
   If yes, please specify type and brand
   ……………………………………………………………………………………………………..

☐ Eligible  ☐ Not eligible  Participant number………..
INFORMATION SHEET

Project title:- Effect of vitamin D₃ supplementation on markers of glycaemia and oxidative stress in Saudi women with Type 2 Diabetes Mellitus

Dear Participant,

This information sheet is to provide you with information about a project being conducted as a PhD in Nutritional Physiology at Manchester Metropolitan University. This study will provide valuable information about whether vitamin D supplements may help control type two diabetes.

Study requirements:-

- **Questionnaires**
  This study will involve two questionnaires, one at the start and one after 16 weeks which should take approximately 5-10 minutes to be completed.

- **Anthropometric data**
  For the purpose of the study, your weight, height and waist circumference will be measured at the start of the study and after 16 weeks.

- **Blood samples**
  As part of the research blood will be taken at pre-screening, the start of the study and after 16 weeks.

- **Vitamin D Supplements**
  This study requires participants to take one tablet on a daily basis for 16 weeks. You will also need to record the days in which supplements were taken and whether any supplements were missed.

Confidentiality and data protection

You may decide not to answer some or any of the questions provided. You may also decide to withdraw from this study at any time. The information you provide will be considered confidential, except that with your permission anonymised quotations may be used. If you request confidentiality, beyond anonymised quotations, the information you provide will be treated only as a source of background research, alongside books and web-based research and the information presented by others. All the information you provide will be handled in line with the Data Protection Act and will not be used for anything other than this research study. Your name and any other personal identifying information will not appear in the course project paper resulting from this study; neither
will there be anything to identify your address. Only the study team will have access to
the data itself.

If you have any questions regarding this study or would like additional information
please contact the email below. Thank you for your assistance in this project.

Alaa Hatim Qadhi

E-mail  10997250@stu.mmu.ac.uk

Consent form

Have you read the information sheet?

Do you understand what the project is about?

Are you aware that you will be asked to complete a medical background questionnaire?

Are you aware that four blood samples will be taken?

Are you aware that these blood samples will be analysed?

Are you aware that you need to consume a tablet on a daily basis?

Are you aware that you can stop participating in the study at any time?

Are you willing for your blood to be used for further analysis?

If you are willing to participate please sign your name below.

I ............................................................... agree to take part in the above research study.
I am aware that all information will be kept confidential in line with the Data Protection Act and that I can withdraw at any time.

Signed..............................................................................

Date....................................................................................

Should you have any questions about this research project please contact Alaa Qadhi on this E-mail 10997250@stu.mmu.ac.uk
Pre-study Screening Questionnaire

A. Participant Section:

Background

Name:……………………………………………... Today’s date: ____/_____/_____
Address:………………………………………………………………………………………….
Telephone:…………………………………………………………………………………..

1- Are you male or female?
(1) Male (2) Female

2- How old are you?
(1) Less than 18 (2) 18 - 24 (3) 25 -34 (4) 35 - 44 (5) 45 - 5 (6) 55-60
(7) 61 and over
Date of birth: ------/------/-------

3- Are you currently?
(1) Single (2) Married (3) Divorced (4) Widowed

4- What is your ethnicity?
(1) Arab (2) Caucasian (3) African
(4) Indian subcontinent (5) Other. Please specify…………………………….

5- Please circle the highest year of school completed:
(1) No schooling (2) Primary school (3) Secondary school (4) High school
(5) Diploma (6) Bachelor’s degree (7) Post-graduate degree

6- Are you currently?
(1) Student (2) Employed (3) Unemployed (4) Retired

7- Are you pregnant?
(1) Yes (2) No

8- Do you currently smoke cigarettes or any other tobacco products on a daily basis?
(1) Yes (2) No

9- Are you on a special diet?
(1) Yes (2) No If Yes Please specify: . . . . . . . . . . . . .
Medications

10- How long have you been diagnosed with type 2 diabetes?
   (1) 1-2 years  (2) 3-5 years  (3) 6-8 years  (4) over 8 years

11- Has either of your parents, or any of your brothers or sisters been diagnosed with diabetes?
   (1) Yes  (2) No

12- Do you have any diabetic complications?
   (1) Yes  (2) No  (3) Don’t know

   If yes please give details of relevant diabetic complications:
   (1) Nephropathy  (2) Neuropathy  (3) Diabetic cataract
   (4) Cardiovascular disease  (5) Dyslipidemia  (6) Retinopathy

   Other…………………………………………………………………………………………

13- Do you have any of the following medical conditions? Circle every relevant condition.
   (1) Renal failure  (2) Gastrointestinal Malabsorption (e.g. Crohn’s disease, Colitis)
   (3) Parathyroid disease  (4) Hypercalcaemia  (5) Liver disease

14- Are you currently taking prescribed medications?

<table>
<thead>
<tr>
<th>Name of Drug</th>
<th>Dose</th>
<th>How many times a day?</th>
</tr>
</thead>
<tbody>
<tr>
<td>e.g. Metformin</td>
<td>1 tablet of 1g</td>
<td>2 times with meals</td>
</tr>
</tbody>
</table>

15- Do you take vitamin supplements?
   (1) Yes  (2) No
   If yes, please specify type and brand
   ...........................................................................................................
16- Have you taken vitamin supplements 3 months prior to today’s date?
(1) No       (2) Yes
If yes, please specify type and brand

B. Researcher Section - Anthropometric Measurements
- Participant’s Height? ____________cm
- Participant’s Weight? ____________kg
- Calculated BMI ____________m²
- Current HbA1c ____________%
- Calcium level ____________mmol/l
- Date tested ____/____/____

☐ Eligible  ☐ Not eligible
Debriefing Questionnaire

C. Participant Section:

Background

Name: .................................................... Today’s date: ____/_____/_____
Address: ............................................................................................................
Telephone: .........................................................................................................

1- I know it must be difficult to take medications regularly. How often did you miss
taking the vitamin tablet?
(1) Once a week (2) twice a week (3) three or more times a week (4) never took them

2- How many times did you forget to record your pill taking on the calendar?
(1) Once a week (2) twice a week (3) three or more times a week (4) never recorded

3- Have you been prescribed any new medications since you started taking the
vitamin supplements, if so please specify?
(1) No (2) Yes
If yes, please specify
........................................................................................................................................
........................................................................................................................................
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4- Have you stopped taking any prescribed medications since you started taking the
tablets?
(1) No (2) Yes
If yes, please explain
........................................................................................................................................
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5- Have the doses of your medications changed since you started taking the tablets?
(1) No (2) Yes
If yes, please explain
........................................................................................................................................
........................................................................................................................................
6- Have you taken vitamin supplements other than those supplied during the study?
(1) No (2) Yes
If yes, please explain

7- Did you suffer from any of the following medical conditions in the last 16 weeks?
Circle every relevant condition.
(1) Renal failure (2) Gastrointestinal Malabsorption (e.g. Crohn’s disease, Colitis) (3) Parathyroid disease (4) Hypercalcaemia (5) other, please specify

8- Are you pregnant?
(1) No (2) Yes

9- Did you start any special diets?
(1) No (2) Yes
If yes, please explain

10- Overall, how would you say the control of your diabetes has been over the last 16 weeks?
(1) Better than before (2) Worse than before (3) No change

11- Have you been diagnosed with any other conditions?

D. Researcher Section - Anthropometric Measurements
• Participant’s Height? ____________ cm
• Participant’s Weight? ____________ kg
• Calculated BMI ____________ m^2
• AGE reading ____________ units

Participant number……………………………

Checklist:
<table>
<thead>
<tr>
<th>Task</th>
<th>Completed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Questionnaire complete</td>
<td>[ ]</td>
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<tr>
<td>Tablets returned</td>
<td>[ ]</td>
</tr>
<tr>
<td>Calendar returned</td>
<td>[ ]</td>
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<tr>
<td>Anthropometric measures</td>
<td>[ ]</td>
</tr>
<tr>
<td>Blood sample taken</td>
<td>[ ]</td>
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</tbody>
</table>
Pill Record

Name ……………………………………………

Participant number …………………………….

<table>
<thead>
<tr>
<th>Week</th>
<th>Sunday</th>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
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</tbody>
</table>

If you took the pill on a particular day please mark the appropriate box with a tick. If you did not take a pill on a certain day please mark this with a cross.
استبيان لمرحلة المسح ل 240 مشاركة

اسم: ........................................................................
رقم الملف: ..............................................................
عنوان: ..............................................................................................
هاتف: ..................................................................................................................................
تاريخ اليوم: ______ / ______ / ______
تاريخ الموعد: .........................................................

1- العمر؟
(1) دون 18 سنة (2) 18-24 سنة (3) 25-34 سنة (4) 35-44 سنة (5) 45-54 سنة (6) 55-60 سنة (7) أكثر من 61 سنة

2- هل تعاني المريضة من السكري النوع الثاني؟
(1) نعم (2) لا

3- نسبة الهيموغلوبين السكري الحالية

4- مؤشر كتلة الجسم: _______ م٢

5- طول المشاركة: _________ سم

6- وزن المشاركة: _________ كجم

7- مستوى الكالسيوم: _________ mg/dL

8- هل تعاني المريضة من أي من الحالات الطبية التالية؟
(1) الفشل الكلوي
(2) سوء الأيض الدهني (مثل مرض كرون والتهاب القولون)
(3) مرض الغدة الدرقية والجار درقية
(4) فرط كالسيوم الدم
(5) أمراض الكبد

4- هل تتناول المريضة مكملات الفيتامينات؟ ومن أي تاريخ بدأت تتناولها؟
(1) نعم
(2) لا

5- هل تتناول المريضة فيتامين D؟
(1) نعم
(2) لا

رقم المشاركة: ..........................................................
مناسبة ................................. غير مناسبة ..........................
ورقة معلومات
عنوان المشروع: أثر مكملات فيتامين D على مستويات السكر في الدم وإجهاد الأكسدة لدى النساء السعوديات اللاتي تعانون من داء السكري النوع الثاني

عزيزتنا المشاركة
يقصد بهذه الورقة التي بين أيدينا موافتك بمعلومات عن المشروع المنفذ في إطار رسالة دكتوراة في مجال فسيولوجيا التغذية بجامعة مانشستر متروبوليتان. حيث تطرح الدراسة معلومات قيمة عما إذا كانت مكملات فيتامين D تساعد في التحكم في النمط الثاني من داء السكري أم لا.

أدوات الدراسة:
• استبيانات

• بيانات ذات صلة بالجسم

• عينات الدم

• مكملات فيتامين D

المشاركة في الدراسة: 
تشمل هذه الدراسة أجراء استبيانين: أحدهما في بداية الدراسة والأخر بعد مرور 16 أسبوعًا من بداية الدراسة، ويستغرق كل استبيان حوالي 5-10 دقائق لاستكماله.

بيانات ذات صلة بالجسم

لأغراض الدراسة ستستخدمين لقياس وزنك وطولك ومحيط الورك في بداية الدراسة وكذلك بعد مرور 16 أسبوعًا من بداية الدراسة.

عينات الدم

يجري فحص الدم قبل بدء الدراسة كجزء من البحث، ويجري نفس الفحص مجددا بعد مرور 16 أسبوعًا من بدئها.

مكملات فيتامين D

تتطلب هذه الدراسة من المشاركات تناول قرصًا واحدًا يوميًا لمدة 16 أسبوعًا، ومن ثم فإنكِ ستحتاجين لتدوين الأيام التي تتناولين فيها المكملات وما إذا انقطعتِ عن استخدام أي مكملات أخرى أم لا.

السرية وحماية البيانات

في حالة رغبتك في الاستماع عن إجابة بعض الأسئلة المطروحة أو الاستجابة عن السؤال من الدراسة كليًا في أي وقت كان ضمن ذلك سيرية معلوماتك، فيما عدا بعض الأسئلات التي يمكن استجوابها بتصريح منك دون الإصراحة عن هوية قائلها، فإذ كنت راغبة في الاحتفاظ بسرية معلوماتك، دون هذه الأسئلات التي لا نفصحي فيها عن هويتك، فسوف تعمل المعلومات التي تأتي فيها لمعالجة مصدرا البحث الأصولية التي تضفي إلى كتب وأبحاث الشبكة والمعلومات التي يقدمها الآخرون، فكافة المعلومات التي تأتي بها يجب التعامل معها وفقاً لقانون حماية البيانات ولا نستخدم أي أعراض أخرى غير أعراض هذه الدراسة، ولا يظهر اسمك أو أي معلومات تتعلق شخصية أخرى في ورقة المشروع التي تطرحها هذه الدراسة، كما أنها لن تشمل أية بيانات تعنوك ببعضها؛ حيث أننا من المسؤولين في قاعدة البيانات.

إذا كانت لديك أي استفسارات تتعلق بالدراسة أو إذا كنت ترغب في الحصول على أي معلومات إضافية بشأنها فرجى التواصل عبر البريد الإلكتروني الموضوع أسفله، نشكر لك تعاونك معنا في هذا المشروع.

البريد الإلكتروني: 10997250@stu.mmu.ac.uk

الإله حاتم قاضي
نموذج الموافقة

<table>
<thead>
<tr>
<th>عنوان السؤال</th>
<th>إجابة</th>
</tr>
</thead>
<tbody>
<tr>
<td>هل اطلعت على ورقة المعلومات؟</td>
<td></td>
</tr>
<tr>
<td>هل تشعرين أنك ملمة بموضوع المشروع؟</td>
<td></td>
</tr>
<tr>
<td>هل تعلمين أنه سيطلب منك تعبئة استبيان عن تاريخك الطبي؟</td>
<td></td>
</tr>
<tr>
<td>هل تعرين أنه سيؤخذ منك أربعة عينات دم؟</td>
<td></td>
</tr>
<tr>
<td>هل تعرين أن هذه العينات ستختضع للتحليل؟</td>
<td></td>
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<tr>
<td>هل تعرين أنه سيكون عليكتناول فرضا واحدا يوميا؟</td>
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<tr>
<td>هل تعلمين أنه بإمكانك الانسحاب من الدراسة في أي وقت ترغبين في ذلك؟</td>
<td></td>
</tr>
<tr>
<td>هل ترحبين باستخدام عينة الدم الخاصة بك في مزيد من التحاليل؟</td>
<td></td>
</tr>
</tbody>
</table>

إذا كنت ترغبين في المشاركة يرجى التوقيع باسمك أدناه:

أوافق أنا ............................. على المشاركة في الدراسة البحثية المذكورة أعلاه.

وأقر بعلمي بالحفاظ على سرية كلية البيانات وفقاً لقانون حماية البيانات وبأنه يوعي الانسحاب من الدراسة في أي وقت أرغب فيه في ذلك.

التوقيع: .............................
التاريخ: .............................

إذا كانت لديك أي استفسارات بشأن مشروع هذا البحث يرجى التواصل مع الآية قاضي عبر البريد الإلكتروني:

10997250@stu.mmu.ac.uk
استبيان المقابلة الأولى

أ- خاص بالمشارك:

معلومات أساسية:

الاسم: ............................................................
رقم المشاركة:.......
العنوان:.......................................................................................
الهاتف: ..............................................................
تاريخ اليوم: __/__/___ : 

هل أنت ذكر أم أنثى؟
(1) ذكر
(2) أنثى

كم عمرك؟
(1) دون 18 سنة (2) بين 18-24 سنة (3) بين 25-34 سنة (4) بين 35-44 سنة (5) بين 45 – 54 سنة (6) بين 55 - 60 سنة (7) أكثر من 61 سنة

تحديد الجنسية التي تحملينها؟
(1) سعودية
(2) غير سعودية

براجا وضع دائرة على أعلى شهادة دراسية حصلت عليها؟
(1) لم التحق بالمدرسة
(2) الشهادة الابتدائية
(3) شهادة المتوسطة
(4) شهادة الثانوية
(5) شهادة الدبلوم
(6) شهادة البكالوريوس
(7) دراسات عليا

هل أنت طالبة?
(1) نعم
(2) موظفة
(3) لا تعملين
(4) متقاعدة

هل تدخنين في الوقت الحالي أو تستخدمين أحد منتجات التبغ يوميًا؟
(1) نعم
(2) لا

هل تتبعين حمية غذائية خاصة؟
(1) نعم
(2) لا

أسئلة طبية ودوانية

منذ متى جرى تشخيص حالتك على أنها حالة داء السكري النوع الثاني؟
(1) سنة واحدة - ستين (2) 3-5 سنوات (3) 6-8 سنوات (4) أكثر من 8 سنوات

هل أصيب أحد والديك أو أحد إخوتك أو أخواتك بداء السكري؟
(1) نعم
(2) لا
هل تعاني من أي مضاعفات لداء السكري؟
(1) نعم (2) لا (3) لا أعلم
إذا كانت الإجابة بنعم برجراء تقديم تفاصيل عن مضاعفات داء السكري التي تعاني منها:
(1) اعتلال الكلى (2) الاعتلال العصبي (3) إعتام عدسة العين الناتج عن الإصابة بداء السكري
(4) أمراض القلب والأوعية الدموية (5) دسلبيديما (ارتفاع نسبة الدهون) (6) اعتلال الشبكية
مضاعفات أخرى:

بصفة عامة، كيف تصفين حالة داء السكري لديك؟
(1) أفضل من ذي قبل (2) أسوأ من ذي قبل (3) لم تتغير
هل تعاني من أي من الحالات الطبية التالية؟ ضع دائرة على كل حالة تعاني منها:
(1) الفشل الكلوي (2) سوء الامتصاص المعوي (مثل مرض كرون والتهاب القولون) (3) مرض الغدة الدرقية والجار (4) فرط كالسيوم الدم (5) أمراض القلب والأوعية الدموية

هل تتناول حاليا أي أدوية موصوفة لك؟ يمكنك استخدام هذه الصور للتعرف على الأدوية

<table>
<thead>
<tr>
<th>الجرعة</th>
<th>اسم الدواء</th>
<th>كم مرة في اليوم؟</th>
</tr>
</thead>
<tbody>
<tr>
<td>منيرين ، مع وجبات الطعام</td>
<td>قرص واحد، 1 جرام</td>
<td>على سبيل المثال، الميتروفين</td>
</tr>
</tbody>
</table>

هل تتناولين مكملات ال维生素؟
(1) نعم (2) لا
إذا كانت الإجابة بنعم، برجراء تحديد نوع الفيتامين والعلامة التجارية له

هل تناولت الفيتامينات قبل 3 أشهر من تاريخ اليوم؟
(1) نعم (2) لا
إذا كانت الإجابة بنعم، برجراء تحديد نوع الفيتامين والعلامة التجارية له

ب - خاص بالباحثة - قياسات الجسم

طول المشاركة __________________سم
وزن المشاركة __________________كم
حساس مؤشر كتلة الجسم __________________م2
نسبة الهموغلوبين السكري الحالية ________________
مستوى الكالسيوم __________________mg/dL
تاريخ الاختبار __________________

رقم المشاركة ________________ غير مناسبة ________________ مناسبة ________________
استبيان المعلومات للمرحلة الثانية

المقابلة الثانية

أ- خاص بالمشاركة:

معلومات أساسية

الاسم:........................................................
رقم المشاركة:................
عنوان:......................................................................................................
هاتف:............................................................ تاريخ اليوم: ....../....../..........

1- نحن نعلم أنه من الصعب تناول الأدوية بانتظام، كم عدد المرات التي لم تتناولين فيها أقراص الفيتامين؟

(1) مرة في الأسبوع (2) مرتان في الأسبوع (3) 3 مرات أو أكثر في الأسبوع (4) لم أتناولها مطلقًا

2- كم عدد المرات التي لم تتذكرين فيها تسجيل تناول أقراص الدواء الخاص بك في المفكرة؟

(1) مرة في الأسبوع (2) مرتان في الأسبوع (3) 3 مرات أو أكثر في الأسبوع (4) لم أسجلها مطلقًا

3- هل تم وصف أي دواء جديده لك منذ أن بدأت في تناول مكملات الفيتامينات، وإذا كان الأمر كذلك،

يرجى تحديد الأدوية الموصوفة؟

(1) لا (2) نعم

إذا كانت الإجابة بنعم، يرجى التوضيح
............................................................................................................
............................................................................................................
............................................................................................................

4- هل توقفتين عن تناول أية دواء موصوفة منذ أن بدأتين في تناول الأقراص؟

(1) لا (2) نعم

إذا كانت الإجابة بنعم، يرجى التوضيح
............................................................................................................
............................................................................................................
............................................................................................................

5- هل تغيرت جرعات الدواء التي تتناوليها منذ أن بدأتين في تناول الأقراص؟

(1) لا (2) نعم

إذا كانت الإجابة بنعم، يرجى التوضيح
............................................................................................................
............................................................................................................
............................................................................................................

6- هل تغيرت جرعات الدواء التي تتناوليها منذ أن بدأتين في تناول الأقراص؟

(1) لا (2) نعم

إذا كانت الإجابة بنعم، يرجى التوضيح
............................................................................................................
............................................................................................................
............................................................................................................
6- هل تناولتين مكملات فيتامين بخلاف تلك المقدمة أثناء الدراسة؟
(1) لا (2) نعم
إذا كانت الإجابة نعم، يرجى التوضيح

7- هل عانيت من أي من الحالات الطبية التالية في الأسابيع الـ16 الأخيرة؟ ضعي دائرة حول أي حالة تعاني منها.
(1) الفشل الكلوي (2) سوء الأيض المعوي (مثل مرض كرون، والتهاب القولون) (3) مرض الغدة الدرقية
(4) فرط كالسيوم الدم (5) أمراض أخرى، يرجى تصددها

8- هل أنت حامل؟
(1) لا (2) نعم

9- هل بدأتي أي جميات غذائية خاصة؟
(1) لا (2) نعم
إذا كانت الإجابة نعم، يرجى التوضيح

10- بصفة عامة، كيف تصфин حالة داء السكري خلال الأسابيع الـ16 الأخيرة؟
(1) أفضل من ذي قبل (2) أسوأ من ذي قبل (3) لم يتغير

11- هل هناك تشخيص لأي حالات أخرى لديكي؟

ب- خاص بالبحث- قياسات الجسم

- وزن المشاركة؟ ___________________________ كجم
- وزن المشاركة؟ ___________________________ كجم
- وزن المشاركة؟ ___________________________ كجم
- وزن المشاركة؟ ___________________________ كجم
• طول المشاركة: __________________ سم
• حساب مؤشر كتلة الجسم: ___________ م²
• معدل الـ AGES

رقم المشاركة: _______________________

قائمة المراجعة:

☐ إكمال الاستبيان
☐ إعادة الأقراس
☐ إعادة الجدول
☐ قياسات الجسم
☐ أخذ عينة دم