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The Effect of Vitamin D$_3$ Supplementation on Markers of Glycaemia, Lipidaemia and Oxidative Stress in Saudi Men with Poorly-Controlled Type-2 Diabetes Mellitus

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

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School of Healthcare Science
Manchester Metropolitan University
PhD 2018
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Abstract

The pathogenesis of diabetes mellitus type 2 (T2DM) has been associated with vitamin D deficiency which plays a role in impaired insulin action in humans. The purpose of this research is to investigate the effect of vitamin D₃ supplementation on biomarkers of glycaemia, lipidaemia and oxidative stress in Saudi men aged >18 years with poorly controlled T2DM. A double-blind, randomised, placebo-controlled, parallel trial was used to investigate 128 Saudi men with poorly-controlled T2DM randomised to receive: 1) a placebo supplement, 2) 50µg/day vitamin D₃ or 3) 100µg/day vitamin D₃ as capsules matched in shape and size over a 16-week period. Fasting glucose, HbA₁c, fasting insulin, lipid profile, serum 25(OH)D, and total antioxidant status were measured, and advanced glycation end products (AGEs) in skin were also measured using an AGE-reader. Vitamin D supplementation of either 100µg or 50µg per day were found to give a significant improvement in the HbA₁c (p<0.001) and a reduction in glucose levels (p<0.001) after 16-weeks’ intervention as compared to the placebo group in both supplementation groups. Significant reductions in total cholesterol (p<0.001) and improvements in HDL levels (p<0.001) after 16-weeks’ intervention were seen compared to the placebo group in both treatment groups. Triglycerides were significantly reduced after 16-weeks’ intervention (p<0.001) only with the higher dose of vitamin D₃ (100µg/day) without any change in the 50µg/day vitamin D₃ group. Vitamin D supplementation failed to demonstrate any improvement in insulin resistance, insulin secretion or oxidative stress such as total antioxidant levels or AGEs as compared to the placebo group after 16-weeks’ intervention. Vitamin D status seems to have a significant role in controlling the development and treatment of diabetes. It is likely that optimised levels of serum vitamin D may be variable between those at high risk.
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.
List of achievements derived from this project

1. Hend Alharbi, Naji Aljohani, Mohammad Maswood, Emma Derbyshire, Nessar Ahmed (2016) Effect of Vitamin D supplementation on markers of dyslipidaemia in Saudi males with poorly-controlled Type 2 Diabetes Mellitus. The 4th International Conference Prehypertension, Hypertension and Cardio Metabolic Syndrome, Venice, Italy (3-6 March 2016).

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGEs</td>
<td>Advanced glycation end products</td>
</tr>
<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>
</tr>
<tr>
<td>BCG</td>
<td>Bromocresol green</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<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 D₃</td>
</tr>
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<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DBP</td>
<td>vitamin D–binding protein</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Ergocalciferol</td>
<td>Vitamin D₂</td>
</tr>
<tr>
<td>FBS</td>
<td>Fasting blood sugar</td>
</tr>
<tr>
<td>FGIR</td>
<td>Fasting glucose/insulin ratio</td>
</tr>
<tr>
<td>g/L</td>
<td>grammes per/litre</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>GLM</td>
<td>General Linear Model</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
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</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostasis model assessment for insulin resistance</td>
</tr>
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<td>HSL</td>
<td>Hormone-sensitive lipase</td>
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<td>Intramuscular injection</td>
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<tr>
<td>IR</td>
<td>Insulin resistance</td>
</tr>
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<td>International unit</td>
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<td>LDL</td>
<td>Low density lipoprotein</td>
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<td>MDA</td>
<td>Malondialdehyde</td>
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</tr>
<tr>
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<td>millimolar</td>
</tr>
<tr>
<td>mmol/L</td>
<td>millimoles per litre</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappaB</td>
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<tr>
<td>ng/ml</td>
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</tr>
<tr>
<td>nmoL/L</td>
<td>nanomoles per litre</td>
</tr>
<tr>
<td>NPDR</td>
<td>Non-proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
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<td>Oxidised LDL</td>
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<td>Pro-oxidants protein carbonyl</td>
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<tr>
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<td>Full Form</td>
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<tr>
<td>---------</td>
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</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>RAGE</td>
<td>Receptors for advanced glycation end products</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised control trial</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>Acid X receptor</td>
</tr>
<tr>
<td>sdLDL</td>
<td>Small dense low density lipoprotein</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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</tr>
<tr>
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<td>Triglycerides</td>
</tr>
<tr>
<td>µg</td>
<td>microgramme</td>
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</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</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 Organization</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>1,25-Dihydroxycholecalciferol</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>25-Hydroxyvitamin-D</td>
</tr>
<tr>
<td>25-OHase</td>
<td>Vitamin D-25-hydroxylase</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction
1.1. Diabetes mellitus

Diabetes is a group of metabolic diseases characterised by hyperglycaemia, which is a common effect of uncontrolled blood glucose concentration and, therefore, of long-term of diabetes, causes serious complications both macrovascular and microvascular, and associated with failure of the eyes, kidneys, and nerve (American Diabetes Association, 2017). These complications result in a significant cause of mortality and morbidity (Ozfirat and Chowdhury, 2010). There are three common types of diabetes, including type 1 diabetes, type 2 diabetes and gestational diabetes (Rhodes et al., 2007).

Diabetes are observed in about 422 million people worldwide (Roglic and the World Health Organization, 2016) and estimates are that 90 % cases suffer from type 2 diabetes (T2DM) (WHO, 2014). The prevalence of diabetes is about 4.9% in UK, 8.3% in Germany, 9.2% in USA, 19% in United Arab Emirates and 24.0% in Saudi Arabia (Guariguata et al., 2014). About 1.6 million deaths are attributed to diabetes each year, and by 2030, it expected that diabetes would be the seventh cause of global deaths (Roglic and the World Health Organization, 2016).

Diabetes, especially T2DM, is a complex disorder, which genetically means there is not a single genetic cause, so there are multiple gene effects in combination with lifestyle and environmental factors (Andersen et al., 2016). Complex disorders often cluster in families, which do not have a clear-cut pattern of inheritance (Flannick & Florez, 2016). The genetic variations are likely to act together with health and lifestyle factors to influence the patient’s overall risk of T2DM (Mohlke & Boehnke, 2015).

T2DM has become a major public health problem in recent years, which increases the risk for developing coronary heart disease, vascular disease, stroke, and also they have a high risk of having hypertension, dyslipidaemia, and obesity (Tuomilehto et al., 2003). Obesity has a significant risk of impaired glucose tolerance and a higher prevalence of obesity with 80% to 90% of people diagnosed with T2DM (Daousi et al., 2006). A study of 14252 Saudi
diabetic patients reported that the majority of the obese diabetics were diagnosed with poor glycaemic control (Shahrani & Al-Khaldi, 2013). Another study, in Kuwait, showed that 48% and 77% obese males and females (respectively) were diabetics (Alarouj et al., 2013). Obesity has been increasing to over 650 million adults worldwide (WHO, 2016). The prevalence of obesity in the UK is about 26% (NHS, 2018), and in Saudi Arabia was 52.9% in 2017 and projected to reach 59.5% by 2022 (Alqarni, 2016). Increasing health awareness, especially in diabetes and its complications, is essential in the prevention of developing diabetes (Aljoudi & Taha, 2009). Thus, an increased health awareness level will help to expand the knowledge in health and diabetic education and that can help to reduce the risk factors for diabetes. It would achieve the self-care, healthy diet and improvement in physical activity to control the development of diabetes (Shrivastava et al., 2013; Agrawal, 2016).

1.1.1. Prevalence of diabetes in Saudi Arabia

The prevalence of diabetes has been increasing since 1987 in all regions of Saudi Arabia. From 1987 to 1999, the prevalence of diabetic males was 2.9% to 9.7% (Fatani et al., 1987; El-Hazmi et al., 1999). Then, between 2004 and 2007, the prevalence of diabetic males increased from 24.2% to 26.2% (Al Osaimi, 2007; Al-Nozha et al., 2004). In 2011 and 2014, the prevalence of diabetes increased from 30.8% to 43.1% (Alqurashi et al., 2011; Al-Quwaidhi et al., 2014). Saudi Arabia had the 7th highest prevalence of diabetes (24.0%) worldwide in 2013 and is anticipated to be the 6th in 2035 (24.5%) for both genders (Guariguata et al., 2014).

The region-specific prevalence of diabetes among the Saudi population is reported to be 27.9% in the Northern region, 26.4% in the Eastern region followed by the Southern region where the prevalence reported to be 18.2% (Al-Nozha, et al., 2004). Although it is well documented that life style contributes significantly to the increased prevalence of T2DM, consanguineous marriage has proved to be a contributing factor to increasing the prevalence of T2DM among Arabs (Bittles, 2001). Anokute (1992) reported a positive correlation between consanguinity and T2DM among the Saudi
population where 80% of all related marriages had a positive family history of T2DM as compared to 20% in non-consanguineous marriages. This could be explained in the lens of genetics, e.g. the SNP marker (rs4812829) (located at 20q13 in the intronic region of HNF4A (Hepatocyte nuclear factor 4 alpha) is linked to an increased susceptibility to T2DM among the Saudi population (Al-Daghri et al., 2013, Al-Daghri et al., 2014). rs4812829 is also linked to T2DM in the South Asia population and in the Japanese population in which five other risk alleles have been identified (Kooner et al., 2011; Fukuda et al., 2012). The rs4812829 marker encodes a transcription factor that binds DNA as a homodimer (Battle et al., 2006). HNF4α is known to control the expression of several genes as the HNF1α (hepatocyte nuclear factor 1 alpha), another transcription factor that in turn regulates hepatic gene expression (Shankar et al., 2013). Nevertheless, mutations in HNF4α have been reported previously to cause mature-onset type 1 diabetes (Eeckhoute et al., 2001).

Another SNP (rs10965250) of the Cyclin-dependent kinase inhibitor-2A/B (CDKN2A/B) has been reported among the Saudi population where the CDKN2A/B has an important role in β-cell function and regeneration (Al-Daghri et al., 2013; Al-Daghri et al., 2014). The rs5215 marker in the potassium inwardly-rectifying channel, subfamily J, member 11 (KNJ11) gene was linked to be associated with type 1 diabetes in a study by Al-Daghri, et al., (2014).
### Table 1.1. The prevalence of diabetes in Saudi Arabia

<table>
<thead>
<tr>
<th>Study</th>
<th>Age</th>
<th>Gender</th>
<th>Prevalence of DM (%)</th>
<th>Methods</th>
<th>Region of Saudi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatani et al., 1987</td>
<td>14–55</td>
<td>male</td>
<td>2.9</td>
<td>Survey</td>
<td>Western Region</td>
</tr>
<tr>
<td>Al-Nuaim, 1997</td>
<td>&gt;15</td>
<td>male</td>
<td>12 Urban; 7 Rural</td>
<td>All Regions</td>
<td></td>
</tr>
<tr>
<td>El-Hazmi et al., 1998</td>
<td>2–77 years</td>
<td>male</td>
<td>5.86</td>
<td>All Regions</td>
<td></td>
</tr>
<tr>
<td>(Warsy &amp; El-Hazmi, 1999)</td>
<td>&gt;14 years</td>
<td>male</td>
<td>9.7</td>
<td>All Regions</td>
<td></td>
</tr>
<tr>
<td>Al-Nozha et al., 2004</td>
<td>30–70 years</td>
<td>male</td>
<td>26.2</td>
<td>Health survey</td>
<td>All Regions</td>
</tr>
<tr>
<td>Al Osaimi, 2007</td>
<td>18–60</td>
<td>male</td>
<td>24.2</td>
<td>Cross sectional study</td>
<td>Riyadh</td>
</tr>
<tr>
<td>Al-Daghri et al., 2011</td>
<td>7–80</td>
<td>male and female</td>
<td>23.1</td>
<td>Cohort study</td>
<td>Riyadh</td>
</tr>
<tr>
<td>Alqurashi et al., 2011</td>
<td>12≥70</td>
<td>male</td>
<td>34.1</td>
<td>Cross-sectional study</td>
<td>All Regions</td>
</tr>
<tr>
<td>Al-Quwaaidhi et al., 2014</td>
<td>male and female</td>
<td>30.8 in 2013 39.5 in 2022</td>
<td></td>
<td>All Regions</td>
<td></td>
</tr>
</tbody>
</table>

#### 1.1.2. Type 2 Diabetes

Type 2 diabetes is known as non-insulin-dependent diabetes and develops because of insufficient production of insulin from β-cells in the pancreas and/or insulin resistance, in contrast to type 1 diabetes, which is characterised by an absolute deficiency of insulin (American Diabetes Association, 2017). Type 2 diabetes can be characterised by insulin resistance. Insulin resistance is a pathological condition, in which the body’s cells are unable to respond effectively to normal levels of insulin (American Diabetes Association, 2009; Cubbon et al., 2016). Therefore, reduced insulin production or insulin resistance leads to the accumulation of glucose in the bloodstream. Insulin acts as a key, unlocking the cells so that they take up glucose, so if there is insufficient insulin, or if it is not working properly, the cells are not completely unlocked or they remain fully locked and glucose accumulates in the bloodstream (Muller-Wieland et al., 2016).

Type 2 diabetes can be diagnosed by fasting blood glucose and glycated hemoglobin (HbA1c) measurements. HbA1c indicates the average level of blood glucose over the previous 3 months and fasting blood glucose tests confirm the diagnosis of diabetes (WHO, 2006; WHO, 2011). Fasting blood glucose test results of ≥ 7.0 mmol/L indicate a diagnosis of diabetes; and a
result of 6.1–7.0 mmol/L or more indicates a high risk of diabetes (WHO, 2006). HbA$_1c$ of 6.5% is the cut-off point for identifying diabetes. A level of HbA$_1c$ ≥ or 8% indicates poorly controlled type 2 diabetes (Rotchford et al., 2002) (Table 1.2).

Table 1.2. The clinical indicators for diabetes diagnosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Fasting blood glucose</th>
<th>HbA$_1c$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>High risk of diabetes</td>
<td>6.1–7.0 mmol/L</td>
<td>6–6.4%</td>
<td>(WHO, 2006; WHO, 2011)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>≥7.0 mmol/L</td>
<td>6.5–7.5%</td>
<td></td>
</tr>
<tr>
<td>Poorly controlled diabetes</td>
<td>≥10 mmol/L</td>
<td>≥8%</td>
<td></td>
</tr>
</tbody>
</table>

1.1.3. Pathophysiology of type 2 diabetes

The pathophysiology of type 2 diabetes is caused by a complex interplay between genetic and environmental factors such as poor dietary intake, obesity and a sedentary lifestyle (Jia et al., 2016).

The changes in glucose metabolism by skeletal muscle and liver can affect normal glucose homeostasis (Lowell & Shulmanz, 2005). Hyperglycaemia can also increase the production of advanced glycation end products (AGEs) which are proteins or lipids that are glycated post exposure to sugars (Bos et al., 2011). AGEs and diabetes are associated with several complications, including oxidative stress (Codoñer-Franch et al., 2012).

Insulin regulates carbohydrate and fat metabolism in the body and triggers cells in the liver, fat tissue and muscle to take up glucose from the blood and store it as glycogen (American Diabetes Association, 2017). Therefore, insulin is secreted in the body in precise amounts to remove excess glucose from the blood, which otherwise would become toxic (Figure 1.1). Excess glucose in the blood for a prolonged duration can lead to accumulation of toxic end products in the eye which will eventually lead to vision impairment. In addition, damage to blood vessels increase the risks of cardiovascular disease, which is a major complication associated with higher blood glucose levels (Jenssen et al., 2017).
1.2. Diabetic complications

Long-term exposure to high levels of sugar and genetically determined susceptibility factors, such as high blood pressure and dyslipidaemia, accelerate the development of diabetic complications (Xu et al., 2014).

The main conditions caused by complications of diabetes are microvascular diseases such as retinopathy, nephropathy, and neuropathy. In addition, macrovascular disease is associated with arterial accelerated atherosclerosis that affect the blood supply to the heart, brain and limbs (Ullah et al., 2016). Figure 1.2 provide a schematic overview of type 2 diabetic complications.
1.3. The pathogenesis of diabetic complications

Hyperglycaemia can be recognised as a major risk of diabetic complications. Several mechanisms have been proposed for pathological alterations related to hyperglycaemia in diabetic patients. These include: aldose reductase (polyol) pathway, protein kinase C (PKC) pathway, the oxidative stress and advanced glycation end products (AGEs) pathway (Giacco & Brownlee, 2010) (Figure 1.3). All of these pathways contribute to oxidative stress.
1.3.1. Aldose reductase (polyol) pathway

Two enzymatic reactions are involved in the aldose reductase (polyol) pathway. The first reaction entails reducing glucose molecules to sorbitol while the second reaction entails oxidising sorbitol to fructose (Chang et al., 2015). In diabetes, activation of the polyol pathway is increased resulting in nicotinamide adenine dinucleotide phosphate depletion, which leads to a reduction in cellular levels of glutathione (Berlanga-Acosta et al., 2013).

1.3.2. Protein kinase C pathway

Multiple isoforms of PKC such as (PKC-α, -β1, -β2, and -γ). These isoforms are activated by phorbol esters, calcium, DAG and phosphatidylycerine (Yu et al., 2013). Diabetes changes the multiple PKC isoforms resulting in many vascular and cellular processes that are abnormal (Giacco & Brownlee, 2010). For instance, the basement membrane becomes thick, changes occur in vessel dilation, and there are endothelial dysfunctions among many others (Saberi et al., 2014).
1.3.3. Oxidative stress

Oxidative stress is an imbalance between oxidants and antioxidants, and causes a reduction in antioxidant capacity (Ceriello, 2000). However, increased in oxidative stress could be related to complications in patients with diabetes such as oxidative DNA damage and insulin resistance (Furukawa et al., 2004; Tiwari et al., 2013). Insulin resistance leads to hyperglycaemia, therefore, acute hyperglycaemia increases the production of reactive oxygen species (ROS) (Choudhury et al., 2017).

1.3.4. Advanced glycation end products (AGEs)

AGEs are irreversibly formed during glycation reactions between sugar and protein (Singh et al., 2014). Levels of AGEs in the circulation could be a related to several diabetic complications (Goh & Cooper, 2008; Singh et al., 2014; Vikram et al., 2014). In type 2 diabetes, the majority of glycation and AGE formation is from glucose (Ahmed, 2005).

1.3.4.1 Glycation Reaction

The glycation reaction pathway starts when glucose binds to proteins forming glycosylation products (Babizhayev et al., 2015). The products are known as Schiff bases and Amadori adducts (Meerwaldt et al., 2008). As time goes by, these adducts go through slow and complex rearrangements that form AGEs (Ott et al., 2014).

Protein glycation occurs when a protein binds covalently to a sugar molecule like glucose or fructose without any enzyme controlling the reaction (Ahmed, 2005; Vlassara & Uribarri, 2014). A compound that is unstable (Schiff base) is formed from the reaction between a sugar molecule like glucose and a free amino group of biological amines (Goh & Cooper, 2008). The Schiff base then goes through rearrangement to form a compound that is more stable called an Amadori product (Bohlooli et al., 2016).
In intermediate stage, there is a breakdown of the Amadori product to a range of dicarbonyl compounds like methylglyoxal, glyoxal and deoxyglucosones that are reactive (Singh et al., 2014). This breakdown of the Amadori product occurs through dehydration and oxidation (Younus & Anwar, 2016). In the advanced stage, oxidation, dehydration and cyclisation reactions result in the formation of AGEs which are compounds that are stable and the reaction is irreversible (Narender et al., 2011). AGEs accumulate over time and interfere with normal physiological functions (Singh et al., 2014) (Figure 1.4).

Figure 1.4. The three stages of glycation
Adapted from (Ahmed, 2005)

1.3.4.2. Autoxidative Glycation

The autoxidative glycation pathway exist glucose with their enediol in equilibrium (Prabhakar et al., 2016). Glucose undergoes autoxidation in the presence of transition metals to form an enediol radical (Ahmed, 2005). This radical reduces molecular oxygen to generate the superoxide radical (O$_2^-$) and becomes oxidised itself to a dicarbonyl ketoaldehyde (Phaniendra et al., 2015). That reacts with protein amino groups forming a ketoimine, which is similar to or more reactive than Amadori products, and participates in AGE formation (Ahmed, 2005). Oxidation reactions are also involved in AGE formation, which is accelerated in the presence of oxygen and reduced under anaerobic conditions (Singh et al., 2014) (figure 1.5).
1.3.4.3. AGE Receptors

The AGEs interact with their cellular receptors to play an important role in developing diabetic complications (Giacco & Brownlee, 2010). AGEs possess cell receptors including macrophage scavenger receptors (Ahmed, 2005; Ott et al., 2014). The AGEs’ receptor (RAGE) is a multi-ligand receptor and a member of the immunoglobulin superfamily of cell surface molecules, and is found on smooth muscle cells, endothelial cells and astrocytes (Ramasamy et al., 2011; Yagihashi et al., 2011).

Studies have shown that RAGE stimulation upregulates key transcription factors implicated in inflammatory responses such as NF-κB (Shamoon et al., 1993; Chang et al., 2011). NF-κB modulates gene transcription for endothelin-1, tissue factors and the generation of pro-inflammatory cytokines (Ahmed, 2005; Yagihashi et al., 2011). There is also enhanced expression of adhesion molecules including vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. The intracellular signalling pathways following activation of RAGE by AGEs (Ramasamy et al., 2011), such as the binding of AGEs with
RAGE in endothelial cells, show the importance of oxidative stress markers as NF-κB becomes activated (Shamoon et al., 1993; Ahmed, 2005) (figure 1.6).

In chronic inflammation of tissues in diabetic patients, RAGE is implicated in the sustained activation of NF-κB that is likely to contribute to the chronicity and unrelenting nature of diabetic target cell stress and dysfunction (Yan et al., 2009). To examine the roles of RAGE in inflammatory mechanisms, experiments have been performed to illustrate the effects of RAGE deletion or antagonism in a range of infectious settings (Ramasamy et al., 2011). Blocking RAGE action was shown to be beneficial and resulted in either improved survival or markedly reduced tissue damage (Ramasamy et al., 2011).

Figure 1.6. Interaction of AGE with RAGE on microphages induces oxidative stress and activation of intracellular signalling causing inflammation
Adapted from (Ahmed, 2005; Yagihashi et al., 2011)

1.4. Role of AGEs in the pathogenesis of diabetic complications

AGEs are usually formed as a result of chronic hyperglycaemia (American Diabetes Association, 2017). Thus, with time, they accumulate in the body and affect the tissues leading to the development of microvascular complications such as diabetic neuropathy, nephropathy and retinopathy (Bos et al., 2011). Additionally, macrovascular complications like atherosclerosis can develop as well (Basta et al., 2004) Figure 1. 7.
1.4.1. Diabetic Retinopathy

Diabetic retinopathy is a complication, which could develop through AGEs. AGEs usually find their way into the retinal vessels of patients with diabetes. Once inside, they cause the basement membrane to thicken and the inner blood-retinal barrier to break down (Goh & Cooper, 2008). Moreover, abnormalities in the endothelial nitric oxide synthase expression are usually observed in retinal vascular endothelial cells that have come into contact with AGEs (Tiwari et al., 2013). This may be the factor contributing to several vaso-regularity abnormalities that occur in the retinal microcirculation in diabetes. AGEs also favor the neovascularisation of the retina and cause the permeability to proteins across the barrier of the retina to increase (Ullah et al., 2016). Microaneurysm formation has also been associated with AGEs in various studies (Giacco & Brownlee, 2010).
1.4.2. Diabetic Nephropathy

Diabetic nephropathy could develop through AGEs. AGEs in the kidneys form complex cross-links which may promote renal damage by stimulating the growth of fibrotic factors (Shera et al., 2004). AGEs also cause the structure of the glomerulus to change by making the basement membrane thicken, thus affecting normal filtration in the kidneys (Giacco & Brownlee, 2010).

1.4.3. Diabetic Neuropathy

Neuropathy in diabetes complication could also develop through AGEs, as they also find their way to the nerves and damage them. For instance, monocytes and endothelial cells are induced to increase cytokines and adhesion molecule production when the AGEs act on specific receptors (Babizhayev et al., 2015). AGEs also affect matrix metalloproteinases leading to damage of nerve fibres. The AGE-RAGE axis plays a role in some mechanisms that cause neuropathy, particularly in sensory deficits. Not only do AGEs contribute to the development of neuropathy, but they also make diabetic neuropathy to become worse (Singh et al., 2014), by reducing the sensorimotor conduction velocity. They also decrease flow of blood to the peripheral nerves (Babizhayev et al., 2015).

1.4.4. Dyslipidaemia

Dyslipidaemia is an essential part of the underlying insulin resistance. Defects in insulin action and hyperglycaemia lead to dyslipidaemia in patients with diabetes (Verges, 2015). In the case of in type 2 diabetes, the obesity/insulin-resistant state that is at the basis of the development of this disease can lead to lipid abnormalities independently of hyperglycemia (Gaggini et al., 2013). The lipid changes associated with diabetes mellitus are attributed to increased free fatty acid flux secondary to insulin resistance (Verges, 2015).

Several factors are likely to be responsible for diabetic dyslipidaemia: insulin effects on liver apoprotein production, regulation of lipoprotein lipase,
of cholesteryl ester transfer protein, and peripheral actions of insulin on adipose and muscle (Filippatos et al., 2017). The release of stored fatty acids from adipocytes requires conversion of stored triglyceride into fatty acids and mono-glycerides that can be transferred across the plasma membrane of the cell (Szalat et al., 2016).

AGEs are prevalent in the diabetic vasculature and contribute to the development of atherosclerosis (Basta et al., 2004). AGEs accumulate in many different cell types that affect the extracellular and intracellular structure and function (Goldin et al., 2006). They lead to macrovascular complications via engagement with the receptor for advanced glycation end products (RAGE) (Goldin et al., 2006). AGEs-bind to the RAGE and increase the endothelial permeability to macromolecules, promoting endothelial dysfunction and thus causing the levels of vascular low-density lipoproteins to go up (Chang et al., 2011). High levels of LDL occur because AGEs reduce their uptake and hence promote the destabilisation of plaques. Therefore, the development of atherosclerosis is accelerated (Schofield et al., 2016).

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**Insulin Resistance**

![Insulin Resistance Diagram](image)

Figure 1.8. The pathogenesis of dyslipidaemia (Adapted from Gaggini et al., 2013)
1.5. Antioxidants

Antioxidants play a vital role in preventing the complications of diabetes. A total antioxidant capacity test in plasma is a marker to assess the situation and the potential of oxidative stress in the body (Aouacheri et al., 2015). Plasma contains many compounds that indicate oxidation in the body and thus protect cells and biological molecules from cellular damage (Rizvi & Maurya, 2007). Low antioxidant capacity can be related to complications in patients with diabetes type diabetes 2 (Kharroubi et al., 2015). Oxidative stress in diabetes develops with a decrease in antioxidant status, which can increase the harmful effects of free radicals (Tiwari et al., 2013).

1.6. Vitamin D

Epidemiological data has shown that an inadequate amount of vitamin D in the body is commonly associated not only with diseases such as rickets, osteomalacia and osteoporosis, but also with cancer, heart disease and type 1 and type 2 diabetes (Martin & Campbell, 2011). A recent report stated that insufficient vitamin D is associated with impaired insulin synthesis as well as insulin secretion (Durmaz et al., 2017). Vitamin D has two forms that are commonly consumed; ergocalciferol (vitamin D$_2$) and cholecalciferol (vitamin D$_3$) (Christakos et al., 2016).

1.6.1. Vitamin D synthesis

The synthesis of vitamin D is through pre-vitamin D$_3$, which is unstable and swiftly changes through a process that is temperature regulated, so that it can become vitamin D$_3$. After turning into vitamin D$_3$, it is released from the skin cells into the extracellular space, where it goes to the dermal capillary bed through the vitamin D–binding protein (DBP) (Holick, 2004). Vitamin D enters the bloodstream and binds with DBP and lipoproteins (Holick, 2004). Vitamin D is discharged from DBP to the liver and undergoes hydroxylation through the vitamin D-25-hydroxylases (25-OHase) to 25-hydroxyvitamin-D (25(OH)D) (Holick, 2004). 25(OH)D is the primary circulating form of vitamin D that can
be used to measure a person’s vitamin D level because, it has a half-life in the circulation of 2 weeks and it correlates with secondary hyperparathyroidism, rickets, and osteomalacia (Holick, 2006).

25(OH)D is linked with DBP, and this complex links further to megalin on the plasma membrane of the renal tubule cell and is transferred into the cell (Holick, 2004). While inside the cell, 25(OH)D is released and is changed in the mitochondria by the 25-hydroxyvitamin D-1α-hydroxylase to generate 25-dihydroxyvitamin D, or 1,25(OH)2D, which is the biologically active form of vitamin D responsible for maintaining calcium and phosphorus homeostasis (Holick, 2006). It attains this through interacting with the nuclear receptor, the vitamin D receptors (VDR) that are located in the cells of the small intestines (Holick, 2006). The 1,25(OH)2D–VDR structure combines with the retinoic acid X receptor (RXR) in the nucleus to create the vitamin D complex. The 1,25(OH)2D-VDR-RXR complex merges with the vitamin D-responsive element (VDRE) for the epithelial calcium channel (Holick, 2006). The augmented expression of the calcium channel allows more calcium to enter the cell, where the vitamin D–dependent calcium-binding protein calbindin 9K assists calcium’s movement into the bloodstream (Holick, 2006).

Figure 1.9. Vitamin D synthesis
(Adapted from Bikle, 2012)
1.6.2. Vitamin D Deficiency in Saudi Arabia

The prevalence of vitamin D deficiency is high among Saudi Arabian men. Vitamin D deficiency was 72.4% in men (Ardawi et al., 2012). Vitamin D deficiency was determined by levels of 25-hydroxy vitamin D (25OHD): The studies of Saudi Arabian men define deficiency as a 25(OH)D serum concentration of ≤ 20 ng/mL, insufficiency as a serum level between > 20 ng/mL and < 30 ng/mL and normal as a serum level ≥ 30 ng/mL (Sadat-Ali et al., 2009).

Table 1.3. Cut-off points of Vitamin D concentration

<table>
<thead>
<tr>
<th>Vitamin D concentration (ng/mL)</th>
<th>Vitamin D concentration (nmol/L)</th>
<th>Classification</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20</td>
<td>&lt;50</td>
<td>Deficiency</td>
<td>(Hollis, 2005; Alshahrani &amp; Aljohani, 2013)</td>
</tr>
<tr>
<td>20-32</td>
<td>50–80</td>
<td>Insufficiency</td>
<td></td>
</tr>
<tr>
<td>32-54</td>
<td>80-135</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>54-90</td>
<td>135–225</td>
<td>Normal in sunny countries</td>
<td></td>
</tr>
<tr>
<td>&gt;100</td>
<td>&gt;250</td>
<td>Excess</td>
<td></td>
</tr>
<tr>
<td>&gt;150</td>
<td>&gt;325</td>
<td>Intoxication</td>
<td></td>
</tr>
</tbody>
</table>

1.6.3. Role of Vitamin D in diabetes

Vitamin D affects β-cell function (there are particular vitamin D receptors in pancreatic cells, the 1α-hydroxylase enzyme exists in pancreatic cells and there is a vitamin D reaction element in the human insulin gene promoter) (González-Molero et al., 2012). Another pathway is that vitamin D can adjust insulin sensitivity (vitamin D triggers the expression of insulin receptor and enhances insulin responsiveness for glucose transport in vitro and contributes to the normalisation of extracellular calcium by ensuring normal calcium influx through the cell membranes) (Kulie et al., 2009). Vitamin D stops systemic inflammation by combining with the promoter region of cytokine genes to impede nuclear transcription factors that can lead to cytokine generation and action) (González-Molero et al., 2012). An active form of vitamin D (dihydroxy vitamin D₃), in the blood, binds to the vitamin D receptor on pancreatic β-cells and boosts insulin receptor expression, which can enhance insulin sensitivity (Thacher & Clarke, 2011).
Table 1.4. The role of vitamin D

<table>
<thead>
<tr>
<th>Gene</th>
<th>Action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDR</td>
<td>Transcription factor when bound to 1,25-dihydroxyvitamin and affects insulin resistance, both with regards to insulin secretion</td>
<td>(Sung et al., 2012; Vangoitsenhoven et al., 2016; Nakashima et al., 2016; Angellotti &amp; Pittas, 2017)</td>
</tr>
<tr>
<td>DBP</td>
<td>The serum carrier of vitamin D metabolites</td>
<td></td>
</tr>
<tr>
<td>1α-hydroxylase</td>
<td>Involved in the metabolism of vitamin D, may influence the susceptibility to type 2 diabetes</td>
<td></td>
</tr>
</tbody>
</table>

1.6.4. The potential mechanisms of vitamin D in type 2 diabetes

The potential mechanisms of vitamin D are that deficiency of both vitamin D and calcium intake may alter the balance of normal insulin release, especially in response to glucose load, which may interfere with extra-cellular and intracellular β-cell calcium pools (Pittas et al., 2007b). The beneficial effects of vitamin D have been demonstrated in both β-cell function and insulin sensitivity studies (Kampmann et al., 2014). It is suspected that vitamin D deficiency might induce higher inflammatory responses and the associated insulin resistance. Therefore, this study supports the assumption that a decrease in vitamin D levels in the body can elevate the chances of type 2 diabetes developing.

The effects of vitamin D and calcium on glucose control in patients with type 2 diabetes has been reported by Pittas et al., (2007b), who conducted a MEDLINE review in January 2007 of clinical trials and observational studies in adults, where the outcome was related to glucose homeostasis. They conducted a meta-analysis study and proposed that a lack of vitamin D and calcium interrupts or pauses glucose control and that nutrient supplementation could be essential to improving glucose management. This suggests that calcium and/or vitamin D may have a role in the future management of type 2 diabetes. In a randomised trial in the UK, Sugden et al. (2008) studied the impact of a single dose of 2500 µg vitamin D$_2$ or placebo for participants with type 2 diabetes. Sampling had a mean age of 64 years and a baseline intake of 25(OH)D of 38 nmol/L. They observed that vitamin D$_2$ enhanced endothelial function, and noted the absence of considerable variation between groups for glycaemic control or insulin sensitivity.
A) Insulin resistance

Vitamin D may have a beneficial effect on insulin action (Sung et al., 2012), directly, by stimulating the expression of insulin receptors and thus enhancing the ability to respond to insulin to transport glucose (Maestro et al., 2000). Indirectly, through its role in extracellular calcium regulation, vitamin D ensures the normal flow of calcium through the cell membranes and cytosolic calcium pools because calcium is necessary for the operation of cell-mediated insulin responses in tissues such as skeletal muscle and adipose tissue (Alvarez & Ashraf, 2010).

B) β-cell

The role of vitamin D in pancreatic β-cell function has direct and indirect effects (Al-Shoumer & Al-Essa, 2015). The direct effect is where vitamin D binds directly to the β-cell vitamin D receptor. The indirect effect is through calcium regulation and the influx of extracellular calcium in β-cells (Wolden-Kirk et al., 2011; Sung et al., 2012).

Figure 1.10. The effect of vitamin D on insulin resistance and β-cell function (Adapted from Van Belle et al., 2013)
1.6.5. Intervention studies of vitamin D and type 2 diabetes

Intervention studies have shown that vitamin D supplementation may increase insulin resistance in patients with type 2 diabetes and may improve their diabetic metabolic control (Martin & Campbell, 2011; Sugden et al., 2008; Al-Shoumer & Al-Essa, 2015), table 5.1.

A 50µg / day dose of vitamin D₃ for 16 weeks in 92 American adults produced a slight improvement in β-cell function (Mitri et al., 2011). A total of 59 non-diabetic, overweight and obese Saudi adults were recruited (31 men, 28 women), who took a similar dose of vitamin D₃ for 18 months (Al-Daghri et al., 2012a) had a favourable outcome in the HDL/LDL ratio and HOMA-β function, which were found to be beneficial for insulin levels. Another study comprising 100 Iranians (70 women and 30 men) who took 1250 µg / week of vitamin D₃ for 8 weeks (Talaei et al., 2013) found that this supplementation level had a significant effect on insulin resistance, and the fasting plasma glucose was significantly reduced.

Nazarian et al., (2011) studied the outcomes of high-dose vitamin D₃ supplements on insulin sensitivity in subjects with vitamin D deficiency and impaired fasting glucose levels, eight of whom were classed as pre-diabetic. Participants were given 250 µg / day of vitamin D for 4 weeks. The findings showed that the acute insulin response to glucose was reduced significantly and insulin sensitivity was enhanced. The authors concluded that high-dose vitamin D₃ supplementation improved insulin sensitivity in subjects with impaired fasting glucose. Thus, in relation to public health, it has been recommended that high-dose vitamin D₃ might deliver an economic means of averting the transition from impaired fasting glucose to diabetes (Nazarian et al., 2011). However, an intake of 17.5 µg / day of vitamin D₃ and 500 mg / day of calcium citrate prevented an increase in plasma glucose and insulin resistance in patients with impaired fasting glucose levels compared with patients treated with a placebo, and found that fasting glucose was unchanged in non–glucose-impaired subjects (Pittas et al., 2007). Support for the findings, of no effects being evident on blood glucose and/or insulin metabolism in non-diabetic subjects when vitamin D levels were corrected has come from Tai et
It has also been that an intake of 20µg / day of vitamin D does not prevent the development of type 2 diabetes (Avenell et al., 2009).

Table 1.5. Intervention studies of vitamin D on glycaemia in diabetes

<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>sample</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugden et al., (2008)</td>
<td>2500µg/week vitamin D₃ for 8 weeks</td>
<td>34 subjects</td>
<td>Significantly improved</td>
</tr>
<tr>
<td>Tai et al., (2008)</td>
<td>250µg vitamin D₃ over 2 weeks</td>
<td>33 subjects</td>
<td>No effect on glycaemia</td>
</tr>
<tr>
<td>Jorde et al., (2009)</td>
<td>1000µg/week Vitamin D₃ or placebo for 6 months</td>
<td>36 subjects with T2DM</td>
<td>No significant effects on glucose metabolism</td>
</tr>
<tr>
<td>Gallagher et al., (2009)</td>
<td>20µg vitamin D for 24 to 62 months</td>
<td>5,292 subjects in England and Scotland</td>
<td>No improvement in glycaemia</td>
</tr>
<tr>
<td>Nagpal et al., (2009)</td>
<td>3000µg of vitamin D₃ over 6 weeks</td>
<td>41 male subjects</td>
<td>Increased insulin sensitivity</td>
</tr>
<tr>
<td>Nazarian et al., (2011)</td>
<td>250µg/day vitamin D₃</td>
<td>8 subjects (3 men, 5 women)</td>
<td>Improved insulin sensitivity in subjects with impaired fasting glucose</td>
</tr>
<tr>
<td>Nikooyeh et al., (2011)</td>
<td>Fortified yogurt drink (25 µg /d vitamin D₃), or fortified yogurt drink (25 µg /d and calcium 500 mg/d) or plain yogurt drink for 12 weeks</td>
<td>90 patients with T2DM</td>
<td>Fasting serum glucose, HOMA-IR and HbA₁c decreased significantly more in both vitamin D groups than in the plain yogurt group</td>
</tr>
<tr>
<td>Mitri et al., (2011)</td>
<td>50µg/day vitamin D₃ For 16 weeks</td>
<td>92 American adults</td>
<td>Slight improvement in β-cell function</td>
</tr>
<tr>
<td>Al-Daghri et al., (2012a)</td>
<td>50µg vitamin D for 18 months</td>
<td>92 type 2 Saudi diabetics (34 men, 58 women)</td>
<td>Improved insulin levels more in women than men.</td>
</tr>
<tr>
<td>Breslavsky et al., (2013)</td>
<td>25µg/day vitamin D₃ or placebo for 12 months</td>
<td>32 subjects</td>
<td>No improvement in glucose homeostasis parameters</td>
</tr>
<tr>
<td>Talaei et al., (2013)</td>
<td>1250µg/week vitamin D₃ for 8 weeks</td>
<td>100 Iranians (70 women and 30 men)</td>
<td>Significant effect on insulin resistance fasting plasma glucose</td>
</tr>
<tr>
<td>Elkassaby et al., (2014)</td>
<td>150 µg /d vitamin D₃ or placebo 6 months</td>
<td>50 patients with type 2 diabetes</td>
<td>Improvement of fasting plasma glucose after 3 months that not shown after 6 months</td>
</tr>
<tr>
<td>Ryu et al., (2014)</td>
<td>25 µg /d vitamin D₃ or placebo after 24 weeks</td>
<td>158 Korean patients with T2DM</td>
<td>No effect on glycaemic control</td>
</tr>
<tr>
<td>Jehle et al., (2014)</td>
<td>7500 µg vitamin D₃ or placebo for 6 months</td>
<td>55 patients with T2DM</td>
<td>Insulin sensitivity (HOMA-IR) and HbA₁c improved in the vitamin D group</td>
</tr>
<tr>
<td>Tabesh et al., (2014)</td>
<td>1250 µg /week vitamin D₃, calcium</td>
<td>118 patients with T2DM</td>
<td>Significant decrease in serum insulin, HbA₁c,</td>
</tr>
<tr>
<td>Study</td>
<td>Intervention</td>
<td>Participants</td>
<td>Outcomes</td>
</tr>
<tr>
<td>-------</td>
<td>--------------</td>
<td>--------------</td>
<td>----------</td>
</tr>
<tr>
<td>Sadiya et al., (2015)</td>
<td>1000 mg/d, or placebo for 8 weeks</td>
<td>87 vitamin D deficient obese patients with T2DM</td>
<td>No effects on fasting blood glucose, HbA1c</td>
</tr>
<tr>
<td>Krul-Poel et al., (2015b)</td>
<td>150 µg/d vitamin D₃ for 3 months followed by 75 µg/d for 3 months or placebo for 6 months</td>
<td>261 non-insulin dependent T2DM</td>
<td>Significant decrease of HbA1c after 6 months in the vitamin D group compared with the placebo group</td>
</tr>
</tbody>
</table>

### 1.6.6. Intervention studies of vitamin D and dyslipidaemia

Intervention studies have found an association between vitamin D supplementation and improvement in dyslipidaemia (Alkharfy et al., 2013; Mohamad et al., 2016). Dyslipidaemia is a major risk factor in developing cardiovascular disease (Paciaroni et al., 2007). Vitamin D deficiency has been related to an increase in total cholesterol and increase in triglycerides (Martins et al., 2007; Karhapaa et al., 2010; Chaudhuri et al., 2013), Table 1.6.
Table 1.6. Effect of vitamin D intervention studies on dyslipidaemia in diabetes

<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>sample</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jorde &amp; Figenschau (2009)</td>
<td>1000 µg / week of vitamin D for 6 months</td>
<td>36 T2DM patients</td>
<td>No change in lipid profiles</td>
</tr>
<tr>
<td>Ponda et al. (2012)</td>
<td>1250 µg / week of vitamin D for 8 weeks</td>
<td>151 subjects with high risk of cardiovascular disease</td>
<td>No effect on the lipid profile</td>
</tr>
<tr>
<td>Talaei et al. (2013)</td>
<td>1250 µg / week of vitamin D for 8 weeks</td>
<td>100 Iranian T2DM patients</td>
<td>No change in the levels of serum total cholesterol, LDL, HDL or triglycerides</td>
</tr>
<tr>
<td>Alkharfy et al. (2013)</td>
<td>50µg vitamin D daily for 12 months</td>
<td>499 Saudi T2DM patients</td>
<td>Significant improvements in serum triglycerides and total cholesterol, as well as HDL in men</td>
</tr>
<tr>
<td>Breslavsky et al. (2013)</td>
<td>25µg / day of vitamin D for 12 months</td>
<td>24 T2DM subjects</td>
<td>No significant result in serum total cholesterol, LDL, HDL and triglycerides levels.</td>
</tr>
<tr>
<td>Eftekhari et al. (2014)</td>
<td>0.25 µg of vitamin D twice per day or placebo tablets for 12 weeks</td>
<td>70 Iranian T2DM subjects</td>
<td>No statistically significant changes between all the variables of groups</td>
</tr>
<tr>
<td>Ramiro-Lozano &amp; Calvo-Romero, (2015)</td>
<td>400 µg of vitamin D per week for 2 months</td>
<td>28 T2DM patients</td>
<td>Not statistically significant changes</td>
</tr>
<tr>
<td>Mohamad et al. (2016)</td>
<td>112.5µg/day of vitamin D for 8 weeks</td>
<td>100 T2DM patients</td>
<td>Significant increase in serum HDL and significant decrease in total cholesterol and LDL levels</td>
</tr>
<tr>
<td>Sadiya et al. (2015)</td>
<td>150 µg / day of vitamin D for 12 weeks, then another 75 µg / day for 12 weeks</td>
<td>87 Emirati obese T2DM patients</td>
<td>No effect on the lipid profile</td>
</tr>
</tbody>
</table>

1.6.7. Intervention studies of vitamin D and oxidative stress

Evidence of the action of vitamin D against oxidative stress has been established. A study by Salum et al. (2013) compared diabetic rats that received 12.5 µg/day of vitamin D₃ for 10 weeks with untreated diabetic rats. The researchers found there was a significant improvement in the total antioxidants in diabetic rats with an intake of vitamin D₃ compared with untreated diabetic rats ($P < 0.001$). A Turkish study of 23 subjects deficient in vitamin D who took 250 µg/day of vitamin D₃ for 12 weeks, determined that vitamin D had a significant effect on reducing oxidative stress (Tarcin et al., 2009).
Table 1.7. Effect of vitamin D intervention studies on oxidative stress in diabetes

<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>sample</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salum et al. (2013)</td>
<td>12.5µg/day of vitamin D&lt;sub&gt;3&lt;/sub&gt; for 10 week</td>
<td>Diabetic rats</td>
<td>Significant improvement in the total antioxidants P&lt;0.001</td>
</tr>
<tr>
<td>Tarcin et al., (2009)</td>
<td>250µg/day of vitamin D&lt;sub&gt;3&lt;/sub&gt; for 12 weeks</td>
<td>23 Turkish subjects</td>
<td>Significant effect on reducing oxidative stress</td>
</tr>
<tr>
<td>Yiu et al., (2013)</td>
<td>125µg/day vitamin D&lt;sub&gt;3&lt;/sub&gt; for 12 weeks</td>
<td>100 Hong Kong subjects</td>
<td>No significant effect on serum biomarkers of inflammation and oxidative stress with T2DM</td>
</tr>
<tr>
<td>Eftekhari et al., (2014)</td>
<td>0.25 µg of vitamin D twice per day or placebo tablets for 12 weeks</td>
<td>70 T2DM patients</td>
<td>Reduction of malondialdehyde, but not statistically significant</td>
</tr>
<tr>
<td>Nikooyeh et al., (2014)</td>
<td>Fortified yogurt drink (25 µg/d vitamin D&lt;sub&gt;3&lt;/sub&gt;), or (25 µg/d and calcium 500 mg/d) or plain yogurt drink for 12 weeks</td>
<td>90 T2DM subjects aged 30-50 years</td>
<td>Significant decrease in serum AGEs and increased levels of the antioxidant superoxide dismutase</td>
</tr>
<tr>
<td>Krul-PoeI, Agca, et al., (2015a)</td>
<td>1250µg/month or a placebo for 6 months</td>
<td>245 patients with T2DM</td>
<td>No effect observed on skin AGE accumulation compared to the placebo</td>
</tr>
<tr>
<td>Shab-Bidar et al., (2015)</td>
<td>Fortified yogurt containing 25µg of vitamin D&lt;sub&gt;3&lt;/sub&gt; and after 12 weeks</td>
<td>100 T2DM patients</td>
<td>Significant improvement in serum total antioxidant capacity and malondialdehyde</td>
</tr>
</tbody>
</table>

1.7. Aims and Objectives

1.7.1. Aims

The purpose of this study is to test whether vitamin D<sub>3</sub> supplementation can reduce the parameters of glycaemia, oxidative stress and lipoedema in Saudi men aged 18 to 60 years with poorly controlled T2DM. Study participants were divided into three study groups and evaluated for 16 weeks. One group received a placebo, the second group a vitamin D supplement with a dose of 50µg and the third group a supplement with a dose of 100µg.
1.7.2. Objectives

- To study the effect of vitamin D₃ supplementation on glycaemia, including blood glucose levels, insulin levels and glycated haemoglobin (HbA₁c).

- To study the effect of vitamin D₃ supplementation on lipidaemia, including low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides and total cholesterol.

- To study the effect of vitamin D₃ supplementation on oxidative stress, including total antioxidant levels and advanced glycation end products (AGEs).
Chapter 2 Methods and materials
2.1. Materials, equipment and software

2.1.1. Materials

- Microcrystalline cellulose placebo; 50µg vitamin D₃; 100µg vitamin D₃ (Metabolics Ltd, Devizes, UK)
- 96-well microtitre plate format (Eppendorf, Germany)
- 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 (BCP) kit (Konelab i20, Finland)
- HbA₁c DCA Vantage Analyzer (Siemens, Germany)
- Calcium kit (Konelab i20, Finland)
- Glucose (GOD-POD) kit (Konelab i20, Finland)
- Cholesterol kit (Konelab i20, Finland)
- Triglycerides kit (Konelab i20, Finland)
- HDL-Cholesterol Plus kit (Konelab i20, Finland)
- Insulin immunoassay kit (Roche Diagnostics, USA)
- Total vitamin D immunoassay kit (Roche Diagnostics, USA)
- Eppendorf tubes (Eppendorf company, Germany)
- Sterile Gloves (Ansell, Canada)
- Deionised water (Local store, Saudi Arabia)

2.1.2 Equipment

- Automated electrochemiluminescence assays (Roche Cobas E401, USA)
- OxiSelect TAC assay kit (Cell Biolabs, USA)
- Pipettes (Eppendorf, Germany)
- Spectrophotometric microplate reader (BioTek, USA)
- AGE reader (Diagnoptics Technologies B.V., Groningen, The Netherlands)
- 50uL to 300uL adjustable single and multichannel micropipettes (Eppendorf, Germany)
- Standard tape measure (Seca, Germany)
- Free-standing stadiometer (Seca, Germany)
- Weighing scales (Seca, Germany)
- 21G syringe needles (BD, USA)
- EDTA, serum and lithium heparin tubes for blood collection (BD Vacutainer®, USA)
- Centrifuge 5702 (Eppendorf, Germany)
- Laboratory freezer – 20°C (Philip Kirsch, Germany)
- Laboratory freezer - 80°C (Philip Kirsch, Germany)
- Laboratory fridge (Philip Kirsch, Germany)
- Orbital shaker (Labtech, 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)
2.2. Ethical approval

Ethical approval was granted by the Faculty Research Degrees office of Manchester Metropolitan University on 1st of May 2014 (SE121327A1) appendix 1. The research was approved by King Fahad Medical City (KFMC) on 1st April 2014 in Riyadh appendix 2. The clinical laboratory was approved to analyse the blood samples by Prince Mutaib, Chair for Biomarkers of Osteoporosis, King Saud University, Riyadh, KSA on 15th May 2014 (4/67/429706) appendix 3.

In addition, the study had to be registered in the Saudi clinical trials registry (SCTR) appendix 4, application #14062303, in order to clear the supplement products for entry to Saudi by the Saudi Food and Drug Authority (SFDA) on 18th August 2014 appendix 5. These ethics approvals can be found as appendices 1-5.

2.3. Study design

The study was conducted over 16 weeks and took the form of a randomised double-blind clinical trial in order to avoid any potential bias. If researchers know that they are administering a placebo, they may convey doubts about its effectiveness to the subject (Schulz & Grimes, 2002). In double-blind clinical trials, it is often necessary to blind products using over-encapsulation. Double-blind designs can also increase the number of units a patient needs to take each day.
Figure 2.1. Flowchart of the parallel-randomised trial of the three groups
Individuals in the vitamin D groups received 100µg or 50µg vitamin D₃ per day, and those in placebo received a cellulose tablet per day as controls, at study baseline to the 16 weeks of the intervention.

2.4. Total sample size

A power analysis was conducted using G*Power 3 software (Faul et al., 2009) to determine the minimum total sample size for conducting two mixed ANOVA repeated measurements between the groups in this study. For this study, the
measurements were repeated at two different times (baseline and after 16 weeks) between the three groups (A, B and C).

G*Power 3 computed that a minimum total sample size of n=120 patients was necessary to correctly test the hypotheses of two mixed ANOVA repeated measurements between the groups at the conventional α=0.05 significance level and an effect size=0.25. To make allowances for the expected loss of patients and missing patients between the baseline and final measurements, a further 20% was added, so that the total sample size at the baseline should be about n=144. Had the loss of patients during the trial been more than 20%, then the results would have been invalid and not been publishable (Schulz & Grimes, 2002; Thoma et al., 2010).

2.5. Intervention trial

The patients were randomly assigned to receive one of three oral treatments: 100µg/day of vitamin D₃ (Group A), 50µg/day of vitamin D₃ (Group B), or a placebo (Group C) as control. This placebo contained only microcrystalline cellulose as used previous studies (Pittas et al., 2007; Zhu et al., 2012; Asemi et al., 2016). Cellulose was chosen as the placebo because it cannot be absorbed by the body and it binds the ingredients of the supplement together as well as being used as a filler (Grossmann & Tangpricha, 2010; Kaptchuk et al., 2010). Including a placebo group in a trial provides a normal baseline of serum vitamin D and shows any fluctuations throughout the seasons. This type of study has been particularly that the doses of vitamin D you have used have been successful in previous studies to raise blood concentrations of vitamin D (Nagpal et al., 2009; Mitri et al., 2011; Beilfuss et al., 2012; Aljohani, 2016).

The patients were randomly allocated to one of the three intervention groups to avoid selection bias (Misra, 2012), and were recruited by requests introduced at their follow-up appointment with the diabetic specialist. These arrangements were distributed by an unprejudiced third party (Biostatistician Specialist at KFMC) to this investigation by block randomisation methods (Suresh, 2011).
Block randomisation design was used to allocate a balanced number of subjects to each group by Excel (Kim & Shin, 2014). It is recommended to apply this method when total sample numbers are more than 100 (Kim & Shin, 2014). The allocator must conceal the block size from the executer and utilise randomly blended block sizes (Kim & Shin, 2014). In this study, 13 blocks, each of 12 patients, were used for a total for 156 patients (appendix 11). This randomisation was done by a third party at the clinical pharmacy of KFMC, and disguised the allocation to the groups from the diabetic consultant and researcher, subsequently reducing the bias further.

A clinical pharmacist prepared tablets “A”, “B” or “C” and placed them into bottles as indicated by the distribution orders. In addition, the diabetic consultant, researcher and patients were all blinded to which meeting would be allocated with which supplement dosage or placebo tablets, all tablets and packaging appearing identical. Blinding has been a key feature of trials examining vitamin D₃ supplementation on controlling diabetes with a particular effort to avoid bias (Pittas et al., 2007; Nagpal et al., 2009; Mitri et al., 2011; Krul-Poel et al., 2015b; Sadiya et al., 2015).

### 2.6. Safety doses of vitamin D

A safety margin of vitamin D is recommended to avoid toxicity as consequence of hypercalcaemia, though, this is problem is very rare (Ozkan et al., 2012). There is an upper safety limit of 250 µg/day for vitamin D supplementation to avoid unfavourable results (Hathcock et al., 2007; Vogiatzi et al., 2014). In our study, the doses of vitamin D₃ supplementation were 50µg or 100µg/day so all supplementation was within the safe dose limit. Corrected calcium levels were measured before the intervention commenced and patients who had a higher corrected calcium level were excluded from the intervention study to avoid hypercalcaemia.
2.7. Recruitment and setting of the trial

The study was conducted in the outpatient clinic of King Fahad Medical City in Riyadh, Saudi Arabia. Patients with T2DM, aged 18–60 years old, were recruited to take part in the study over a 16-week period, following invitations to consecutive clinical attenders.

The researchers and nurses then conducted interviews so the consent forms and the medical questionnaires could be completed, and made the anthropometric measurements and took AGE readings for each patient who was included in the study. Subjects were randomly assigned to individual recalls based on their subject number and follow-up appointment. Following collection of all data, all participants were given presentations about the study by the researchers, who then helped them to fill out a questionnaire, and explained how to fill in a supplement-recoding calendar at baseline. Then, patients collected their intervention supplement tables from the pharmacy window after the consultant prescribed the blinded supplements for this study by special code of this trial in KFMC the system. These codes were made available to the clinical pharmacy by a special system in KFMC. At the end of the study, the researchers and nurses filled out a debriefing questionnaire, and recorded the anthropometric measurements and AGE readings for each patient.

Patients’ demographic data and medication before and after supplementation was recorded. They were advised to maintain their usual diet and about other habits that could affect their level of vitamin D and metabolism, as well as being advised to avoid taking any supplements during the study. Blood samples were taken both at baseline and at the end of the study by a phlebotomist.

Laboratory tests were carried out by trained laboratory staff at the clinical laboratory in the Prince Mutaib Chair for Biomarkers of Osteoporosis, King Saud University, Riyadh, Saudi Arabia. Study coordination, statistical analysis and subsequent work were undertaken at Manchester Metropolitan University.
2.8. Inclusion and exclusion criteria

All participants were Saudi men with T2DM with the following inclusion criteria: a glycated haemoglobin (HbA1c) > 8 mmol/L (Talaei et al., 2013), a BMI > 25, an age between 18 and 60 years (Mitri et al., 2011) Al-Daghri et al., 2012) and being of Saudi Arabian origin.

All subjects with renal disease, diabetes mellitus type 1, major systemic illnesses such as gastrointestinal malabsorption, parathyroid disease, hypercalcaemia, or diabetes requiring medication, were excluded from the present study. Participants who do not use all the capsules were defined as non-compliant and excluded from the study (Table 2.1).

Table 2.1. Inclusion and exclusion criteria of the intervention study

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 - 60 years</td>
<td>Major systemic illness such as renal disease</td>
</tr>
<tr>
<td>HbA1c &gt; 8%</td>
<td>Diabetes mellitus type 1</td>
</tr>
<tr>
<td>BMI &gt; 25</td>
<td>Diabetes requiring medication including vitamin D supplements</td>
</tr>
<tr>
<td>Saudi citizen</td>
<td>Participants who may have altered their hypoglycaemic medication during study</td>
</tr>
</tbody>
</table>

2.9. Consent forms

The consent form sheets showed the trial information to make sure all subjects were convinced to be a part of the intervention (Hernandez et al., 2014). Consent forms should be written in simple language to avoid misunderstanding, particularly with literacy levels (Gupta, 2013). The best consent forms demonstrate a brief overview of a study with the focus on important points (Grady et al., 2017). In this study, the consent form sheets were given to subjects and the researcher explained to them how the intervention study worked. Subjects had a one-to-one interview with researchers to fully understand the study pathway at all stages. Before the subjects signed the consent sheet to be part of this study, researcher asked them to make sure if they accepted or declined (Appendix 7).
2.10. Pre-study screening questionnaire

Potential participants attended a meeting with the researcher where their eligibility for the study was assessed. The researcher sat with the potential patients and first checked if they met the inclusion criteria, then those who did, completed the pre-study screening questionnaire with the researcher.

The pre-study screening questionnaire asked questions were simple and translated so as to be understandable. It was recommended to be not too formal and not too medical. (Sullivan, 2011). The present study questionnaire was in two categories. The first category was the personal information, were the questions were closed and the participant chose the appropriate answer (Tonna et al., 2007). Some questions were open-ended but these are hard to analysis. (O'Cathain & Thomas, 2004)

The questionnaires were conducted via a face-to-face interview with the researcher to make sure that any misunderstanding that could lead to unanswered questions could be solved (Patterson et al., 2015). The questionnaires covered basic background to indicate the patient’s gender, age, marital status, education, employment and smoking status. It also indicated the medical condition and the patient’s intake of vitamin D for the three months prior the study. (Appendix 8).

2.11. Anthropometric measurements

Body mass index (BMI) is a strong tool to predict the percentage of body fat and incidence of T2DM (Schulze et al., 2012). It measures the body fat based on height and weight that applies to adults and is universally expressed in units of kg/m² (Gorstein & Akre, 1988).

2.12. Advanced glycation end products (AGEs)

A skin autofluorescence reader was used to measure tissue AGEs. Autofluorescence has been validated against AGE measurements by skin
biopsies from the site of skin autofluorescence measurements (Meerwaldt et al., 2005; Bos et al., 2011; Krul-Poel et al., 2015b).

This is a method of assessing AGE levels within the skin in less than 30 seconds and is a non-invasive procedure. 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 wavelengths of auto-fluorescence produced by the AGEs, and its intensity, correlate to the quantity of AGEs present within the skin tissue (Diagnoptics, 2012).

2.13. Blood collection

Subjects were asked to fast overnight (at least 8 hours with no food or beverage). Venous blood samples were required for each patient and were taken by registered phlebotomists in the procedure room in KFMC next to the clinic, using an aseptic technique. A normal 4 mL vacutainer tube was used to collect the sample for serum insulin and vitamin D tests and a 2 mL vacutainer tube with EDTA was used to get whole blood for glycated haemoglobin (HbA1c) tests. A 4 mL vacutainer containing lithium and heparin was to collect the blood sample to test lipids, fasting glucose, calcium, albumin and antioxidant levels. All blood samples were obtained within a consistent time period and the patient’s information was recorded on these containers as Name, Date of Birth and Hospital number (or equivalent) for identification. The withdrawn blood was taken to lab to be centrifuged and kept in the freezer at -20°C until end of each week. All samples were then transferred to store off-site at the laboratory of the Prince Mutaib Chair for Biomarkers of Osteoporosis, King Saud University at a temperature of -80°C until the intervention study had finished. The researcher along with clinical technicians ran all the samples to analyse them.
2.14. Supplement compliance

In this intervention study, patients were followed up using a supplement calendar sheet (Jimmy & Jose, 2011) (Appendix 10). It is technique helpful to measure compliance at the end of a study (Hubbard et al., 2012). Previous intervention studies have used the same technique of supplement calendar sheets (von Hurst et al., 2010). There were follow-up phone messages to remind the patients to take their medication as directed. Patients were asked to take their vitamin D or placebo capsules after a meal with water.

Compliance was assessed through interviews and the number of unused capsules that were returned in the tablet bottle to the clinical pharmacy. Participants who did not use 80% of the capsules were defined as noncompliant and excluded from the study (Dodd & Webb, 2000). Subjects reported difficulty in taking 4 capsules per day for 6 months (von Hurst et al., 2010). This was taken into account in the present study to reduce the number of capsules, as this could negatively affect compliance.

2.15. Debriefing questionnaire

The debriefing questionnaire contained questions about the need for assistance and the presence of questionnaire items which were confusing, or difficult to answer. The questionnaire was used assess how easy the participants found it to comply with the study, any changes in medical status or medication, and any changes to diet. It also invited comments for any other information or opinions patients might wish to give (Bjordal et al., 2000). (Appendix 9).

2.16. Blood analysis

All methods were in accordance with the procedures in place and the clinical laboratory and information were received at the Prince Mutaib Chair for Biomarkers of Osteoporosis, King Saud University, Riyadh, Saudi Arabia.
Storage and deposition of blood samples followed the Human Tissue Act and Manchester Metropolitan University guidelines.

Figure 2.2. Blood analysis flowchart

2.16.1. Measurement of vitamin D concentration

The technique that was used in this study was a competitive electrochemiluminescent protein-binding assay (Orwoll et al., 1994; Pittas et al., 2007). Analysis was carried out using a Roche Cobas E411 analyser. Controls for the various concentration ranges were run individually at least once every 24 hours when the test is in use, once per reagent kit, and following each calibration. This was designed to measure total vitamin D concentration (25-OH-vitamin D) in serum or plasma (see the insert sheet in Appendix 12).
2.16.2. Measurement of the corrected calcium concentration

Corrected calcium concentration was calculated after analysis of the calcium and albumin blood tests using a chemical analyser by Konelab 20. It was a fully open system which allows for a fully flexible solution to meet any colorimetric or enzymatic requirements (see insert sheets in Appendix 13 and 14). The calibration was by an automated series dilution from a stock calibrator.

Corrected calcium was calculated using the formula of Jain et al. (2008):

\[
\text{Corrected total calcium (mmol/L)} = \text{total calcium (mmol/L)} + 0.01 \left[30 \left( \frac{g}{L} \right) - \text{albumin (g/L)} \right]
\]

2.16.3. Measurement of fasting blood glucose concentration

Fasting blood glucose was measured using a chemical analyser (Konelab) (see insert sheet in Appendix 15). Calibration was by an automated series dilution from a stock calibrator. Previous studies used the same technique (Al-Daghri et al. 2012; Al-Othman et al. 2012). Cut-off points for fasting blood glucose are listed in section 1.2.2., Table 1.2. (see insert sheet in Appendix 15)

2.16.4. Measurement of glycated haemoglobin (HbA\textsubscript{1c})

This study measured HbA\textsubscript{1c} using a Siemens DCA Vantage immunoassay analyser. HbA\textsubscript{1c} sample volume required was 1µL whole blood. Preparation of the sample required no pipetting or pretreatment. Automatic calibration was with every cartridge traceable to International Federation of Clinical Chemistry reference materials and test methods for measurement of HbA\textsubscript{1c}, and results came within 6 minutes (see insert sheet in Appendix 16). Cut-off points for HbA\textsubscript{1c} are in section 1.1.2., Table 1.2.
2.16.5. Measurement of fasting insulin

Fasting insulin in similar studies has been determined using a number of different techniques, most notably radioimmunoassay (Raghuramulu et al., 1992; Pittas et al., 2007; Mitri et al., 2011) and enzymatic methods (von Hurst et al., 2010; Al-Daghrí et al., 2012a). Insulin levels were measured using the electrochemiluminescent immunoassay, as used by Nagpal et al., (2009); Tarcin et al., (2009); Shab-Bidar et al., (2011). The Roche electrochemiluminescent immunoassay was used and analysed using a Cobas E401 analysis system (see insert sheet in Appendix 17). Controls for the various concentration ranges were run individually at least once every 24 hours when the test was use, once per reagent kit, and following each calibration and stored at 2-8 °C.

Table 2.2. Cut-off points of fasting insulin

<table>
<thead>
<tr>
<th>Classification</th>
<th>Cut-off</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal level</td>
<td>5 to 11 μU/mL</td>
<td>Lee et al., 2006</td>
</tr>
<tr>
<td>Standard level</td>
<td>&lt;12 μU/mL</td>
<td></td>
</tr>
<tr>
<td>Insulin resistance levels</td>
<td>12.94 to 17 μU/mL</td>
<td></td>
</tr>
</tbody>
</table>

2.16.6. Calculating the homeostasis model assessment

Insulin resistance and β-cell dysfunction have been shown to be vital in the pathogenesis of T2DM (Lee et al., 2016). Insulin resistance has been assessed by the homeostasis model (HOMA-IR) (Niemczyk et al., 2013). HOMA-IR is a frequently used marker in clinical research studies (Katsuki et al., 2001; Wallace et al., 2004). β-cell function and insulin resistance can be estimated by the homeostasis model assessment (HOMA), derived from calculating the balance between hepatic glucose secretion and insulin secretion from fasting levels of glucose (in mmol/L) and insulin using the following formulas (Matthews et al., 1985; Wallace et al., 2004):

\[
HOMA - \beta = 20 \times \frac{[\text{Insulin}]}{([\text{Glucose mmol/L}] - 3.5)}
\]

\[
HOMA - IR = \frac{[\text{Glucose mmol/L}] \times [\text{Insulin}]}{22.5}
\]
2.16.7. Measurement of lipid concentrations

Lipid profiles were measured using a chemical analyser (Konelab, Espoo, Finland) for total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL), (see insert sheet in Appendices 18, 19 and 20). Calibration was by an automated series dilution from a stock calibrator. Previous studies have used the same analysis (Al-Daghri et al., 2012a; Al-Othman et al., 2012).

Low-density lipoprotein cholesterol (LDL) levels were calculated using the Friedewald Formula as follows, (all measurements were in mmol/L). It was estimated to be most accurate for triglyceride concentrations below 4.5 mmol/L (de Cordova and de Cordova, 2013). It was calculated as:

\[
LDL = Total\ cholesterol - HDL - (Total\ triglyceride \div 2.19)
\]

Table 2.3. The American Heart Association reference range of lipid profiles and classifications

<table>
<thead>
<tr>
<th>Lipid profile</th>
<th>Cut-off</th>
<th>Classification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>Below 5.2 mmol/L</td>
<td>Desirable</td>
<td>(The American Heart Association, 2013)</td>
</tr>
<tr>
<td></td>
<td>5.2-6.2 mmol/L</td>
<td>Borderline high</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Above 6.2 mmol/L</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>Below 1.7 mmol/L</td>
<td>Desirable</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.7-2.2 mmol/L</td>
<td>Borderline high</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.3-5.6 mmol/L</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Above 5.6 mmol/L</td>
<td>Very high</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>Below 1 mmol/L</td>
<td>Poor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-1.5 mmol/L</td>
<td>Better</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Above 1.5 mmol/L</td>
<td>Best</td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>Below 1.8 mmol/L</td>
<td>Best for people who have heart disease or diabetes</td>
<td>(The American Heart Association, 2013)</td>
</tr>
<tr>
<td></td>
<td>Below 2.6 mmol/L</td>
<td>Optimal for people at risk of heart disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.6-3.3 mmol/L</td>
<td>Near optimal if there is no heart disease. High if there is heart disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4-4.1 mmol/L</td>
<td>Borderline high if there is no heart disease. High if there is heart disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.1-4.9 mmol/L</td>
<td>High if there is no heart disease. Very high if there is heart disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Above 4.9 mmol/L</td>
<td>Very high</td>
<td></td>
</tr>
</tbody>
</table>

2.16.8. Measurement of total antioxidant capacity

Total antioxidants were measured using a colorimetric method with the commercially available OxiSelect (TAC) assay kit (Antibodies-online, 2013).
The principal of this kit was to compare the samples with a known concentration of uric acid standard within a 96-well microtitre plate. Samples and standards were diluted with a reaction reagent and, upon the addition of copper, the reaction proceeded for a few minutes. The reaction was stopped and the samples read with a standard spectrophotometric microplate reader at 490 nm. Antioxidant capacity was determined by comparison with the uric acid standards. TAC has been demonstrated to be a reliable method for measuring oxidative stress, with a lower TAC value representing higher levels of oxidative stress (Ceriello et al., 1997), (see insert sheet in Appendix 21).

2.17. Data protection

A MRC data protection certificate had been obtained prior to start of the trial from the researcher. All baseline and follow-up data were protected by a special coded and encrypted file with a password. All subjects’ names and medical patient numbers were with a third party.

2.18. Data handling and cleaning

The data cleaning process demanded careful consideration, as it could significantly affect the final statistical results. The entire process was guided by the preliminary plan of data analysis, which was formulated in the research design phase. Cleaning the data required consistency checks and treatment of missing responses, generally done through SPSS. Missing responses pose problems if their proportion to the total is significant (more than 10 percent) (Wilson et al., 2013). The following practices were implemented:

- Use of study codes on data documents (questionnaire, results, etc) instead of recording identifying information and keeping a separate document that linked the study code to subjects’ identifying information locked in a separate location with restricted access to this document (sole primary investigator access);
- Encryption of identifiable data;
• Removal of face sheets containing identifiers (names and addresses) from survey instruments containing data after receipt from study participants;
• Proper disposal, destruction, or deletion of study data/documents;
• Limited access to identifiable information;
• Secure storage of data documentation within locked locations;
• Security codes assigned to computerised records.

2.19. Statistical analysis

In this study, we were interested in examining the effects of vitamin D on a number of parameters; we did not give vitamin D in the pre-test to the group of men who were randomly assigned to three independent groups. Two levels of vitamin D were given for the period of 16 weeks to only two groups, then a measure of parameters was obtained again post-test. This design consisted of one subject variable (test), with two times (pre and post), and one between subjects variable (therapy), with three groups (control, 100μg and 50μg vitamin D).

Mixed ANOVA was also applied to examine if there was an interaction between these two factors (time and groups) on the dependent variable (parameter). For example, the researcher used a mixed ANOVA to determine whether any change in calcium (i.e., the dependent variable) was the result of the interaction between the type of treatment (i.e., the 100 or 50μg level of vitamin D; that is, the "conditions", which was the "between-subjects" factor) and "time" (i.e., the within-subjects factor, consisting of two time points). If there was no interaction, follow-up tests could still be performed to determine whether any change in calcium was simply due to one of the factors (i.e., groups or time). Mixed ANOVA was additionally applied to test if there was cooperation between these two components (time and groups) on the reliant variable (parameter). For instance, the specialist utilised a Mixed ANOVA to decide if any adjustment in calcium (i.e., the dependent variable) was the consequence of the correlation between the kind of treatment (i.e., the 100 or
50μg of vitamin D; that is, the "conditions", which is the "between-subjects" factor) and "time" (i.e., the inside subjects factor, comprising of two time points). In the event that there was no correlation, follow-up tests could in any case be performed to decide if any adjustment in calcium was just because of one of the variables (i.e., groups or time).

The F-test was used by ANOVA to examine significant difference, namely if the p-value of the test was less than 0.05, the difference was statistically significant.

2.2. Normality

The normality assumption was checked, and all the measurements were approximately normally distributed. The values for asymmetry and kurtosis between -2 and +2 are considered standard in order to prove normal univariate distribution (George & Mallery, 2010).

Table 2.4. The normality of data

<table>
<thead>
<tr>
<th>Group</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Std. Error</td>
</tr>
<tr>
<td>A</td>
<td>BMI pre</td>
<td>0.972</td>
</tr>
<tr>
<td></td>
<td>BMI post</td>
<td>1.316</td>
</tr>
<tr>
<td></td>
<td>Vit D (nmol/l) pre</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>Vit D (nmol/l) post</td>
<td>0.774</td>
</tr>
<tr>
<td></td>
<td>Ca (mmol/l) pre</td>
<td>-0.105</td>
</tr>
<tr>
<td></td>
<td>Ca (mmol/l) post</td>
<td>0.502</td>
</tr>
<tr>
<td></td>
<td>ALB IT (g/L) pre</td>
<td>1.175</td>
</tr>
<tr>
<td></td>
<td>ALB IT (g/L) post</td>
<td>1.203</td>
</tr>
<tr>
<td></td>
<td>Corrected Ca (mmol/L) pre</td>
<td>0.461</td>
</tr>
<tr>
<td></td>
<td>Corrected Ca (mmol/L) post</td>
<td>0.551</td>
</tr>
<tr>
<td></td>
<td>glucose (mmol/l) pre</td>
<td>0.774</td>
</tr>
<tr>
<td></td>
<td>glucose (mmol/l) post</td>
<td>0.901</td>
</tr>
<tr>
<td></td>
<td>HbA1c (%) pre</td>
<td>1.562</td>
</tr>
<tr>
<td></td>
<td>HbA1c (%) post</td>
<td>-0.316</td>
</tr>
<tr>
<td>B</td>
<td>BMI pre</td>
<td>2.244</td>
</tr>
<tr>
<td></td>
<td>BMI post</td>
<td>2.065</td>
</tr>
<tr>
<td></td>
<td>Vit D (nmol/l) pre</td>
<td>0.421</td>
</tr>
<tr>
<td></td>
<td>Vit D (nmol/l) post</td>
<td>0.394</td>
</tr>
<tr>
<td></td>
<td>pre</td>
<td>post</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------</td>
<td>------------</td>
</tr>
<tr>
<td>Ca (mmol/l) _pre</td>
<td>-0.317</td>
<td>0.365</td>
</tr>
<tr>
<td>Ca (mmol/l) _post</td>
<td>0.915</td>
<td>0.365</td>
</tr>
<tr>
<td>ALB IT (g/L) _pre</td>
<td>0.518</td>
<td>0.365</td>
</tr>
<tr>
<td>ALB IT (g/L) _post</td>
<td>0.823</td>
<td>0.365</td>
</tr>
<tr>
<td>Corrected Ca (mmol/L) _pre</td>
<td>-0.099</td>
<td>0.365</td>
</tr>
<tr>
<td>Corrected Ca (mmol/L) _post</td>
<td>0.873</td>
<td>0.365</td>
</tr>
<tr>
<td>glucose (mmol/l) _pre</td>
<td>0.856</td>
<td>0.365</td>
</tr>
<tr>
<td>glucose (mmol/l) _post</td>
<td>1.099</td>
<td>0.365</td>
</tr>
<tr>
<td>HbA1c (%) _pre</td>
<td>1.532</td>
<td>0.365</td>
</tr>
<tr>
<td>HbA1c (%) _post</td>
<td>1.193</td>
<td>0.365</td>
</tr>
<tr>
<td>BMI pre</td>
<td>0.672</td>
<td>0.361</td>
</tr>
<tr>
<td>BMI _post</td>
<td>0.611</td>
<td>0.361</td>
</tr>
<tr>
<td>Vit D (nmol/l) _pre</td>
<td>-0.043</td>
<td>0.361</td>
</tr>
<tr>
<td>Vit D (nmol/l) _post</td>
<td>-0.039</td>
<td>0.361</td>
</tr>
<tr>
<td>Ca (mmol/l) _pre</td>
<td>0.321</td>
<td>0.361</td>
</tr>
<tr>
<td>Ca (mmol/l) _post</td>
<td>1.566</td>
<td>0.361</td>
</tr>
<tr>
<td>ALB IT (g/L) _pre</td>
<td>0.285</td>
<td>0.361</td>
</tr>
<tr>
<td>ALB IT (g/L) _post</td>
<td>0.469</td>
<td>0.361</td>
</tr>
<tr>
<td>Corrected Ca (mmol/L) _pre</td>
<td>0.831</td>
<td>0.361</td>
</tr>
<tr>
<td>Corrected Ca (mmol/L) _post</td>
<td>1.825</td>
<td>0.361</td>
</tr>
<tr>
<td>glucose (mmol/l) _pre</td>
<td>0.546</td>
<td>0.361</td>
</tr>
<tr>
<td>glucose (mmol/l) _post</td>
<td>0.893</td>
<td>0.361</td>
</tr>
<tr>
<td>HbA1c (%) _pre</td>
<td>2.050</td>
<td>0.361</td>
</tr>
<tr>
<td>HbA1c (%) _post</td>
<td>0.342</td>
<td>0.361</td>
</tr>
</tbody>
</table>

Valid N (listwise)
Chapter 3 Demographic characteristics
3.1. Introduction

More than 422 Million people who suffer from diabetes mellitus around the world, and about 90% of these suffer with T2DM (Roglic & World Health Organization, 2016). During the last two decades, the prevalence of T2DM has spread globally (WHO, 2011). The Middle East and North Africa has the second highest of increase in the prevalence of T2DM. It has been predicted that people who have diabetes will increase by 96.2% in 2035 (Guariguata et al., 2014). Based on the International Diabetes Federation estimates, the prevalence of T2DM in Saudi Arabia is in the top ten countries worldwide (Khan & Hamdy, 2017). In the 1980’s, studies in Saudi Arabia determined the prevalence of T2DM as being between 2.4% to 4.3% (Abuyassin & Laher, 2016), while its prevalence dramatically increased estimated to 25.4% in a recent study (Al-Rubeaan et al., 2015).

The prevalence of T2DM has been documented with increasing age (Thibault et al., 2016). Ageing is the gradual deterioration of bodily functions over the lifetime of the individual, which occurs at different rates among individual organs and tissues in the body. Advancing age is linked to changes in glucose intolerance functions (Kalyani & Egan, 2013); thus, facilities for diabetic screening of older adults in hospitals is necessary (Al Saif & Alsenany, 2015). In this study, the age range was 18-60 years to enable comparison with the studies of Mitri et al. (2011) and Al-Daghri, et al., (2012b).

Family history and increasing the risk of diabetes in the population have been shown to have a positive association (Hariri et al., 2006; Zhang et al., 2015). Genetic factors play an important role in increasing the risk of having diabetes. Also, lifestyle factors such as smoking, diet and lack of physical activity have been linked to a higher risk of having diabetes (Jankowich et al., 2011). Lack of physical activity will increase the chance of obesity. Obesity carries a significant risk of impaired glucose tolerance and a higher prevalence of obesity with 80% to 90% of people diagnosed with T2DM (Daousi et al., 2006). Thus, increased educational level will help to expand the perceptions in health and diabetic education. It assists in reducing risk factors for diabetes and
achieves the self-care, healthy diet and doing physical activity to control the possibility of diabetes (Shrivastava et al., 2013; Agrawal, 2016).

The aim of this chapter was to identify the relevant adjustable and non-adjustable risk factors and to control for their effects. The following parameters were measured: age; duration of T2DM; family history; educational level; smoking status across the two intervention groups A (100 µg vitamin D₃) and B (50 µg vitamin D₃), and compared against the control group.

3.2. Methods

This was a parallel, double-blind, randomised control trial of 156 men with poorly controlled T2DM with HbA₁c levels greater than 8.0%. Ethical approval was granted by the Manchester Metropolitan University Ethics Committee as stated in section 2.2. All participants were recruited from King Fahad Medical City hospital in Saudi Arabia, and informed consent was obtained as detailed in sections 2.7. There were 128 subjects who met the inclusion criteria who were randomly allocated to one of the three test groups. These were as follows: 50µg vitamin D per day, 100µg vitamin D per day, or a placebo, as described in section 2.5. 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 2.15.

3.3. Results

A total of 128 male patients with T2DM were included in this study, after recruitment of 156 participants at baseline. 28 T2DM male patients were not included in the study. The reasons for this attrition between the pre-test (baseline measurements) and post-test (16 weeks after the baseline) sample are listed in Table 3.1. Non-compliance in taking the vitamin D supplements was seen in about quarter of each group (13.2%-17.6%). Supplement non-compliance was classed as those who had taken less than 80% of the tablets and these subjects were excluded from the study. Compliance was encouraged in the present study by reducing the number of capsules
(Maningat et al., 2013). This method of compliance calculation has been used to determine the acceptable level of supplements for compliance (Osterberg & Blaschke, 2005; Mitri et al., 2011).

Participants excluded from the intervention study in group A were 9 subjects; one subject had their diabetic medication changed during the intervention, Non-compliance with taking vitamin D supplements and took additional vitamin D during the study. Three subjects had their diabetic medication changed. One subject had a medical condition; and one subject had a medical condition and took additional vitamin D during the study. Three subjects were non-compliant about taking vitamin D supplements and one of them took additional vitamin D during the study. In group B, 9 participants were excluded. Five subjects were non-compliant about taking vitamin D supplements and took additional vitamin D during the study. Two subjects changed diabetic medication during the intervention and were non-compliant about taking vitamin D supplements. One subject’s diabetic medication was changed during the intervention as well as having a medical condition and being non-compliant about taking vitamin D supplements. One subject had a medical condition and was non-compliant about taking vitamin D supplements. In group C, 10 subjects were excluded from the study. One subject was non-compliant about taking vitamin D supplements. Four subjects were non-compliant about taking vitamin D supplements and took additional vitamin D during the study. Two subjects had diabetic medication changed during the intervention and were non-compliant about taking vitamin D supplements. Two subjects had a medical condition, changed diabetic medication during the intervention and were non-compliant about taking vitamin D supplements and one of them took additional vitamin D during the study. In the end, there were 43 participants in group A, 42 participants in group B and 43 participants in group C, as shown in Table 3.1.
Table 3.1. The reasons for excluding subjects between the pre-test measurements and post-test (after 16 weeks)

<table>
<thead>
<tr>
<th>Reason</th>
<th>Group A (100 µg)</th>
<th>Group B (50 µg)</th>
<th>Group C (placebo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Non-compliance with taking vitamin D supplements</td>
<td>9</td>
<td>17.3</td>
<td>9</td>
</tr>
<tr>
<td>Contracted gastrointestinal malabsorption during the intervention</td>
<td>2</td>
<td>3.8</td>
<td>2</td>
</tr>
<tr>
<td>Changed diabetic medication during the intervention</td>
<td>4</td>
<td>7.7</td>
<td>3</td>
</tr>
<tr>
<td>Took additional vitamin D during the study</td>
<td>3</td>
<td>5.8</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3.2. Distribution of sample number of the three groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of participants</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (100 µg vitamin D₃)</td>
<td>Included 43</td>
<td>82.7%</td>
</tr>
<tr>
<td></td>
<td>Excluded 9</td>
<td>17.3%</td>
</tr>
<tr>
<td></td>
<td>Total 52</td>
<td>100.0%</td>
</tr>
<tr>
<td>B (50 µg vitamin D₃)</td>
<td>Included 42</td>
<td>82.4%</td>
</tr>
<tr>
<td></td>
<td>Excluded 9</td>
<td>17.6%</td>
</tr>
<tr>
<td></td>
<td>Total 51</td>
<td>100.0%</td>
</tr>
<tr>
<td>C (placebo)</td>
<td>Included 43</td>
<td>81.1%</td>
</tr>
<tr>
<td></td>
<td>Excluded 10</td>
<td>18.9%</td>
</tr>
<tr>
<td></td>
<td>Total 53</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

3.3.1. Demographics

The demographic characteristics of the 128 participants groups A, B, and C, as reported in the pre-test questionnaire are shown in Table 3.3. It was seen that all selected patients were Saudi men. The majority of participants were married, which are represented as 97.7% in group A, 90.5% in group B and 95.3% in group C. The average age range for the participants was more than 45 years. The highest percentage of a similar age group was about 86.1% of 45 to 60 years in group A. Then, the age group of 45 to 55 was more than half (59.5%) of group B and the age group of 55-60 years was also more than half (58.1%) of group C. The average ages were very similar between the three groups. It was 51.67+/-7.48 years for group A, was 52.29+/-7.127 years for group B and was 50.33+/-9.54 years for group C. Statistically, there was no significant difference in age between the three groups (F=0.649, p-value=0.524).
The educational level between the three groups covered high school, diploma and bachelor’s degree. Specifically, these levels were represented by more than half (58.2%) of group A, the majority (81%) of group B, and more than half (60.5%) of group C. Regarding the occupation, the participants were observed to be employed (60.5% in group A, 59.5% in group B and 44.2% in group C). This was followed by the retired (32.6%, 35.7% and 46.5% for A, B and C, respectively), who were fewer in number than the employed participants.

Table 3.3. Distribution of demographic characteristics of participants of the three groups (n = 128)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Category</th>
<th>Group</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A (100 µg)</td>
<td>B (50 µg)</td>
<td>C (placebo)</td>
<td></td>
</tr>
<tr>
<td>Nationality</td>
<td>Saudi</td>
<td>43</td>
<td>41</td>
<td>97.6</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Age</td>
<td>18-24 years</td>
<td>1</td>
<td>2</td>
<td>2.3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>25-34 years</td>
<td>1</td>
<td>2</td>
<td>2.3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>35-44 years</td>
<td>4</td>
<td>12</td>
<td>9.3</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>45-55</td>
<td>18</td>
<td>25</td>
<td>41.9</td>
<td>59.5</td>
</tr>
<tr>
<td></td>
<td>55-60 years</td>
<td>19</td>
<td>25</td>
<td>44.2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Mean+/-SD</td>
<td>51.67 +/- 7.48</td>
<td>52.29 +/- 7.127</td>
<td>50.33 +/- 9.54</td>
<td></td>
</tr>
<tr>
<td>Marital Status</td>
<td>Single</td>
<td>1</td>
<td>3</td>
<td>2.3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Married</td>
<td>42</td>
<td>38</td>
<td>97.7</td>
<td>90.5</td>
</tr>
<tr>
<td>Education</td>
<td>No schooling</td>
<td>5</td>
<td>1</td>
<td>11.6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Primary school</td>
<td>4</td>
<td>2</td>
<td>9.3</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>Secondary school</td>
<td>4</td>
<td>4</td>
<td>9.3</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>High school</td>
<td>10</td>
<td>16</td>
<td>23.3</td>
<td>38.1</td>
</tr>
<tr>
<td></td>
<td>Diploma</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>Bachelor’s degree</td>
<td>12</td>
<td>12</td>
<td>27.9</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>Postgraduate degree</td>
<td>5</td>
<td>1</td>
<td>11.6</td>
<td>2.4</td>
</tr>
<tr>
<td>Employment</td>
<td>Student</td>
<td>1</td>
<td>1</td>
<td>2.3</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Employed</td>
<td>26</td>
<td>25</td>
<td>60.5</td>
<td>59.5</td>
</tr>
<tr>
<td></td>
<td>Unemployed</td>
<td>2</td>
<td>1</td>
<td>4.7</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Retired</td>
<td>14</td>
<td>15</td>
<td>32.6</td>
<td>35.7</td>
</tr>
</tbody>
</table>

3.3.2. Health information

The health characteristics of the 128 participants, classified A, B, and C, as reported in the pre-test questionnaire, are summarised in Table 3.4. Most the participants were found to be non-smokers, and they did not follow any particular diet. About two-third of the participants in each group (65.1% in A,
71.4% in B and 69.8% in C) took multi-vitamin supplements (not containing vitamin D but containing calcium or iron) before or during the study.

The family history of diabetes ranged from 81% to 86% within the three groups. Duration of T2DM was seen to be more than eight years for more than half of participants (62.8% in group A, 71.4% in group B and 79.1% in control group). For health complications, nephropathy was 2.3% for group A, while it was not seen for the other groups. Also, neuropathy (30%-58%), diabetic cataract (26.2%-32.6%), cardiovascular disease (39.5%-45.2%), dyslipidaemia (60.5%-67.4%) and retinopathy (14%-18.6%) was observed in the sample.

Table 3.4. Health characteristics of participants at baseline

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Category</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (100μg)</td>
<td>B (50μg)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>No</td>
<td>5</td>
<td>11.6</td>
</tr>
<tr>
<td>Diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No special diet</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>Duration of T2DM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2 years</td>
<td>3</td>
<td>7.0</td>
</tr>
<tr>
<td>3-5 years</td>
<td>7</td>
<td>16.3</td>
</tr>
<tr>
<td>6-8 years</td>
<td>6</td>
<td>14.0</td>
</tr>
<tr>
<td>&gt; 8 years</td>
<td>27</td>
<td>62.8</td>
</tr>
<tr>
<td>Family history of diabetes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>36</td>
<td>83.7</td>
</tr>
<tr>
<td>Health Complications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>Nephropathy</td>
<td>1</td>
<td>2.3</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>13</td>
<td>30</td>
</tr>
<tr>
<td>Diabetic Cataract</td>
<td>14</td>
<td>32.6</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>17</td>
<td>39.5</td>
</tr>
<tr>
<td>Dyslipidaemia</td>
<td>26</td>
<td>60.5</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>Medical condition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid and parathyroid</td>
<td>4</td>
<td>9.3</td>
</tr>
<tr>
<td>disease doesn't contain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vitamin D</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Taking vitamin D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>supplements three months</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>before study date</td>
<td>43</td>
<td>100</td>
</tr>
</tbody>
</table>

3.4. Discussion

As T2DM has the highest incidence rate, several biological and lifestyle factors are believed to increase the possibility of developing T2DM even earlier in life.
as these factors may influence the genetic causes of T2DM. These factors include age, duration of diabetes, family history, educational level, and smoking were discussed.

3.4.1 Age

Epidemiological studies have shown that the risk of developing diabetes mellitus increases with age (Maruthur, 2013). The average age in this study was similar between the three groups. The mean ± SD was 51.67±7.48 years in group A and about 86.1% were in the age group 45 - 60 years. The mean ±SD was 52.29±7.127 years for group B, of which more than half (59.5%) were around 45 - 55 years in age. The mean ± SD was 50.33±9.54 years for group C of which 58.1% were in the age group 55 - 60 years. Statistically, there was no significant difference in age between the three groups (F=0.649, p-value=0.524)

The risk of developing T2DM is reported to be increased by 40% by the age of 40-49 years (Thibault et al., 2016). The Decode study group found that the risk of developing T2DM was higher in men aged between 40 and 59 years old (Tuomilehto et al., 2003). The prevalence of diabetes mellitus was higher in men over 50 years in age when compared with women of a similar age range in a Saudi community (Alqurashi et al., 2011). Another study found an increased in the prevalence of diabetes in people aged 58.5 years or older (AlSaif & Alsenany, 2015)

The incidence of T2DM is predicted to increase by 44.1% in 2022 among Saudi young adults age >25 years (Al-Quwaidhi et al., 2014). An epidemiological study of diabetes stated that the incidence of diabetes would significantly increase by 4.4% in 2030 (Wild et al., 2004). This change in the incidence rate of T2DM could be due to hormonal changes caused by age, obesity and the modernisation of lifestyles (Alqurashi et al., 2011).
3.4.2. Duration of diabetes

Poorly controlled of diabetes is linked to the long duration of diabetes. The long-term of diabetes increases the glycedated haemoglobin (HbA1c) or insulin levels (Verma et al., 2006; Sherwani et al., 2016). Long-term duration diabetes increases the insulin levels with insulin sensitivity linked negatively with fasting insulin and insulin levels.

In our study, male patients with poorly controlled T2DM had long-term duration of diabetes (eight years or more). They were more than half (62.8% in group A, 71.4% in group B and 79.1% in group C) of the study population. Another study found that 178 Libyan men who were suffering from poorly-controlled diabetes showed a significant association between the long term of duration diabetes and HbA1c (Bastos et al., 2016). In a further study, there was a significant relationship between the duration of diabetes and 500 patients who had HbA1c over 8% (Shera et al., 2004).

3.4.3. Family history

The link between the risk of diabetes and family medical history has been demonstrated in the literature (Akhuemonkhan & Lazo, 2017). Family medical history is essential to clear a basic approach of health prospective (Tarini & McInerney, 2013). Family history is useful to assess for diabetes risk and other medical conditions (Hariri et al., 2006).

In this study, the family history of diabetes was high and similar between the interventions groups and control group (83.7% group A, 81% group B and 86% control group). Regular physical activity for those with a diabetic family history might reduce the development of hyperglycaemia and T2DM (Valdez et al., 2007). Knowing the family history with regard to diabetes not only highlights genetic or environmental factors but it also helps to build health strategies to prevent diabetes (Das et al., 2012).
3.4.3. Educational level

Educational level is essential to controlling the risk of developing diabetes (Yoon et al., 2013). Educational level also helps individuals to understand the health information that affects health and reduces the diabetes epidemic (Powers et al., 2017). In this study, the educational level was between high school, diploma and bachelor’s degree. Specifically, these educational levels were represented by more than half (58.2%) of group A, the majority (81%) of group B, and more than half (60.5%) of group C. Specifically, Bachelor’s degrees (n = 12 about 27.9%) in group A, in group B were n=12 (about 28.6%), and n = 11 (about 25.6%) in group C. Postgraduate degrees were n= 5 (about 11.6%) in group A, n= 1 (about 2.4%) in group B and n= 3 (about 7.0%) in group C.

3.4.4. Smoking levels

Smoking has a critical effect on increasing the development of diabetes (Chang et al., 2012). Smokers are associated with insulin resistance, inflammation and dyslipidaemia (Chang et al., 2012). Smoking affects the stimulation of insulin, thereby effecting glucose transport into the cell by 10% to 40% in men who smoked compared with men who were non-smokers. (Chang et al., 2012; Sharip et al., 2017). One hour after smoking, insulin resistance was significantly increased (Wang et al., 2015). Diabetics who smoked had a high level of triglycerides as well a low level of HDL (Freeman et al., 1993; Tirosh et al., 2008). Lastly, smoking increases inflammation and oxidative stress (Lee et al., 2012) directly damages β-cell function (Woynillowicz et al., 2012) and impairs endothelial function (Ozaki et al., 2010).

However, in this study, the participants were found to be almost all non-smokers. The low prevalence of male smokers in this study may be explained by cultural factors (Abdalla et al., 2007; Mohammadnezhad et al., 2015). Most of the patients answered that they did not smoke because it would affect their social prospects in Saudi Arabia (Abdelwahab et al., 2013).
3.5. Conclusion

This chapter examined the demographic characteristics of patients with poorly controlled diabetes. The significant findings to emerge from this study are that the incidence of poorly controlled diabetes in middle-aged, educated, working patients was linked to family medical history. The most important limitation lies in the fact that their non-smoking status is due to Saudi cultural factors.
Chapter 4 The effect of vitamin D₃ supplementation on vitamin D concentration and BMI in men with poorly-controlled type-2 diabetes after 16 weeks
4.1. Introduction

Vitamin D is essential to prevent cardiovascular disease and cancer (Martin & Campbell, 2011), and it helps the body to absorb and use calcium by helping with its storage and making use of calcium in bones (Ahmadieh & Arabi, 2011). The ideal serum vitamin D concentration is 50 nmol/L or above (Holick et al., 2011). Vitamin D deficiency is defined as ≤ 50 nmol/L, and insufficiency is defined as a serum level between 50 nmol/L and 80 nmol/L (Sadat-Ali et al., 2009; Wakayo et al., 2016). Vitamin D deficiency is prevalent among Saudi Arabian men, with a rate of 72.4% (Alsuwadia et al., 2013; Alfawaz et al., 2014). This could be related to melanin, which causes darker skin to respond less to sun exposure, affecting the skin’s ability to make vitamin D (Nair & Maseeh, 2012).

Current dietary reference intake recommendations state that adults should take 20 µg of vitamin D per day (Institute of Medicine, 2011). Vitamin D₃ supplementation should be prescribed in subjects who need to achieve the serum of vitamin D level to 100 nmol/L or 150 nmol/L (Martin & Campbell, 2011). High supplement doses of vitamin D₃ are more potent than vitamin D₂ (Binkley et al., 2007). A daily 50 µg of vitamin D₃ is recommended for Arab patients with T2DM, a protective effect for cardiologic health indices having been found at this vitamin D intake (Al-Daghri, et al., 2012a). The tolerable upper limit for vitamin D has been raised to 250 µg/day (Hathcock et al., 2007). High doses of vitamin D supplementation above 250 µg lead to toxic hypercalcaemia (Alshahrani & Aljohani, 2013; Vogiatzi et al., 2014). Hypercalcaemia is responsible for vitamin D toxicity. The early symptoms of vitamin D toxicity are anorexia, diarrhoea, constipation, nausea, and vomiting. Then, other symptoms can appear within a few days or weeks such as frequent urination, extreme thirst, nervousness and kidney stones (Alshahrani & Aljohani, 2013). The vitamin D metabolism can be excreted through the bile into the faeces, and very few are eliminated through the urine, due to renal reuptake of vitamin D metabolites bound to DBP, as mediated by the cubilin-megalin receptor system (Nykjaer et al., 2005). For this reason, this
study measured calcium and serum vitamin D concentrations before and after supplementation to ensure subject safety throughout the study.

Vitamin D deficiency is linked with obesity and it is the main risk factor in the development of T2DM (Wakayo et al., 2016). Saudi Arabia has the world’s 5th highest rate of diabetes, with 20% of the population being affected and 26% of males having obese weight (Abuyassin & Laher, 2015). Obesity and being overweight have a number of contributing factors that up lead to them, which include metabolic, genetic, environmental, and behavioural influences (Alqarni, 2016). The rapid increases in obesity rates are directly contributed by environmental and behavioural factors, rather than the biological factors (Alqarni, 2016). People in urban areas have higher obesity rates as compared people in rural areas, possibly due to the intake of high-fat diets and more sedentary lifestyles (Alqarni, 2016). Saudi Arabia has of the highest obesity and overweight prevalence rates (De Nicola et al., 2015), where 7 out of 10 people experience the problem (Memish, 2013). There is a lack of literature on average body mass index (BMI) in Saudi Arabia. However, in Palestinian and Lebanese people the average BMI in diabetics was 33.7 kg/m2 and 30.8 kg/m2, respectively (Abuyassin & Laher, 2016).

In the published literature, low concentrations of vitamin D have been found in obese patients, and obese patients need a higher intake of vitamin D than those of normal weight (Ekwaru et al., 2014). Vitamin D deficiency increases parathyroid hormone concentrations (Sadat-Ali et al., 2015), which increase cytosolic calcium level (Christakos et al., 2011). The optimal concentration of vitamin D might enhance the mobilisation of fat, increasing lipolysis and thus decreasing intracellular fat accumulation (Chang & Kim, 2016).

The aim of this study was to examine the effect of vitamin D₃ supplementation on serum vitamin D concentration and BMI in men with poorly controlled T2DM after 16 weeks.
4.2. Methods

This was a parallel, double-blind, randomised control trial of 128 participants. Blood samples were collected from all participants at baseline and at 16 weeks using the aseptic technique described in section 2.11. Vitamin D status was measured using the Roche electrochemiluminescent protein-binding assay COBAS E411, as per section 2.16.1. 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

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D₃), 42 participants in group B (supplementation with 50 µg vitamin D₃), and 43 participants in group C (control, placebo group). The mean concentrations of vitamin D were analysed among the three studied groups: group A, group B, and C, and differences between the pre-test and post-test mean vitamin D concentrations among the groups were analysed. A two-way mixed ANOVA statistical test was used to compare the groups. The results (Table 4.1) showed that there was a statistically significant difference in the mean vitamin D concentrations measured at baseline and at the end of the 16-week-long intervention (F=93.53, p <0.001). Furthermore, there was a significant difference in mean vitamin D concentrations between the intervention and control groups when measured post-test (F=14. 02, p <0.001, Table 4.1).
Table 4.1. Two-way mixed ANOVA comparing the mean differences of serum levels of Vitamin D upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between times (pre- and post-test)</td>
<td>93.53**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction between different doses, intervention period and groups</td>
<td>71.59**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Between groups in per-test</td>
<td>0.810</td>
<td>0.447</td>
</tr>
<tr>
<td>Between groups in post-test</td>
<td>14.02**</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean Vitamin D concentration in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test, **indicates a significant difference at p < 0.01

Table 4.2. Post-hoc comparisons of the mean vitamin D serum concentration (nmol/L) between intervention groups and a control group at baseline and after the intervention period (16 weeks)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>77.85±5.27</td>
<td>115.14±5.082</td>
<td>37.28**</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>B</td>
<td>85.04±5.26</td>
<td>94.58±4.66</td>
<td>9.53**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C</td>
<td>86.03±5.83</td>
<td>81.50±5.70</td>
<td>-4.51</td>
<td>0.074</td>
</tr>
</tbody>
</table>

Intervention groups: A - 100µg vitamin D, and B - 50µg vitamin D; control group (C) - placebo. **indicates a significant difference at p < 0.001; $^b$ indicates adjustment for multiple comparisons; mean ± standard deviation

Comparison of the pre- and post-test results for each group showed that in group A the vitamin D increased significantly after 16 weeks (mean difference=37.28, $p <0.001$, see Table 4.2). For group B, the vitamin D also increased significantly after 16 weeks (mean difference=9.53, $p <0.001$). The results of measurements of the vitamin D concentration among intervention groups A and B and a control group C, at baseline and after the intervention period are also presented in Figure 4.1.
Table 4.3. Post-hoc comparisons of the mean vitamin D concentrations among the intervention and the control groups after 16 weeks

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean difference</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>20.56**</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>33.56**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>13.08*</td>
<td>0.044</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and group B, 50µg vitamin D are compared with control (placebo) group C after 16 weeks of intervention. **indicates a significant difference at p < 0.001; *indicates a significant difference of p < 0.05; <sup>b</sup> indicates adjustment for multiple comparisons.

After 16 weeks, the results (as analysed by two-way mixed ANOVA, Table 4.1), showed that there was a significant difference in vitamin D between the three groups (F=14.02, p <0.001). As the one-way ANOVA statistical analysis of the results of a post-hoc testing showed, the mean vitamin D concentration in group A was significantly higher than in group B (mean difference=20.56, p <0.002, (see Table 4.3). Furthermore, the mean post-intervention concentration of vitamin D in group A was significantly increased than in group C (mean difference=33.56, p <0.001) and the post-intervention mean vitamin D concentration in group B was significantly increased than in group C (mean difference=13.08, p <0.044).
Figure 4.1. Changes in the mean vitamin D status concentrations (nmol/L) in intervention and control groups between the pre-test and post-test measurements. The bar chart shows intervention groups (group A - 100µg vitamin D₃ (n=43) and group B, 50µg vitamin D₃ (n=42) compared with the placebo group (C) as control (n=43) between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences are marked with ** of *p < 0.001* and with * of *p < 0.005*.

4.3.2 Corrected calcium

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D₃), 42 participants in group B (supplementation with 50 µg vitamin D₃) and 43 participants in group C (control, placebo group). The changes in the mean corrected calcium values were analysed among groups A, B and C placebo pre- and post-test, as well as in relation to the duration of treatment, by two-way mixed ANOVA. The results of the two-way mixed ANOVA test (Table 4.4) showed that there was no significant difference in mean corrected calcium between the pre- and post-tests (F=0.052, *p*-value=0.82), nor between the groups post-test (F=1.48, *p*-value=0.23). In addition, the interaction between treatment duration and the effect was not significant (F=0.4, *p*-value=0.669).
Table 4.4. Two-way mixed ANOVA analysis of differences in the mean corrected calcium upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between times (pre- and post-test)</td>
<td>0.052</td>
<td>0.821</td>
</tr>
<tr>
<td>Interaction between different doses, intervention period and groups</td>
<td>0.403</td>
<td>0.669</td>
</tr>
<tr>
<td>Between groups in per-test</td>
<td>1.123</td>
<td>0.329</td>
</tr>
<tr>
<td>Between groups in post-test</td>
<td>1.482</td>
<td>0.231</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean corrected calcium concentration in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test.

Table 4.5. Post-hoc comparisons of the differences between the mean corrected calcium (mmol/L) among groups (intervention and control) at baseline and after the intervention study

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.24±.17</td>
<td>2.24±.15</td>
<td>0.01</td>
<td>0.820</td>
</tr>
<tr>
<td>B</td>
<td>2.24±.14</td>
<td>2.31±.26</td>
<td>0.03</td>
<td>0.481</td>
</tr>
<tr>
<td>C</td>
<td>2.25±.15</td>
<td>2.26±.24</td>
<td>-0.02</td>
<td>0.584</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D₃ supplementation and B, 50µg vitamin D₃ and control (C), placebo group. $^b$ indicates adjustment for multiple comparisons; mean ± SD.

The mean corrected calcium concentrations among the intervention groups and control group from the baseline to after the intervention period were calculated (as summarised in Table 4.5). There was no significant difference in the mean corrected calcium values in groups A, B and C, between baseline and after 16 weeks of treatment (see Table 4.5 and Figure 4.2).
Figure 4.2. Corrected calcium (mmol/L) before and after vitamin D₃ supplementation. The bar chart shows intervention groups A, 100µg vitamin D₃, (n=43) and B, 50µg vitamin D₃, (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements. Corrected calcium remained unchanged after vitamin D₃ supplementation. Results are presented as mean ± SD (n=128).

4.3.3 Body mass index (BMI)

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D₃), 42 participants in group B (supplementation with 50 µg vitamin D₃), and 43 participants in group C (control, placebo group). The changes in the mean percentage of BMI were analysed among groups A, B and C, pre- and post-test, by two-way mixed ANOVA. The results of the mixed ANOVA test (Table 4.6) showed that there was a significant decrease in the mean percentage of BMI between the pre- and post-tests (F=30.34, p-value=0.001, see Table 4.6). In addition, the interaction between treatment duration and treatment effect was statistically significant (F=6.19, p-value=0.001).
Table 4.6. Two-way mixed ANOVA test comparing the mean differences of BMI upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between times (pre- and post-test)</td>
<td>30.34**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction between different doses, intervention period and groups</td>
<td>6.19**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Between groups in pre-test</td>
<td>0.021</td>
<td>0.979</td>
</tr>
<tr>
<td>Between groups in post-test</td>
<td>0.76</td>
<td>0.467</td>
</tr>
</tbody>
</table>

**indicates a significant difference at p < 0.001

The comparisons included: the difference in mean BMI in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test.

Table 4.7. Post-hoc comparisons of the differences between the mean BMI (kg/m²) among groups (intervention and control) at baseline and after the intervention study

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-value</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>30.42±5.27</td>
<td>29.63±5.08</td>
<td>-0.794**</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>30.28±5.26</td>
<td>29.00±4.66</td>
<td>-1.27**</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>30.53±6.53</td>
<td>29.69±6.28</td>
<td>0.22</td>
<td>0.690</td>
<td></td>
</tr>
</tbody>
</table>

Intervention groups: group A, 100µg vitamin D₃ supplementation, and group B, 50µg vitamin D₃ and control (C) placebo group. **indicates a significant difference between the groups at p < 0.001; b indicates adjustment for multiple comparisons; mean ± SD.

The mean levels of BMI % among the intervention groups and control group at the baseline and after the intervention period were calculated (see Table 4.7). Comparison of the pre- and post-test results for each group revealed that in group A BMI decreased significantly after 16 weeks (mean difference = -0.794, p-value<0.001). For group B, the BMI decreased significantly (mean difference= -1.27, p-value<0.001). The differences in mean BMI % in the groups from baseline to 16 weeks post-treatment are also presented in Figure 4.3. However, there were no statistically significant difference compared with control groups in both intervention treatment groups (see Table 4.8).
Table 4.8. Post-hoc comparisons of BMI (kg/m²) between the intervention groups and control after 16 weeks

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean difference</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>0.62</td>
<td>0.594</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>-0.816</td>
<td>0.484</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>-1.44</td>
<td>0.220</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D<sub>3</sub> supplementation, and B, 50µg vitamin D<sub>3</sub> compared with control (placebo) group C after 16 weeks of intervention. <sup>b</sup> indicates adjustment for multiple comparisons.

Figure 4.3. The comparisons of mean BMI (kg/m²) after vitamin D<sub>3</sub> treatment
The bar chart shows intervention groups A, 100µg vitamin D<sub>3</sub> (n=43), and B, 50µg vitamin D<sub>3</sub> (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements. Results are presented as mean ± SD (n=128).

4.4. Discussion

This study found a significant improvement in vitamin D status at both vitamin D doses (100 µg and 50 µg vitamin D<sub>3</sub>), from 77.85 ± 5.27 to 115.14 ± 5.082, and from 85.04 ± 5.26 to 94.58 ± 4.66 nmol/L, respectively. Previous studies that have noted that the concentrations of serum vitamin D or, as it is defined
25(OH)D, increase by approximately 0.7 to 1.0 nmol/L for every 1 µg/day of vitamin D₃ supplementation (Dawson-Hughes et al., 2010). A daily dose of 25 µg of vitamin D₃ over 11 weeks increased the 25(OH)D concentration from 49 to 72 nmol/L, and the vitamin D levels plateaued in participants on that regimen for 6 weeks (Holick, 2008). A weekly dose of 1,250 µg of vitamin D₃, led to a high serum levels of 25(OH)D remaining steady for 2 weeks then dropping gradually (Armas et al., 2004; Alshahrani & Aljohani, 2013). Vitamin D₂ is absorbed as vitamin D₃, however, the vitamin D₂ concentration dropped after 3 days (Alshahrani & Aljohani, 2013).

A study of hip-fracture patients with vitamin D insufficiency who were given 25µg of vitamin D₃, showed that after 3 months, the level of 25(OH)D had increased by 31%–52% (Glendenning et al., 2009). A dose 100 µg/day of vitamin D₃ was observed to effect a 1.7-fold rise in vitamin D level for 2 weeks (Trang et al., 1998; Mastaglia et al., 2006; Alshahrani & Aljohani, 2013). In our study, the mean increase in serum 25(OH)D concentration after vitamin D₃ supplementation increased by 37.28 nmol/L ($p < 0.001$) for 100 µg/day vitamin D₃ supplementation, and by 9.53 nmol/L ($p <0.001$) after 50 µg/day vitamin D₃ for 16 weeks. Our study showed significant improvement in serum vitamin D in both intervention groups, with the majority of participants in group A reaching optimal concentrations of vitamin D after 16 weeks. The current study used safe doses of 100 µg/day and 50 µg/day of vitamin D₃, with no adverse effect on the serum concentration of corrected calcium.

Vitamin D deficiency (< 50 nmol/L) is commonly seen clinically in patients with T2DM (Sugden et al., 2008). Supplementation to achieve higher levels of vitamin D remains a promising adjuvant therapy for T2DM patients (Al-Daghri, et al., 2012a). Nutritional recommendations for correcting deficient vitamin D levels involve treatment doses of 1,250 µg/week of vitamin D₃ for 6–8 weeks, thereafter 20-25 µg/per day of vitamin D₃ (Dawson-Hughes et al., 2010). Secondly, patients with insufficient vitamin D levels (50–75 nmol/L) need a treatment dose with 20-25µg/day of vitamin D₃. This will increase the vitamin D level to 7 nmol/L over 3 months (Ross et al., 2011; Aljohani, 2016). The final
optional recommendation is a dose of 7,500 µg one or two times per year for increasing serum vitamin D level (de Torrente de la Jara et al., 2006).

The absorption of calcium in the small intestine can be adversely affected by low vitamin D levels (about 10%–15% absorption), but calcium absorption rises to about 30%–40% when the optimal vitamin D level is attained (Holick, 2004). In this study, no significant increase was observed in mean corrected calcium concentration in intervention group A (100 µg vitamin D₃) or B (50 µg vitamin D₃). Neither of the intervention groups exceeded the normal range (< 2.5 nmol/L), supporting the safe dose of vitamin D used to avoid hypercalcemia (Sugden et al., 2008). Hypercalcaemia results when the calcium concentration exceeds 3.5 nmol/L (Mirrakhimov, 2015), and serum vitamin D levels above 325 nmol/L are considered as vitamin D toxicity (Ozkan et al., 2012). Hypercalcemia affects cardiac, nervous system, renal, and gastrointestinal functions (Ozkan et al., 2012).

There is an association between vitamin D and adipose tissue (Piccolo et al., 2013). Each 2-unit increase of BMI (kg/m²) is associated with 1.15% decrease in the concentration of vitamin D (Vimaleswaran et al., 2013). In this study, there appeared a link between vitamin D levels and BMI in T2DM patients. It found an improvement in lowering the mean BMI in both groups A (100 µg vitamin D₃) and B (50 µg vitamin D₃) from 30.42 ± 5.27 kg/m² to 29.63 ± 5.08 kg/m² and from 30.28 ± 5.26 kg/m² to 29 ± 4.66 kg/m², respectively. However, no significant difference was observed when comparing the intervention groups with the control after 16 weeks. Supporting this finding there was no significant association with changes in adipose tissue or circulating vitamin D₃ levels and reduced weight (Sebekova et al., 2015).

One of the issues that emerges from these findings is that most of the T2DM patients in King Fahad Medical City were treated by high doses of vitamin D (50 µg/week vitamin D₃). Amongst our exclusion criteria was if to any patients had taken vitamin D 3 months prior the study so as to avoid any confounding results as vitamin D has a circulating half-life of 15 days and a turnover in the body of two months (Aljohani, 2016). Patients were requested to return the vitamin D bottle to the clinical pharmacy to avoid non-compliance. However,
some of those returned the vitamin D calendar sheet. Future trials should set up a reminder for patients to take the capsule to minimise non-compliance. It also recommends increasing the dose of vitamin D so it would be taken once a week, also to reduce non-compliance.

4.5. Conclusion

This study investigated vitamin D and BMI in men with poorly controlled T2DM. The results of this investigation show that intake of vitamin D₃ was associated with a significant increase in the serum concentration of vitamin D ($p < 0.001$) and a reduction in BMI, and also that the supplementation had no adverse link to the corrected calcium levels.
Chapter 5 Effect of vitamin D₃ supplementation on glycaemia in poorly-controlled type-2 diabetes after 16 weeks
5.1. Introduction

T2DM is a chronic metabolic disorder and has a major effect on increasing morbidity and mortality, (Altinok et al., 2016). Controlling blood glucose levels helps to reduce the development of microvascular and macrovascular complications (Chawla et al., 2016). Glycaemic control is indicated by HbA1c, which is the most important laboratory parameter (Kohnert et al., 2015). The general target of HbA1c is ≤7% for glycaemic control, with values over 7% showing poor glycaemic control (Phillips & Leow, 2014). Poor glycaemic control has been related to vitamin D deficiency (Kant et al., 2010; Unadike, 2010).

Vitamin D plays a vital role in dysfunctions of glucose and insulin metabolism, and therefore is conceivably linked with the development of T2DM (Jamka et al., 2015). A systematic review indicates that the risk of diabetes can potentially be modified by vitamin D (Danescu et al., 2009). There is now convincing evidence of the role of vitamin D in insulin secretion from the pancreas and in insulin sensitivity, and therefore the effect it has on the pathogenesis of diabetes (Borissova et al., 2003; Chiu et al., 2004). Vitamin D deficiency may affect glycaemia by impairing insulin secretion and insulin resistance in T2DM (Talaei et al., 2013). Vitamin D receptors are found on pancreatic β-cells, and vitamin D is essential for normal insulin secretion (Johnson et al., 1994; Mitri & Pittas, 2014). Subsequently, vitamin D is a regulator of insulin secretion from pancreatic β-cells (Zeitz et al., 2003). Also, vitamin D stimulates the expression of the insulin receptor (Maestro et al., 2000).

The aims of this study are to determine the effect of vitamin D3 supplementation on controlling glycated haemoglobin, reducing glucose levels, and improving insulin resistance and β-cell function in poorly-controlled type-2 diabetic Saudi men.
5.2. Methods

Blood samples were taken from all 128 participants at baseline and 16 weeks using the aseptic technique described in section 2.13. Fasting insulin was measured using the Roche electrochemiluminescent immunoassay COBAS E411 as described in section 2.16.5. Fasting glucose levels were determined using the Konelab, reference method described in section 2.16.3. β-cell function and insulin resistance were calculated according to the homeostasis assessment model as described in section 2.16.6. Glycated haemoglobin levels were determined using the Siemens DCA Vantage Analyser as per section 2.16.4.

5.3. Result

5.3.1. Analysis of the serum levels of glycated haemoglobin (HbA$_{1c}$) in the intervention and control groups

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D$_3$), 42 participants in group B (supplementation with 50 µg vitamin D$_3$) and 43 participants in group C (control, placebo group). The mean concentrations of HbA$_{1c}$ were analysed for the three study groups, and differences between the pre-test and post-test mean HbA$_{1c}$ concentrations between the groups were analysed. A Two-way mixed ANOVA statistical test was used to compare the groups, and the summary of the results are presented in Table 5.1. The results show that there was statistically significant difference in the mean HbA$_{1c}$ concentrations between the measurement at baseline and at the end of the 16-week-long intervention (F=131.51, p-value<0.001, Table 5.1.). Furthermore, there was a significant correlation between the duration of the intervention (“time”) and treatment effect, indicating that the effect of intervention, in at least one group, depended on the time of treatment (F=34.45, p<0.001). A significant difference in the mean HbA$_{1c}$ concentrations was found between the intervention and control groups when measured post-test (F=14.47, p-value<0.001).
Table 5.1. Two-way mixed ANOVA comparing the mean differences of serum levels of HbA1c upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between baseline and 16-wk intervention</td>
<td>131.51**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction time and groups</td>
<td>34.45**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Between groups in pre-test</td>
<td>2.25</td>
<td>0.110</td>
</tr>
<tr>
<td>Between groups in post-test</td>
<td>14.47**</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean HbA1c concentration in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between the groups in pre-test measurement, and between groups in post-test. **indicates a significant difference at p < 0.001.

Table 5.2. Post-hoc comparisons of the mean HbA1c serum concentration (%) among intervention groups and a control group at baseline and after the intervention period

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-value a</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9.77±1.67</td>
<td>7.67±0.98</td>
<td>-2.10**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B</td>
<td>9.20±1.10</td>
<td>8.24±1.02</td>
<td>-0.95**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C</td>
<td>9.21±1.44</td>
<td>9.01±1.42</td>
<td>-0.20</td>
<td>0.222</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D, and B, 50µg vitamin D and control group (C), placebo. **indicates a significant difference between pre-test and post-test at p < 0.001; a indicates adjustment for multiple comparisons; mean ± SD.

Comparison of the pre- and post-test results for each group showed that in group A the HbA1c decreased significantly after 16 weeks (mean difference =-2.10, p-value<0.001, see Table 5.2). For group B, the HbA1c also decreased significantly after 16 weeks (mean difference=-0.95, p-value=<0.001). The results of measurements of the HbA1c levels among intervention groups A and B and a control group C, at baseline and after the intervention period are also presented in Figure 5.1.

After 16 weeks, the results (as analysed by two-way mixed ANOVA, Table 5.1), showed that there was a significant difference in HbA1c between the three groups (F=14.47, p <0.00, Table 5.1). As the one-way ANOVA statistical analysis of the results of a post-hoc test showed, the mean HbA1c concentration in group A was significantly lower than in group B (mean difference=-0.57, p-value=.023, (see Table 5.3). Furthermore, the mean post-intervention concentration of HbA1c in group A was significantly lower than in group C (mean difference=-1.34, p <0.001) and the post-intervention mean HbA1c concentration in group B was significantly lower than in group C (mean difference=-0.75, p <0.003).
Table 5.3. Post-hoc comparisons of the mean HbA1c concentrations among the intervention and the control groups after 16 weeks

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean difference</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 16 weeks</td>
<td>A</td>
<td>B</td>
<td>-0.57</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>-1.34**</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>-0.75*</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D; are compared with control (placebo) group C after 16 weeks of intervention. **indicates a significant difference of p < 0.001; *indicates a significant difference of p < 0.05.

Figure 5.1. Mean changes of HbA1c concentration with vitamin D treatment
The bar chart shows intervention groups A, 100µg vitamin D<sub>3</sub> (n=43) and B, 50µg vitamin D<sub>3</sub> (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences of p < 0.001 are marked with (**) and of p < 0.05) are marked with (*).

5.3.2. Comparison of fasting blood glucose levels between the intervention and control groups

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D<sub>3</sub>), 42 participants in group B (supplementation with 50 µg vitamin D<sub>3</sub>) and 43 participants in group C (control, placebo group). Analysis of the changes in concentrations of blood glucose between the intervention groups A, B and the control (C) group), pre- and post-intervention, was performed using a two-way
mixed ANOVA test. The results of the analysis are shown in Table 5.4. As the results of the mixed ANOVA showed, there was a highly significant difference in mean glucose concentration between the baseline and the 16-week point (F=67.46, p <0.001, Table 5.4). The interaction between time and treatment was also statistically significant (F=28.07, p <0.001), indicating that the treatment effect in at least one group depended on the duration of the treatment.

Table 5.4. Two-way mixed ANOVA analysis of differences in the mean fasting blood glucose concentrations upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between times (pre- and post-test)</td>
<td>67.46**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction time and groups</td>
<td>28.07**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Between groups in pre-test</td>
<td>1.47</td>
<td>0.232</td>
</tr>
<tr>
<td>Between groups in post-test</td>
<td>10.95**</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean fasting blood glucose concentrations in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test.

Table 5.5. Post-hoc comparisons of the differences between the mean fasting blood glucose concentrations (mmol/L) among the intervention and control groups at baseline and after the intervention study

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11.80±4.31</td>
<td>8.22±2.24</td>
<td>-3.58**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B</td>
<td>10.46±3.30</td>
<td>9.78±2.77</td>
<td>-0.67*</td>
<td>0.044</td>
</tr>
<tr>
<td>C</td>
<td>11.51±3.64</td>
<td>11.07±3.64</td>
<td>-0.44</td>
<td>0.179</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D and control (C), placebo group. **indicates a significant difference between pre-test and post-test of p < 0.001; *indicates a significant difference between pre-test and post-test of p < 0.05; b indicates adjustment for multiple comparisons; mean ± SD.

The results one-way ANOVA analysis of differences in the mean blood glucose concentrations (mmol/L) in the intervention groups and a control group, between the baseline and the measurements after the intervention period are summarised in Table 5.5, showed that in group A the blood glucose concentration decreased significantly after 16 weeks (mean difference=-3.58, p <0.001). The mean blood glucose concentration in group B also decreased significantly after 16 weeks (mean difference=-0.67, p <0.044). The differences in mean blood glucose concentrations (mmol/L) in the groups between baseline and after 16 weeks of treatment are also shown in Figure 5.2.
A two-way mixed ANOVA analysis (results given in Table 5.4) showed that after 16 weeks there were significant differences in the mean blood glucose concentration between the three groups (F=10.95, \(p<0.001\), Table 5.4). Using one-way ANOVA analysis of the results of the post-hoc testing (see Table 5.6 and Figure 5.2), it was established that the mean blood glucose concentration in group A was significantly lower than in group B (mean difference=-1.56, \(p=0.012\)). In addition, the mean blood glucose concentration in group A was significantly lower than in group C (mean difference=-2.85, \(p<0.001\)). The mean blood glucose concentration in group B was also significantly lower than in group C (mean difference=-1.28, \(p=0.038\)).

Table 5.6. Post-hoc comparisons of fasting blood glucose levels (mmol/L) among intervention groups and control after 16 weeks

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean difference</th>
<th>p-value(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 16 weeks</td>
<td>A</td>
<td>B</td>
<td>-1.56(^*)</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>-2.85(^{**})</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>-1.28(^*)</td>
<td>0.038</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D supplementation are compared with control (placebo) group C after 16 weeks of intervention. \(^{**}\)indicates a significant difference between the groups of \(p<0.001\); \(^*\)indicates a significant difference between the groups of \(p<0.05\); \(^b\)indicates adjustment for multiple comparisons.
Figure 5.2. Changes in the mean fasting blood glucose concentrations (mmol/L) in intervention and control groups between pre-test and post-test measurements. The bar chart shows intervention groups A, 100µg vitamin D₃ (n=43) and B, 50µg vitamin D₃ (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences of p < 0.001 are marked with (**) and of p < 0.005) are marked with (*).

5.3.3. Analysis of differences in the insulin levels between the intervention and control groups

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D₃), 42 participants in group B (supplementation with 50 µg vitamin D₃) and 43 participants in group C (control, placebo group). The changes in insulin levels (µU/mL) were analysed among groups A, B and C pre-test as well as post-test, by two-way mixed ANOVA statistical analysis. The results (summarised in Table 5.7) show that no statistically significant difference (of p≤0.05) was observed between the pre- and post-hoc insulin levels, nor between the groups either pre- or post-test. Furthermore, no significant correlation between
the duration of the intervention and the effect on insulin levels was observed in the studied groups.

Table 5.7. Two-way mixed ANOVA test comparing the mean differences of insulin levels upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between times</td>
<td>0.26</td>
<td>0.610</td>
</tr>
<tr>
<td>Interaction time and groups</td>
<td>0.06</td>
<td>0.941</td>
</tr>
<tr>
<td>Between groups in pre-test</td>
<td>0.01</td>
<td>0.985</td>
</tr>
<tr>
<td>Between groups in post-test</td>
<td>0.04</td>
<td>0.961</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean insulin levels in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test.

Table 5.8. Post-hoc comparisons of insulin (µU/mL) levels among the intervention and control groups, at baseline and after the intervention

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-value&lt;sub&gt;b&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>16.23±2.81</td>
<td>17.79±2.64</td>
<td>1.57</td>
<td>0.581</td>
</tr>
<tr>
<td>B</td>
<td>16.73±2.85</td>
<td>16.88±2.67</td>
<td>0.15</td>
<td>0.956</td>
</tr>
<tr>
<td>C</td>
<td>16.06±2.71</td>
<td>16.86±2.6</td>
<td>0.79</td>
<td>0.779</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D and control (C) - placebo group. <sup>b</sup> indicates adjustment for multiple comparisons; mean ±SD.

Furthermore, the post-hoc comparison between the mean insulin levels (µU/mL) in the intervention groups and control, and between the baseline and after the intervention period, using the mixed ANOVA test are shown in Table 5.8, and Figure 5.3), did not reveal any statistically significant changes (of p<0.05) in insulin levels between the pre- and post-test (F=0.26, p-value=0.610, Table 5.7). Similarly, the interaction between duration of the treatment and effect on the group was not significant (F=0.06, p-value=0.941), nor were there differences between the groups pre- and post-test.
Figure 5.3. Mean changes in insulin level (µU/mL) in the intervention groups A and B, and the control group C (placebo)
The bar chart shows intervention groups A, 100µg vitamin D₃ (n=43) and B, 50µg vitamin D₃ (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements were not statistically significant. Results are presented as mean ± SD (n=128).

5.3.4. Insulin resistance (HOMA-IR) analysis before and after the intervention

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D₃), 42 participants in group B (supplementation with 50 µg vitamin D₃) and 43 participants in group C (control, placebo group). The homeostatic model assessment (HOMA) was used to quantify insulin resistance (IR) in the studied patient groups. The changes in the mean calculated HOMA-IR values were analysed among groups A, B and C pre- and post-test, as well as in relation to the duration of treatment, by two-way mixed ANOVA as summarised in Table 5.9). The results of the two-way mixed ANOVA test showed that there was no significant difference in mean HOMA-IR between the pre- and post-tests (F=0.590, p-value=0.444, Table 5.9), nor between the groups in pre- or post-test (F=0.105, p-value=0.9, and F=0.349, p-value=0.706, respectively). In addition, the interaction between treatment duration and the effect was not significant (F=0.199, p-value=0.900).
Table 5.9. Two-way mixed ANOVA comparing the mean differences of HOMA-IR upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between times</td>
<td>0.590</td>
<td>0.444</td>
</tr>
<tr>
<td>Interaction time and the three groups</td>
<td>0.199</td>
<td>0.820</td>
</tr>
<tr>
<td>Between the three groups in pre-test</td>
<td>0.105</td>
<td>0.900</td>
</tr>
<tr>
<td>Between the three groups in post-test</td>
<td>0.349</td>
<td>0.706</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean HOMA-IR in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test.

Table 5.10. Post-hoc comparisons of HOMA-IR between groups (intervention and control) at baseline and after the intervention study

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8.18±1.48</td>
<td>6.62±1.35</td>
<td>-1.56</td>
<td>0.339</td>
</tr>
<tr>
<td>B</td>
<td>7.50±1.50</td>
<td>16.88±1.37</td>
<td>-0.35</td>
<td>0.828</td>
</tr>
<tr>
<td>C</td>
<td>8.44±1.49</td>
<td>16.86±1.35</td>
<td>0.25</td>
<td>0.876</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D; control group (C) placebo. <sup>b</sup> Indicates adjustment for multiple comparisons; mean ±SD.

The mean HOMA-IR concentrations among the intervention groups and control group from the baseline to after the intervention period were calculated (as summarised in Table 5.10). The small decreases in mean HOMA-IR values in groups A, B and C, between baseline and after 16 weeks of treatment, were not statistically significant (see Table 5.10 and Figure 5.4).

Figure 5.4. Mean changes in insulin resistance (HOMA-IR) in the intervention groups A and B, and the control group C (placebo)
The bar chart shows intervention groups A, 100µg vitamin D<sub>3</sub> (n=43), and B, 50µg vitamin D<sub>3</sub> (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-
test measurements were not statistically significant. Results are presented as mean ± SD (n=128).

5.3.5. β-cell function (HOMA-β) analysis pre- and post-intervention

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D₃), 42 participants in group B (supplementation with 50 µg vitamin D₃) and 43 participants in group C (control, placebo group). The homeostatic model assessment (HOMA) was used to quantify β-cell function in the patient groups studied. The changes in the mean percentage of β-cell function (HOMA-β) were analysed among groups A, B and C pre- and post-test, by two-way mixed ANOVA, as summarised in Table 5.11. The results of the mixed ANOVA test showed that there was a significant increase in mean percentage of β-cell function between the pre- and post-tests (F=8.03, p-value=0.005, Table 5.11). In addition, the interaction between treatment duration and treatment effect was statistically significant (F=3.85, p-value=0.024).

Table 5.11. Two-way mixed ANOVA comparing the mean differences in HOMA-β upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between times</td>
<td>8.03**</td>
<td>0.005</td>
</tr>
<tr>
<td>Interaction time and the three groups</td>
<td>3.85*</td>
<td>0.024</td>
</tr>
<tr>
<td>Between the three groups in per-test</td>
<td>0.533</td>
<td>0.588</td>
</tr>
<tr>
<td>Between the three groups in post-test</td>
<td>2.66</td>
<td>0.073</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean HOMA-β in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test. **indicates a significant difference at p < 0.01 with * of p < 0.005.

Table 5.12. Post-hoc comparisons of HOMA-β (%) between the groups (intervention and control) at baseline and after the intervention study

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50±9.89</td>
<td>96.96±13.72</td>
<td>46.45**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B</td>
<td>60.50±9.99</td>
<td>65.54±13.92</td>
<td>5.04</td>
<td>0.675</td>
</tr>
<tr>
<td>C</td>
<td>46.38±9.87</td>
<td>53.37±13.76</td>
<td>6.99</td>
<td>0.557</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D; control group (C) – placebo. **indicates a significant difference p < 0.001 b indicates adjustment for multiple comparisons; mean ±SD.

The mean levels of HOMA-β % among the intervention groups and control group at the baseline and after the intervention period were calculated (see Table 5.12). Comparison of the pre- and post-test results for each group
revealed, that in group A β-cell activity increased significantly after 16 weeks (mean difference = 46.45, p-value<0.001). For group B, the HOMA-β increased slightly, but the result was not statistically significant (mean difference=5.04, p-value=0.675). The differences in mean HOMA-β % in the groups from baseline to 16 weeks post-treatment are also presented in Figure 5.5.

Figure 5.5. Mean changes in β-cell function (HOMA-β) in the intervention groups A and B, and the control group C (placebo)
The bar chart shows intervention groups A, 100µg vitamin D₃ (n=43) and B, 50µg vitamin D₃ (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences of p < 0.001 are marked with **

5.4. Discussion

The relationship between vitamin D deficiency, glucose metabolism and insulin resistance is inconsistent with the literature. This study has found that vitamin D supplementation with either 100µg or 50µg per day significantly improves the HbA₁c and reduces glucose levels as compared to the placebo group. In contrast, there were no significant changes in insulin resistance. In addition, there was a slight, but not significant, increase in insulin and HOMA-β at both doses of vitamin D, with 100µg/day producing a larger increase than 50µg/day compared to the placebo group. These findings support previous studies (Yiu et al., 2013; Ryu et al., 2014).
This study found a significant improvement in HbA\(_1c\) in those supplemented with vitamin D. After 16 weeks of vitamin D supplementation for group A, the HbA\(_1c\) decreased significantly (mean difference = -2.10, p-value < 0.001). For group B, the HbA\(_1c\) also decreased significantly after 16 weeks (mean difference = -0.95, p-value < 0.001). Previous studies have found a significant association between Hba\(_1\) and serum vitamin D levels in diabetic subjects (Kositsawat et al., 2010; Tahrani et al., 2010; Mohamad et al., 2016). Tromso (2011) also found a significant association between serum vitamin D levels and HbA\(_1c\) (p < 0.001) after adjusting for gender, age, BMI, physical activity, serum triglycerides, serum calcium, and haemoglobin (Hutchinson et al., 2011). This association seems to be most obvious in the elderly, obese, and in those with the highest triglyceride levels and impaired glucose tolerance risk factors for T2DM. A cross-sectional study of 715 T2DM patients found a significant association between HbA\(_1c\) and serum vitamin D (p=0.003) (Zoppini et al., 2013). Another a cross-sectional study of 233 T2DM patients, who ranged in age from 16 to 96 years and who took vitamin D supplements for a six-month period, found an association between vitamin D and the HbA\(_1c\) concentrations (p=0.039) (Sebekova et al., 2015). However, other authors have failed to find a significant effect on Hba\(_1\) level in subjects with low concentrations of serum vitamin D levels (less than 50 nmol/L) (Luo et al., 2009). Even with glycaemic control, vitamin D supplementation was not found to have any affect (Jorde & Figenschau, 2009; Patel et al., 2010). A randomised double-blind control trial found no change in HbA\(_1c\) levels with a single intramuscular injection of 7500 µg of vitamin D supplemented after three months, in 42 Iranian T2DM patients (Heshmat et al., 2012). That could be due to poor glycaemic control, in which vitamin D ineffective due to the low activity of 25-hydroxylase in the liver (Zoppini et al., 2013).

There is an association between vitamin D supplementation and improved fasting blood glucose levels (Kayaniyil et al., 2010). In our study, there was a significant difference in fasting glucose (p=0.001) among the intervention groups (50µg and 100µg vitamin D\(_3\)) compared to the control group. Fasting plasma glucose has also been shown to differ significantly after an intake of 1250µg vitamin D once a week for eight weeks in 100 Iranian T2DM patients.
(p=0.05) (Talaei et al., 2013). However, a cross-sectional study of 715 ambulatory T2DM patients found no significant association with fasting plasma glucose (p=0.122) (Zoppini et al., 2013). A meta-analysis of several studies of vitamin D supplementation showed no effect on the glucose homeostasis (Seida et al., 2014). A randomised control trial did not find a significant difference between subjects administered with vitamin D and the placebo group on the fasting blood glucose levels after six months’ intake of 1000µg of vitamin D per week in 36 subjects with T2DM (Jorde & Figenschau, 2009). Our finding varies from a larger randomised study with 100 T2DM patients with an intake of 125µg per day of vitamin D or a placebo for 12 weeks (Yiu et al., 2013). This could result from the concentration of vitamin D, which was around 77 nmol/L and above in our study (see Table 4.2). An improvement of glycaemic levels has been shown where the vitamin D concentration is above 80 nmol/L (von Hurst et al., 2010).

In our study, there was no significant difference in fasting insulin levels, HOMA-IR and HOMA-β between the intervention groups (50µg and 100µg vitamin D₃) compared to the control group. Measure insulin level is the best way to calculate insulin resistance (HOMA-IR) and insulin secretion (HOMA-β) (Laakso, 1993). Previous studies had similar results (Bjordal et al., 2000; Jorde & Figenschau, 2009; De Boer, 2008; Heshmat et al., 2012). A randomised study of 61 subjects in two intervention groups and a placebo group, and intakes of 2500µg or 5000µg vitamin D₃ for 16 weeks, did not find an improvement in HOMA-IR and HbA₁c (Witham et al., 2010). These findings have been supported with smaller studies using different supplement dosages and durations, which also demonstrated vitamin D to have an effect on insulin, HOMA-IR and HOMA-β (Heshmat et al., 2012; Breslavsky, et al., 2013; Ryu et al., 2014; Kampmann et al., 2014; Al-Shahwan et al., 2015). Nevertheless, an improvement in glucose control found after vitamin D supplementation in T2DMmellitus (Borissova et al., 2003; Al-Daghri, et al., 2012a; Mohamad et al., 2016). An intervention study in 92 Saudi diabetic patients found a significant difference in insulin levels (p<0.001), and an improvement in HOMA-IR (p<0.001) and HOMA-β (p< 0.002) in 120 diabetes patients taking 50µg/day of vitamin D₃ for 18 months. It showed that this improvement was
more marked in women than in men (Al-Daghri, et al., 2012a). In a randomised controlled trial, an intake of 50µg vitamin D₃ daily for 16 weeks was found to improve β-cell function in those at high risk of diabetes (Mitri et al., 2011). Another interventional study with similar doses of vitamin D (50µg/day) found a significant difference in HOMA-IR (p=0.052) and insulin (p=0.044) in 45 Saudi T2DM patients after 12 months’ intervention (Al-Shahwan et al., 2015). A daily dose of 100µg for six months improved the HOMA-IR when the vitamin D concentration was above 80 nmol/L (von Hurst et al., 2010). Another intervention study also found an improvement in HOMA-IR when the vitamin D concentration was between 100 and 150 nmol/L, but no improvement when the vitamin D concentration was below 100nmol/L (Talaei et al., 2013). Talaei (2013) found a significant effect on insulin and HOMA-IR in 100 Iranian T2DM patients after an intake of 1250 µg/week for eight weeks.

On the other hand, insulin resistance and fasting blood glucose levels were unchanged after three years of vitamin D supplementation at 17.5 µg per day, and both markers had significantly increased in the placebo group (Pittas et al., 2007). This suggests that vitamin D may not improve insulin resistance and fasting blood glucose levels. The difference in outcomes may be related to the different doses used, the low vitamin D concentration, ethnic background, and the sample size. Improvements were seen when vitamin D doses were 50 µg or higher, the vitamin D concentration was above 80 nmol/L, and the sample size was larger. Future studies are essential to understand the relationship between glycaemic control and vitamin D in T2DM. Also, there needs to be clarification on, for instance, the type of anti-diabetic medication taken, as this can lead to confusion.

5.5. Conclusion

This study examined the effects of vitamin D on glycaemia and found it significantly lowered glycated haemoglobin (p <0.001) and fasting glucose levels (p <0.001), in both intervention groups compared with the placebo group. There was no significant effect on insulin levels or homeostasis assessment models in poorly controlled T2DM. This suggests that vitamin D may not be essential to improving the homeostasis assessment model marker,
however, vitamin D could have a role in control the development of diabetic complications.
Chapter 6 Effect of vitamin D₃ supplementation on lipidaemia in poorly-controlled type-2 diabetes after 16 weeks
6.1. Introduction

Vitamin D deficiency is related with atherosclerosis (De Boer et al., 2008), obesity (Wortsman et al., 2000), and diabetes (Scragg, 2008). Dyslipidaemia is a major risk factor in developing cardiovascular disease (Paciaroni et al., 2007). Vitamin D deficiency has been related with an increase in total cholesterol and increase in triglycerides (Martins et al., 2007; Karhapaa et al., 2010; Chaudhuri et al., 2013). Also, lower HDL levels have been linked with vitamin D deficiency (Wang et al., 2008; Choi et al., 2011). However, the relationship between vitamin D and dyslipidaemia is contentious (Filippatos et al., 2017).

Vitamin D deficiency may reduce circulating triglycerides via suppression of hepatic triglyceride formation and increased the hepatic calcium intake (Wimalawansa, 2016). Another mechanism by which vitamin D might increase calcium absorption, reducing fatty acids in the gut, increasing fat absorption and lowering triglyceride levels (Zittermann, 2006; Christensen et al., 2009).

The aim of this study was to investigate the effect of vitamin D on improving dyslipidaemia by increasing high-density lipoprotein, and by lowering the serum levels of total cholesterol, triglycerides and low-density lipoprotein in poorly controlled type-2 diabetic Saudi men.

6.2. Methods

Blood samples were taken from all 128 participants at baseline and 16 weeks using the aseptic technique described in section 2.13. High-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides and total cholesterol were determined using the Siemens DCA Vantage Analyzer as per section 2.16.7.

6.3. Results

6.3.1. Analysis of the serum levels of total cholesterol in the intervention and control groups

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D₃), 42
participants in group B (supplementation with 50 µg vitamin D₃) and 43 participants in group C (control, placebo group). The mean concentrations of total cholesterol were analysed among the three groups A, B and C, and differences between the pre-test and post-test mean total cholesterol concentrations among the groups were analysed. A two-way mixed ANOVA statistical test was used to compare the groups, and the summary of the results is presented in Table 6.1. The results have shown that there was a statistically significant difference in the mean total cholesterol concentrations between the measurement at baseline and at the end of the 16-week long intervention (F=19.038, p-value<0.001, Table 6.1). Furthermore, there was a significant difference in mean total cholesterol concentrations was found between the intervention and control groups when measured post-test (F=5.55, p-value<0.005).

Table 6.1. Two-way mixed ANOVA comparing the mean differences of serum levels of total cholesterol upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between baseline and 16-wk intervention</td>
<td>19.038**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction time and the three groups</td>
<td>7.45**</td>
<td>0.001</td>
</tr>
<tr>
<td>Between the three groups per-test</td>
<td>0.951</td>
<td>0.389</td>
</tr>
<tr>
<td>Between the three groups post-test</td>
<td>5.555*</td>
<td>0.005</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean total cholesterol concentration in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test.**indicates a significant difference at p < 0.01 with * of p < 0.005.

Table 6.2. Post-hoc comparisons of the mean total cholesterol serum concentration (in mmol/L) among intervention groups and a control group at baseline and after the intervention period

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.74±1.38</td>
<td>3.77±0.96</td>
<td>-0.965**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B</td>
<td>4.40±1.22</td>
<td>3.89±1.00</td>
<td>-0.513**</td>
<td>0.008</td>
</tr>
<tr>
<td>C</td>
<td>4.42±1.17</td>
<td>4.48±1.17</td>
<td>0.056</td>
<td>0.765</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D and B, 50µg vitamin D and control group C, placebo. **indicates a significant difference between pre-test and post-test of p < 0.001; a indicates adjustment for multiple comparisons; mean ±SD.

Comparison of the pre- and post-test results for each group showed that in group A the total cholesterol decreased significantly after 16 weeks (mean difference=-0.965, p-value<0.001, see Table 6.2). For group B, the total cholesterol also decreased significantly after 16 weeks (mean difference=
0.513, p-value=<0.008, Table 6.1). The results of measurements of the total cholesterol levels among groups A and B and C, at baseline and after the intervention period are also presented in Figure 6.1.

After 16 weeks, the results (as analysed by two-way mixed ANOVA, presented in Table 6.2), showed that there was a significant difference in total cholesterol between the three groups (F=5.55, p <0.005, Table 6.1). As the one-way ANOVA statistical analysis of the results of a post-hoc testing showed, the mean total cholesterol concentration in group A was lower than in group B (mean difference=-0.12, (see Table 6.2). Furthermore, the mean post-intervention concentration of total cholesterol in group A was significantly lower than in group C (mean difference=-0.71, p <0.002) and the post-intervention mean total cholesterol concentration in group B was significantly lower than in group C (mean difference=-0.59, p <0.01).

Table 6.3. Post-hoc comparisons of the mean total cholesterol concentrations among the intervention and the control groups after 16 weeks

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean difference</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 16 weeks</td>
<td>A</td>
<td>B</td>
<td>-0.12</td>
<td>0.612</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>-0.71**</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>-0.59**</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D are compared with control (placebo) group C after 16 weeks of intervention. **indicates a significant difference of p < 0.001.
6.3.2. Triglycerides

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D₃), 42 participants in group B (supplementation with 50 µg vitamin D₃) and 43 participants in group C (control, placebo group). Analysis of the changes in concentrations of triglycerides between the intervention groups A, B and C, pre- and post-intervention, was performed using a two-way mixed ANOVA test. The results of the analysis are shown in Table 6.4. As the results of the mixed ANOVA showed, there was a highly significant difference in mean triglyceride concentrations between the baseline and a sixteen-week point (F=28.19, p-value<0.001, Table 6.4). The interaction between time and treatment was also statistically significant (F=20.02, p-value<0.001), indicating that the treatment effect in at least one group depended on the duration of the treatment.

Figure 6.1. Mean changes of total cholesterol concentration in vitamin D treatment
The bar chart shows intervention groups A, 100µg vitamin D₃ (n=43) and group B, 50µg vitamin D₃ (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences of p < 0.001 are marked with (**).
Table 6.4. Two-way mixed ANOVA analysis of differences in the mean triglycerides concentrations (mmol/L) upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between times (pre- and post-test)</td>
<td>28.19**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction time and the three groups</td>
<td>20.02**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Between the three groups in per-test</td>
<td>0.100</td>
<td>0.91</td>
</tr>
<tr>
<td>Between the three groups in post-test</td>
<td>8.86**</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean triglycerides concentrations in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test.** indicates a significant difference at p < 0.01.

Table 6.5. Post-hoc comparisons of the differences between the mean triglyceride concentrations (mmol/L) among groups (intervention and control) at baseline and after the intervention study

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.14±0.85</td>
<td>1.3±0.43</td>
<td>-0.833**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B</td>
<td>2.07±1.16</td>
<td>1.97±1.07</td>
<td>-0.09</td>
<td>0.337</td>
</tr>
<tr>
<td>C</td>
<td>2.16±1.01</td>
<td>2.16±1.24</td>
<td>-0.004</td>
<td>0.972</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D and control (C) - placebo group. ** indicates a significant difference between pre-test and post-test of p < 0.001; b indicates adjustment for multiple comparisons; mean ± SD.

The results one-way ANOVA of differences in the mean triglyceride concentrations (mmol/L) between the intervention and control groups, between baseline and post- intervention period measurements are summarised in Table 6.5). These show that in group A the triglyceride concentration decreased significantly after 16 weeks (mean difference= -0.833, p-value<0.001). The mean triglyceride concentration in group B also decreased, but not significantly, after 16 weeks (mean difference= -0.09, p <0.337). The differences in mean triglyceride concentrations (mmol/L) in the groups between baseline and after 16 weeks of treatment are also shown in Figure 6.2.

A two-way mixed ANOVA analysis (Table 6.4) showed that after 16 weeks there were significant differences in the mean triglycerides concentration between the three groups (F=8.86, p <0.001). Using one-way ANOVA analysis of the results of the post-hoc testing (see Table 6.6 and Figure 6.2), it was established that the mean triglycerides concentration in group A was significantly lower than in group B (mean difference= -0.66, p <0.002). In
addition, the mean triglyceride concentration in group A was significantly lower than in group C (mean difference = -0.85, \( p < 0.001 \)). The mean triglyceride concentration in group B was also lower but not significantly different from group C (mean difference = -0.19).

Table 6.6. Post-hoc comparisons of triglyceride levels (mmol/L) between intervention groups and control after 16 weeks

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean difference</th>
<th>p-value(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 16 week</td>
<td>A</td>
<td>B</td>
<td>-0.66**</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>-0.85**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>-0.19</td>
<td>0.377</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation B, 50µg vitamin D compared with control (placebo) group C after 16 weeks of intervention. **indicates a significant difference between the groups of \( p < 0.001 \); \(^b\) indicates adjustment for multiple comparisons.
Figure 6.2. Changes in the mean triglyceride concentrations (mmol/L) in the intervention and control groups pre-test and post-test
The bar chart shows intervention groups A, 100µg vitamin D$_3$ (n=43) and B, 50µg vitamin D$_3$ (n=42) compared with the placebo group (C) as control (n=43) between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences of $p < 0.001$ are marked with **.

6.3.3. High-density lipoprotein (HDL)
A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D$_3$), 42 participants in group B (supplementation with 50 µg vitamin D$_3$) and 43 participants in group C (control, placebo group). The mean concentrations of HDL were analysed among group A, B and C, and differences between the pre-test and post-test mean HDL concentrations among the groups were analysed. A two-way mixed ANOVA statistical test was used to compare the groups, and the summary of the results is presented in Table 6.7. The results show that there was statistically significant difference in the mean HDL concentrations between the measurement at baseline and at the end of the
16-week long intervention (F=79.52, p-value<0.001, Table 6.7). Furthermore, there was a significant difference in mean HDL concentrations between the intervention and control groups when measured post-test (F=43.61, p-value<0.001).

Table 6.7. Two-way mixed ANOVA comparing the mean differences of serum levels of HDL upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between baseline and 16-wk intervention</td>
<td>79.52**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction time and the three groups</td>
<td>39.61**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Between the three groups in pre-test</td>
<td>0.008</td>
<td>0.992</td>
</tr>
<tr>
<td>Between the three groups in post-test</td>
<td>43.61**</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean HDL concentrations in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test.**indicates a significant difference at p < 0.01.

Table 6.8. Post-hoc comparisons of the mean HDL serum concentration (mmol/L) between the intervention groups and control group at baseline and after the intervention period

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-value(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.90±.338</td>
<td>1.60±.461</td>
<td>0.70**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B</td>
<td>0.89±.311</td>
<td>1.08±.347</td>
<td>0.19**</td>
<td>0.002</td>
</tr>
<tr>
<td>C</td>
<td>0.90±.335</td>
<td>0.90±.247</td>
<td>0.004</td>
<td>0.951</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D and B, 50µg vitamin D and control group (C), placebo.**indicates a significant difference between pre-test and post-test of p< 0.001. \(^b\) indicates adjustment for multiple comparisons; ± mean SD.

Comparison of the pre- and post-test results for each group showed that in group A the HDL increased significantly after 16 weeks (mean difference=0.70, p-value<0.001, see Table 6.8). For group B, the HDL also increased significantly after 16 weeks (mean difference=0.19, p-value<0.002). The results of measurements of the HDL levels among intervention groups A and B and a control group C, at baseline and after the intervention period are also presented in Figure 6.3.

After 16 weeks, the results (as analysed by two-way mixed ANOVA, Table 6.9), showed that there was a significant difference in HDL between the three groups (F=79.52, p-value <0.001). As the one-way ANOVA statistical analysis of the results of a post-hoc testing showed, the mean HDL concentration in group A was significantly increased than in group B (mean difference=0.53, p-value<0.001, (see Table 6.9). Furthermore, the mean post-intervention
concentration of HDL in group A was significantly greater than in group C (mean difference= 0.70, p <0.001) and the post-intervention mean HDL concentration in group B was significantly greater than in group C (mean difference=0.18, p <0.03).

Table 6.9. Post-hoc comparisons of the mean HDL concentrations between the intervention and control groups after 16 weeks

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean difference</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 16 weeks</td>
<td>A</td>
<td>B</td>
<td>0.53**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>0.70**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>0.18**</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D are compared with control (placebo) group C after 16 weeks of intervention. **indicates a significant difference of p < 0.001; <sup>b</sup> indicates adjustment for multiple comparisons.

Figure 6.3. Mean changes of HDL concentration in vitamin D treatment

The bar chart shows intervention groups A, 100µg vitamin D<sub>3</sub> (n=43) and B, 50µg vitamin D<sub>3</sub> (n=42) compared with the placebo group (C) as control (n=43) between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences of p < 0.001 are marked with **.
6.3.4. Low-density lipoprotein (LDL)

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D₃), 42 participants in group B (supplementation with 50 µg vitamin D₃) and 43 participants in group C (control, placebo group). Analysis of the changes in concentrations of LDL between groups A, B and C pre- and post-intervention, was performed using a two-way mixed ANOVA test. The results of the analysis are shown in Table 6.10. As the results of the mixed ANOVA show there was a highly significant difference in mean LDL concentration between the baseline and 16-week point (F=103.22, p-value<0.001, Table 6.10). The interaction between time and treatment was also statistically significant (F=26.65, p-value<0.001, Table 6.10), indicating that the treatment effect in at least one group depended on the duration of the treatment.

Table 6.10. Two-way mixed ANOVA analysis of differences in the mean fasting LDL concentrations (mmol/L) upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between times (pre- and post-test)</td>
<td>103.22**</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Interaction between different doses, intervention period and groups</td>
<td>26.65**</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Between the three groups in pre-test</td>
<td>1.88</td>
<td>.157</td>
</tr>
<tr>
<td>Between the three groups in post-test</td>
<td>13.97**</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean LDL concentrations in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test.**indicates a significant difference at p < 0.01.

Table 6.11. Post-hoc comparisons of the differences between the mean LDL concentrations (mmol/L) among groups (intervention and control) at baseline and after the intervention study

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-valueᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.56±1.29</td>
<td>1.88±0.98</td>
<td>-1.68**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B</td>
<td>3.16±1.01</td>
<td>2.49±0.97</td>
<td>-0.68**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C</td>
<td>3.29±1.11</td>
<td>2.43±1.03</td>
<td>-0.21</td>
<td>0.149</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D, and control (C) - placebo group. **indicates a significant difference between pre-test and post-test of p < 0.001; ᵃᵇ indicates adjustment for multiple comparisons; ± mean SD.

The results one-way ANOVA analysis of differences in the mean LDL concentrations (mmol/L) in the intervention groups and a control group, between the baseline and the measurements after the intervention period are
summarised in Table 6.11). These showed that in group A the LDL concentration decreased significantly after 16 weeks (mean difference=-1.68, p-value <0.001). The mean LDL concentration in group B also decreased significantly after 16 weeks (mean difference=-0.68, p-value <0.001). The differences in mean LDL concentrations (mmol/L) in the groups between baseline and after 16 weeks of treatment are also shown in Figure 6.4.

A two-way mixed ANOVA analysis results (see Table 6.10) showed that after 16 weeks there were significant differences in the mean LDL concentration between the three groups (F=13.97, p <0.001). Using one-way ANOVA analysis of the results of the post-hoc testing (see Table 6.12 and Figure 6.4), it was established that the mean LDL concentration in group A was significantly lower than in group B (mean difference=-0.61, p =0.003). In addition, the mean LDL concentration in group A was significantly lower than in group C (mean difference=-1.06, p <0.001). The mean LDL concentration in group B was also significantly lower than in group C (mean difference=-0.453, p =0.03).

Table 6.12. Post-hoc comparisons of LDL levels (mmol/L) among interventions groups and control after 16 weeks

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Group</th>
<th>Mean difference</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 16 week</td>
<td>A</td>
<td>B</td>
<td>-0.61**</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>-1.06**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C</td>
<td>-0.453*</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and group B, 50µg vitamin D are compared with control (placebo) group C after 16 weeks of intervention. **indicates a significant difference between pre-test and post-test of p< 0.001; *indicates a significant difference between pre-test and post-test of p< 0.05; <sup>b</sup> indicates adjustment for multiple comparisons.
Figure 6.4. Changes in the mean fasting LDL concentrations (mmol/L) in the intervention and control groups between the pre-test and post-test measurements. Interventions groups A, 100µg vitamin D₃ and group B, 50µg vitamin D₃ and group C control (placebo). Results are presented as mean ± SD (n=128). Statistically significant differences of p < 0.001) are marked with ** and of p < 0.005) are marked with *.

6.4. Discussion

This study has found that vitamin D supplementation of either 100µg or 50µg per day provides a significant improvement in dyslipidaemia. There were reductions in total cholesterol, triglycerides and LDL levels as compared to the placebo group, and improvements in HDL compared to the placebo group. These findings support previous studies (Alkharfy et al., 2013; Mohamad et al., 2016).

A Middle Eastern intervention study of 92 type 2 diabetic Saudi subjects who had an intake of 50µg of vitamin D for 18 months, presented a mean serum level of vitamin D of 23.5 nmol/L. There was a significant improvement in serum total cholesterol as well as LDL (p < 0.001 and p < 0.004, respectively).
However, there was no change in serum triglycerides and HDL with an increase of 20 nmol/L in the mean serum level of vitamin D across the intervention period (Al-Daghri, et al., 2012a). It has been suggested that an improvement in lipidaemia requires a higher concentration of vitamin D than 70-80 nmol/L (Hossein-nezhad & Holick, 2013). In an intervention study of 499 Saudi type 2 diabetic patients randomly given 50µg vitamin D daily or receiving none as control group (who were advised to increase sun-exposure) for 12 months, found significant improvements in serum triglycerides and total cholesterol, as well as HDL were found in men (Alkharfy et al., 2013). Another Middle East intervention study was of 100 T2DM patients who had an intake of 112.5µg /day of vitamin D for 8 weeks. It found a significant increase in serum HDL and a significant decrease in total cholesterol and LDL levels (Mohamad et al., 2016). It indicated that a concentration of serum vitamin D greater than 49.92 nmol/L was significantly associated with decreased serum LDL cholesterol (Chaudhuri et al., 2013) and it suggested that if the vitamin D concentration were greater than 152 nmol/L, it would improve the diabetic lipid profiles (Mohamad et al., 2016). The mean vitamin D concentration of our study was 77.85 ± 5.27 nmol/L and we had a significant improvement in male diabetic subjects that supports the suggestions of Chaudhuri et al., (2013) and Mohamad et al., (2016).

A Middle Eastern cross-sectional study of 108 Iranian T2DM patients who were aged between 20 and 80 years, found an association between serum vitamin D concentrations and triglycerides (Saedisomeolia, 2014). Another Middle Eastern cross-sectional study of 309 Emirati obese and T2DM patients found a correlation between serum vitamin D and reduced serum triglycerides, LDL ($p<0.01$) and a relationship between serum vitamin D and serum HDL, LDL, total cholesterol and triglycerides which was stronger in men than in women (Sadiya et al., 2014). Serum triglyceride reduction could involve vitamin D and intestinal calcium absorption increases by vitamin D. Then, this calcium might reduce the triglyceride levels via the reduction of hepatic triglyceride formation and secretion through a suppressive effect of vitamin D on the concentration of parathyroid hormone (Eftekhari et al., 2014).
A consecutive study of 28 type 2 diabetic patients who were treated with 400 µg of vitamin D per week for 2 months found a significant decrease in serum total cholesterol \( (p<0.04) \), and decreased serum LDL, cholesterol and triglyceride levels. However, these findings were not statistically significant. There was no change in serum HDL cholesterol levels (Ramiro-Lozano & Calvo-Romero, 2015). The meta-analysis of Wang et al., (2012) conducted on 12 intervention studies of 1,346 patients, found a pooled effect on serum LDL whereas there were no statistically significant effects on serum total cholesterol, HDL and triglyceride levels after an intake of vitamin D (Wang et al., 2012). It was stated that the significant result on serum LDL levels of vitamin D supplementation was seen in obese subjects and in short study interventions, whereas in long trials, a significant effect on serum HDL level was observed (Wang et al., 2012).

Some interventions failed to find significant results in improving lipid metabolism (Yiu et al., 2013; Ryu et al., 2014). An intervention study of 100 Iranian type 2 diabetics with an intake of 1250 µg/week of vitamin D for 8 weeks, indicated that there was no significantly different change in the levels of serum total cholesterol, LDL, HDL or triglycerides (Talaei et al., 2013). This outcome could be because it was a single-blind study without comparison with a placebo. A double-blind randomised clinical trial of 87 Emirati obese type 2 diabetic subjects with an intake of 150 µg/day of vitamin D for 12 weeks, then another 75 µg/day for 12 weeks, showed the mean vitamin D concentration was 61.4 ± 18.8 nmol/L and it found no effect on the lipid profile (Sadiya et al., 2015). This could be because it did not reach sufficiently high vitamin D concentrations of >75 nmol/L. Another double-blind, randomised, placebo-controlled trial had 70 Iranian type 2 diabetics who were aged 30-75 years and given 0.50 µg of vitamin D/day. It was found that there was a significant decrease in serum total cholesterol, triglycerides and LDL levels, \( p< 0.05 \), in both intervention and placebo groups. However, there was no change in serum HDL levels in the intervention group, and there were no statistically significant changes between all the variables of the groups (Eftekhari et al., 2014). In an intervention study of 36 type 2 diabetic patients with normal serum levels of vitamin D and an intake of 1000 µg/week of vitamin D for 6 months, and no
change in lipid profiles was found (Jorde & Figenschau, 2009). Another study was of 151 subjects with a high risk of cardiovascular disease and who had vitamin D deficiency. An intake of 1250 µg/week of vitamin D for 8 weeks was found to have no effect on the lipid profile (Ponda et al., 2012). Also, a study of 24 T2DM subjects with an intake of 25µg/day of vitamin D for 12 months and a mean serum concentration of vitamin D of 29.45 nmol/l, found no significant result on serum total cholesterol, LDL, HDL or triglycerides levels. Furthermore, the mean serum concentration of vitamin D was only 43.92 nmol/l after the intervention study (Breslavsky et al., 2013). Vitamin D concentration was below the sufficient vitamin D concentration level, which confirmed the suggestion that it must be 75 nmol/L to have a good improvement in lipid metabolism.

The effects of vitamin D supplementation studies on improving dyslipidaemia are inconsistent (Eftekhari et al., 2014; Mohamad et al., 2016). It is too complex to draw a conclusion for a variety of reasons such as the designs of the studies, doses, ethnic background, duration of the studies and sample sizes. A significant improvement in dyslipidaemia has been found in Middle Eastern studies when the vitamin D dose is 50µg/day or high, when 70 participants or more are included and when the duration of the study is 16 weeks or greater. Also, if concentrations of serum vitamin D above 152.25 nmol/L have been achieved, they have shown significant improvement in lipidaemia. The limitation of these studies is that they lack comparison of the different T2DM therapies and how they affect vitamin D supplementation and metabolic changes, not only by gender, but also by the presence of the disease itself.

6.5. Conclusion

This study investigated the effects of vitamin D on dyslipidaemia and found a significant improvement in total cholesterol (p <0.005) and triglycerides, HDL and LDL (p <0.001), in both intervention groups compared with the placebo group after the intervention. This was in Saudi men with poorly controlled T2DM. This suggests that vitamin D may be essential to improve the lipid biomarkers’ profile.
Chapter 7 Effect of vitamin D₃ supplementation on oxidative stress in poorly-controlled type-2 diabetes after 16 weeks
7.1. Introduction

Oxidative stress is a major factor facilitating the development and pathogenesis of complications in T2DM mellitus (Cavalcante et al., 2015). In diabetes, persistent hyperglycaemia distorts the endogenous antioxidant defence mechanisms in type-2 diabetic patients (Ceriello, 2000). Hyperglycaemia also increases free radicals (Ullah et al., 2016). The endogenous antioxidant defence system is composed of the non-enzymatic and enzymatic pathways (Sebekova et al., 2015). These pathways play a crucial part in counterbalancing toxic reactive oxygen species (ROS) (Picu et al., 2017). Regulation of oxidising and reducing states is important for various organ functions, and cell proliferation, activation and viability (Shab-Bidar et al., 2015).

Advanced glycation end products, also known as AGEs, are a group of compounds involved in the pathophysiology of diabetic complications (Ahmed, 2005). Vitamin D may minimise various depositions of AGEs in type-2 diabetic patients (Sebekova et al., 2015). Vitamin D may influence oxidative stress as it reduces the formation of plasma free radicals and increases antioxidant defences in diabetic patients (Singh et al., 2002; Saif-Elnasr et al., 2017). The relation between vitamin D and AGEs is limited and it has been established only in diabetic rats (Salum et al., 2013).

The aim of this chapter was to investigate the effect of vitamin D₃ on oxidative stress in improving the antioxidant capacity and reducing the AGEs in poorly-controlled type 2 diabetic Saudi men.

7.2. Methods

Blood samples were taken at baseline and after 16 weeks of vitamin D₃ supplementation (or placebo) from 128 participants using the aseptic technique described in section 2.13. Total antioxidant capacity was determined using OxiSelect™ Total Antioxidant Capacity (TAC) Assay Kits as per section 2.16.8 and AGEs were determined using an AGE skin autofluorescence reader as described in section 2.12.
7.3. Results

7.3.1. Total antioxidant capacity (TAC)

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D₃), 42 participants in group B (supplementation with 50 µg vitamin D₃), and 43 participants in group C (control, placebo group). The changes in total antioxidant capacity (in mM) were analysed among groups A, B and C (control group) pre-test as well as post-test, by two-way mixed ANOVA statistical analysis. The results, summarised in Table 7.1, show that no statistically significant difference (of \( p \leq 0.05 \)) was observed between the pre- and post-hoc total antioxidant capacity, nor between the groups either pre- or post-test. Furthermore, no significant correlation between the duration of the intervention and the effect on insulin levels was observed in the studied groups.

Table 7.1. Two-way mixed ANOVA test comparing the mean differences of total antioxidant capacity upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between times</td>
<td>3.882</td>
<td>0.051</td>
</tr>
<tr>
<td>Interaction between different doses, intervention period and groups</td>
<td>1.17</td>
<td>0.365</td>
</tr>
<tr>
<td>Between the three groups pre-test</td>
<td>0.637</td>
<td>0.530</td>
</tr>
<tr>
<td>Between the three groups post-test</td>
<td>1.02</td>
<td>0.361</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean TAC in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test.

Table 7.2. Post-hoc comparisons of total antioxidant capacity (mM) concentration among the intervention and control groups, at baseline and after the intervention

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.61±0.14</td>
<td>0.66±0.27</td>
<td>0.054</td>
<td>0.077</td>
</tr>
<tr>
<td>B</td>
<td>0.65±0.23</td>
<td>0.71±0.28</td>
<td>0.050</td>
<td>0.103</td>
</tr>
<tr>
<td>C</td>
<td>0.63±0.18</td>
<td>0.63±0.16</td>
<td>-0.001</td>
<td>0.982</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation and group B, 50µg vitamin D and control (C)-placebo group; * indicates adjustment for multiple comparisons; mean ± SD.

Furthermore, the post-hoc comparison between the mean total antioxidant capacity (mM) in the intervention groups and control, between the baseline and after the intervention period, using the mixed ANOVA test (Table 7.2, and Figure 7.1), did not reveal any statistically significant (\( p > 0.05 \)) changes in the total antioxidant capacity between duration of the treatment and the effect on
the group (F=1.17, p-value ≤ 0.36, Table 7.1.), nor were there significant differences between the groups pre- and post-test.

Figure 7.1. Mean changes in plasma total antioxidant capacity (mM) in the intervention groups A and B, and the control group C (placebo). Interventions groups A, 100µg vitamin D₃ and group B, 50µg vitamin D₃, and group C, control (placebo). Mean total antioxidant capacity measured pre- and post-test; Results are presented as mean ± SD (n=128). The calculated differences in mean total antioxidant capacity between pre- and post-test were not statistically significant.

7.3.2. Advanced glycation end products (AGEs)

A total of 128 male patients with T2DM took part in the study. There were 43 participants in group A (supplementation with 100 µg vitamin D₃). 42 participants in group B (supplementation with 50 µg vitamin D₃), and 43 participants in group C (control, placebo group). The changes in the mean AGEs values were analysed among groups A, B and C (placebo) pre- and post-test, as well as in relation to the duration of treatment, by two-way mixed
ANOVA is summarised in Table 7.3. The results of the two-way mixed ANOVA test showed that there was no significant difference in mean skin AGEs between the groups in pre- or post-test (F=.032, p-value > 0.57, and F=0.25, p-value > 0.77, respectively, Table 7.3.). In addition, the interaction between treatment duration and the effect was not significant (F=0.57, p-value ≤ 0.57).

Table 7.3. Two-way mixed ANOVA comparing the mean differences of AGEs upon vitamin D supplementation

<table>
<thead>
<tr>
<th>Source of difference</th>
<th>F-test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between times</td>
<td>4.24</td>
<td>0.050</td>
</tr>
<tr>
<td>Interaction between different doses, intervention period and groups</td>
<td>0.57</td>
<td>0.571</td>
</tr>
<tr>
<td>Between the three groups in pre-test</td>
<td>0.327</td>
<td>0.722</td>
</tr>
<tr>
<td>Between the three groups in post-test</td>
<td>0.258</td>
<td>0.773</td>
</tr>
</tbody>
</table>

The comparisons included: the difference in mean AGEs in vitamin D supplementation groups (group A - 100 µg and group B - 50µg) and a control group (placebo) between baseline and after intervention, between different doses and intervention period, between the groups in pre-test measurement, and between groups in post-test.

Table 7.4. Post-hoc comparisons of AGEs between groups (intervention and control) at baseline and after the intervention study

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>After 16 weeks</th>
<th>Mean difference</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.35±0.49</td>
<td>2.34±0.39</td>
<td>-0.001</td>
<td>0.745</td>
</tr>
<tr>
<td>B</td>
<td>2.36±0.55</td>
<td>2.32±0.52</td>
<td>-0.038</td>
<td>0.113</td>
</tr>
<tr>
<td>C</td>
<td>2.43±0.50</td>
<td>2.39±0.50</td>
<td>-0.039</td>
<td>0.104</td>
</tr>
</tbody>
</table>

Intervention groups A, 100µg vitamin D supplementation, and group B 50µg vitamin D; control group (C) – placebo; <sup>b</sup> indicates adjustment for multiple comparisons; mean +/-SD.

The mean AGEs among the intervention groups and control group from the baseline to after the intervention period were calculated (as summarised in Table 7.4). The small decreases in mean AGE values in groups A, B and C, between baseline and after 16 weeks of treatment, were not statistically significant (see Table 7.4 and Figure 7.2).
Figure 7.2. Mean changes in AGEs (UV) in the intervention groups A and B, and the control group C (placebo). Interventions groups A: 100µg vitamin D₃ and B: 50µg vitamin D₃ and group C: control (placebo). Mean AGEs measured pre- and post-test; Results are presented as mean ± SD (n=128). The calculated AGEs between pre- and post-test were not statistically significant.

7.4. Discussion

In this study, vitamin D supplementation of either 100µg or 50µg per day failed to demonstrate any improvement in oxidative stress as compared to the placebo group. There was a slight increase in total antioxidant capacity for both intervention groups, but it was not statistically significant when compared with the control group. Also, mean skin AGEs were not significantly different between the vitamin D₃ treatment groups and control group.

Clinical findings on total antioxidant functions of vitamin D in diabetes are limited and the previous evidence (Yiu et al., 2013; Shab-Bidar et al., 2015) provided conflicting findings with range of oxidative stress biomarkers having been used. A randomised, controlled trial of 100 T2DM given supplements in
fortified yogurt that contained 25µg of vitamin D₃, showed a significant improvement in serum total antioxidant capacity (p ≤ 0.03) and malondialdehyde (p ≤ 0.002) after 12 weeks (Shab-Bidar et al., 2015). A double blind, randomised, placebo-controlled trial conducted with 40 non-diabetic elderly women receiving 5000µg of vitamin D₃ or a placebo for one month found a significantly increased total antioxidant capacity (p ≤ 0.03) (Cavalcante et al., 2015). In a cross-sectional study conducted in 200 patients with T2DM or healthy controls, a significant, positive association was found with total antioxidant capacity (p ≤ 0.05) compared to a healthy group (Saedisomeilia et al., 2013). However, this finding was contradicted in the literature and it was based on limited evidence (Salum et al., 2013).

Another clinical finding used different biomarkers for testing antioxidant activity. An intervention study of 90 T2DM subjects aged 30-50 years used supplements with fortified yogurt that contained 25µg of vitamin D₃. After 12 weeks, significantly increased levels of the antioxidant superoxide dismutase (p ≤ 0.025) were found (Nikooyeh et al., 2014). In addition, a Turkish study of 23 subjects deficient in vitamin D who took 250µg/day of vitamin D₃ for 12 weeks, determined that vitamin D had a significant effect on reducing oxidative stress (Tarcin et al., 2009). A study by (Salum et al., 2013) compared diabetic rats that received 12.5µg/day of vitamin D₃ for 10 weeks with diabetic rats that did not receive treatment. The researchers found there was a significant improvement in the total antioxidants in diabetic rats with an intake of vitamin D₃ compared with diabetic rats without treatment (p ≤ 0.001). In contrast, no significant change was found in the antioxidant superoxide dismutase in T2DM subjects after an intake of 125µg of vitamin D for 12 weeks (Yiu et al., 2013). They also showed a reduction of malondialdehyde after 0.25µg of vitamin D/day for 12 weeks but this was not statistically significant (Eftekhari et al., 2014).

In addition, one of the oxidative stress biomarkers used in this study was skin AGEs. Skin autofluorescence has been used as a measure of AGEs’ accumulation, and evidence for a relationship between vitamin D₃ and AGEs accumulation is, so far, is scarce. In this study, there was no significant
reduction in skin AGEs and these findings are supported by similar studies conducted in 245 patients with T2DM who were randomly assigned to receive either vitamin D$_3$ 1250µg/month or a placebo for 6 months. There was no effect was observed on skin AGEs’ accumulation compared to the placebo (Krul-Poel et al., 2015a). In a cross-sectional study, in a total of 233 type-2 diabetics, no association between vitamin D status and skin AGEs or plasma AGE-fluorescence was seen (Sebekova et al., 2015). That could have resulted from using an AGE reader to measure the AGEs accumulated in the skin and this may not to be enough to measure all the AGE types such as circulating AGEs and AGEs without fluorescent properties (Krul-Poel, et al., 2015a).

On the other hand, an intervention study that used an alternative biomarker of AGEs in 90 type-2 diabetic subjects aged 30-50 years who took supplements with fortified yogurt that contained 25µg of vitamin D$_3$ for 12 weeks. A significant decrease in serum AGEs ($p \leq 0.003$) was found (Nikooyeh et al., 2014). The literature review demonstrated that the relation between AGEs and vitamin D is limited to having been seen in diabetic rats (Salum et al., 2013; Lee et al., 2014). Vitamin D may reduce the accumulation of AGEs, and vitamin D supplementation provides an important protection from the oxidative damage associated with the development of diabetic vascular complications (Salum et al., 2013). It suggests that using a different way of measuring AGEs, such as serum of AGEs and receptor AGEs would have provided a different result, and that using a variety of biomarkers to measure oxidative stress is warranted. A longer duration of the intervention than the present study is also needed.

7.5. Conclusion

This study investigated the effects of vitamin D on oxidative stress and found no significant improvement in the total antioxidant status and reduction of the AGEs in either intervention group compared with the placebo group in Saudi men with poorly-controlled T2DM. There is a lack of research in the literature as well as studies in the field to assess the effect of vitamin D on oxidative stress. It would be recommended to study a variety of biomarkers of oxidative stress and also to use AGEs in both skin and serum.
Chapter 8 General discussion
8.1. General Discussion

Diabetes is a group of metabolic diseases characterised by hyperglycaemia. Hyperglycaemia is the result of a disorder in insulin secretion and insulin sensitivity and causes disturbances of carbohydrate, fat and protein metabolism (Alotaibi et al., 2017). The incidence of T2DM has increased for the past two decades and this number is estimated to go beyond 435 million worldwide by 2030 (Nguyen et al., 2017). This number is expected to increase to 642 million by 2040 (Ogurtsova et al., 2017). The largest numbers of people with diabetes are in China, about 109.6 million, and India, about 69.2 million. However, the highest prevalence rates of diabetes are found in the Pacific Islands and the Middle East (Unnikrishnan et al., 2017). In Saudi Arabia, the recent prevalence of T2DM is 32.8%, and therefore will expected to increase to 35.37% by 2020 and 45.8% by 2030 (Meo, 2016).

Hyperglycaemia also increases the production of advanced glycation end products (AGEs) (Bos et al., 2011). AGEs and diabetes are associated with several complications, including oxidative stress (Chang et al., 2011). Diabetes mellitus type 2 in combination with a deficiency of vitamin D levels has been associated with increased markers of oxidative stress in literature reviews (Codoñer-Franch et al., 2012; Talaei et al., 2013). In Saudi men, the prevalence of vitamin D deficiency is between 28% and 37% although Saudi is a sunny country (Sadat-Ali et al., 2009). Since dark skin absorbs a reduced amount of ultraviolet radiation B (UVB) light, then it is less able to synthesis vitamin D (Holick, 2004). The presence of vitamin D receptor gene polymorphisms can also impair the production of vitamin D and result in a higher risk of T2DM (Yang et al., 2017).

In the present study, there was no relationship between vitamin D and oxidative stress in Saudi male patients with T2DM, whether in improving the total antioxidant or reducing the accumulation of AGEs. Our finding is contrast with (Cavalcante et al., 2015) who found a significantly increased total antioxidant capacity. The previous finding was limited to T2DM subjects and it was observed only in diabetic rats (Salum et al., 2013). However, vitamin D demonstrates an effect on glycaemic control (Rolim et al., 2016). Prior studies
have observed a significant association between HbA$_{1c}$ and serum vitamin D levels in diabetic subjects (Kositsawat et al., 2010; Tahrani et al., 2010). Our intervention study found a significant reduction in HbA$_{1c}$ in both groups A (100µg vitamin D$_3$) and B 50µg (vitamin D$_3$), with a mean difference = -2.10, $p<0.001$ and mean difference= -0.95, $p<0.001$, respectively. In addition, it found a significantly reduced fasting glucose ($p <0.001$) among the intervention groups (50µg and 100µg vitamin D$_3$) compared to the control group (placebo). An improvement of glycaemic levels has been shown where the vitamin D concentration is above 80 nmol/L and that concentration is in our intervention study (von Hurst et al., 2010).

Vitamin D supplementation plays a role in stimulating insulin release by the vitamin D receptor and the active form of vitamin D (1,25(OH)$_2$D) which is found in the pancreatic β-cell (Wimalawansa, 2016). Support for the hypothesis of an improvement in insulin resistance after vitamin D supplementation has been highlighted by Al-Shahwan et al. (2015). However, in our intervention study, we did not observe a significant change in insulin resistance (HOMA-IR or HOMA-β). Previous studies had similar results for HOMA-IR or β-cell function (HOMA-β) (Bjordal et al., 2000; de Boer, 2008; Jorde & Figenschau, 2009; Heshmat et al., 2012). A high level of vitamin D could be connected with a lower risk of T2DM and a recommendation that maintaining optimal vitamin D status may be a strategy to avert the development of T2DM (Liu et al., 2010).

Dyslipidaemia is a common feature, and one of the most important risks, of atherosclerosis in diabetes (Mooradian, 2009). It consists of increased triglyceride and/or cholesterol levels and decreased HDL cholesterol levels (Martinez-St John et al., 2016). The diabetic dyslipidaemia pathophysiology is not fully understood, although insulin has an important function in regulating the metabolism of lipids (Verges, 2015; Schofield et al., 2016). Vitamin D supplementation plays a role in the improvement in lipid levels by the vitamin D receptor, which enhances the absorption of fat in the gut (Pilz et al., 2016). Vitamin D also reduces the circulating triglycerides via suppression of hepatic triglyceride formation and increases in the hepatic calcium intake.
In the present intervention study, there were reductions in total cholesterol, triglycerides and low-density lipoprotein (LDL) levels, and improvements in high-density lipoproteins (HDL) compared to the placebo group, supporting the suggestions of (Chaudhuri et al., 2013; Mohamad et al., 2016). Serum HDL concentrations were increased in both intervention groups compared to the placebo group ($p<0.001$). The serum concentrations of LDL were reduced after treatment with vitamin D$_3$ by $-1.68$ mmol/L in group A (100µg vitamin D$_3$) and $-0.68$ in group B (50µg vitamin D$_3$). Triglycerides also had a significant reduction ($p < 0.001$) but only at the higher dose of vitamin D group (100µg vitamin D$_3$) with no significant reduction in group B (50µg vitamin D$_3$). Serum concentrations of total cholesterol were significantly reduced after the 16 weeks’ treatment with vitamin D; mean difference $-0.97$ mmol/L in group A (100µg vitamin D$_3$) and $-0.513$ mmol/L in group B (50µg vitamin D$_3$).

Unfortunately, it is difficult to draw a conclusion from this owing to the inconsistent results from previous studies (Kampmann et al., 2014; Mohamad et al., 2016). These studies had a variety of designs, doses, subjects’ ethnic background, duration of the studies and sample sizes. It is recommended that to observe a significant improvement in dyslipidaemia in Middle Eastern subjects, the vitamin D dose should be 50µg/day or higher, include 70 participants or more, that the duration of the study be 16 weeks or longer, and that serum vitamin D concentrations be above 152.25 nmol/L (Al-Daghri, et al., 2012a; Alkharfy et al., 2013; Mohamad et al., 2016).

Vitamin D supplementation may also have diverse positive effects, such as improvement in muscle performance that could lead to augmented physical activity and weight loss and, thus, lessen insulin resistance (Mason et al., 2016). In the present study, an improvement in lowering the mean BMI was found in both groups A (100µg vitamin D$_3$) and B (50µg vitamin D$_3$) from 30.42 ± 5.27 to 29.63 ± 5.08 kg/m$^2$ and from 30.28 ± 5.26 to 29 ± 4.66 kg/m$^2$, respectively. However, it was not statistically different when comparing the intervention groups with the control after 16 weeks, supporting the finding of a double-blind study by (Mason et al., 2014).
Effect of vitamin D$_3$ supplementation

- Lipidaemia
  - Total cholesterol
  - Triglycerides
  - HDL
  - LDL
- Glycaemia
  - HbA$_{1c}$
  - Fasting blood glucose

Figure 8.1: The beneficial changes observed in the biomarkers after vitamin D supplementation in this intervention study. Vitamin D supplementation in this study improved the biomarkers for glycaemia and lipidaemia in both groups compared to control.

8.2. Limitations of the trial

It is plausible that a number of limitations could have influenced the results obtained. To begin with, the length of the intervention was short, which to have an effect on insulin resistance or β-cell function, warrants a longer study duration. In addition, Middle Eastern studies require a high dose of the vitamin D greater than or equal to 50µg per day. Despite this, the present study was unable to investigate a significant relationship of vitamin D and oxidative stress. It suggests that measuring different types of AGEs, such as serum of AGEs and receptor AGEs, would have provided a different result. Also, using a variety of biomarkers to measure oxidative stress is warranted.
8.3. Future work

The findings of this study have a number of important implications for future practice. A further study could assess the long-term effects of vitamin D supplementation on insulin resistance, β-cell function or oxidative stress, for which a longer trial is warranted. Further studies need to be carried out in order to validate which marker is most effective in oxidative stress as well as measuring different types of AGEs, such as serum of AGEs and receptor AGEs. Another possible area of future research would be to investigate how different therapies of T2DM affect vitamin D supplementation and metabolic changes. Also, it recommends conducting research in increasing of diabetes genetically, finding the obesity cut-off points for the risk of diabetes and conducting community studies to increase the awareness of the epidemic of diabetes.

8.4. Conclusion

This study investigated the effects of vitamin D on glycaemia, dyslipidaemia, and oxidative stress. Vitamin D₃ has been shown to control glycaemia significantly and improved lipid metabolism in both intervention groups compared with the placebo group in Saudi men with poorly-controlled T2DM. However, there was no relationship between vitamin D and oxidative stress or insulin sensitivity and secretion. Observational studies mostly showed that vitamin D intake and supplements reduce the risks of developing T2DM. The intervention trials showed mixed results and were inconclusive on this matter. Large doses of vitamin D, nevertheless, have shown some protective factors for those at risk in developing T2DM. Even if the exact functions of vitamin D in helping to control blood glucose remain inadequately understood, vitamin D status seems to have a significant role in controlling the development and treatment of diabetes. It is likely that optimised levels of serum vitamin D may be dissimilar for people at risk for developing diabetes. Vitamin D doses of 1,250 µg/week of vitamin D3 for 6–8 weeks, thereafter 20-25 µg/per day of vitamin D3 are highly recommended (Dawson-Hughes et al., 2010).
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Appendices

Appendix 1

FACULTY OF SCIENCE AND ENGINEERING

MEMORANDUM

TO Hend Alharbi

FROM Elanor Henry

DATE 1st May 2014

SUBJECT Application for Ethical Approval (SE121327A1)

On the 1st May 2014 the Head of Ethics for Science & Engineering considered your amendments to application for Ethical Approval (SE121327A1) entitled “The effect of vitamin D3 on biomarkers of glycaemia and oxidative stress in Saudi males with poorly-controlled type 2 Diabetes Mellitus”. The application has been granted Favourable Opinion and you may now commence the project.

MMU requires that you report any Adverse Event during this study immediately to the Head of Ethics (Prof Bill Gilmore) and the Administrator (Elanor Henry). Adverse Events are adverse reactions to any modality, drug or dietary supplement administered to subjects or any trauma resulting from procedures in the protocol of a study.

An Adverse Event may also be accidental loss of data or loss of sample, particularly human tissue. Loss of human tissue or cells must also be reported to the designated individual for the Human Tissue Authority licence (currently Prof Bill Gilmore).

If you make any changes to the approved protocol these must be approved by the Faculty Head of Ethics. If amendments are required you should complete the attached form and submit it to the Administrator.

Regards

Elanor Henry
Assistant Research Administrator
All Saints North
Appendix 2: King Fahad Medical City ethical approval

IRB Registration Number with KACST, KSA: H-01-R-012
IRB Registration Number with OHRRP/NIH, USA: IRB00003644
Approval Number Federal Wide Assurance NIH, USA: FWA00018774

April 1, 2014
IRB Log Number: 14-034
Department: Diabetes
Category of Approval: FULL

Dear Dr. Naji Aljohani:

I am pleased to inform you that submission dated February 18, 2014 for the study titled ‘The effect of vitamin D3 on biomarkers of glycaemia and oxidative stress in Saudi men with poorly controlled Type 2 Diabetes Mellitus (TZDM) was reviewed at the IRB meeting held on February 24, 2014 and was approved according to Good Clinical Practice guidelines. The list of documents reviewed and members present are attached.

Please be informed that in conducting this study, you as the Principal Investigator are required to abide by the rules and regulations of the Government of Saudi Arabia, the KFMC/IRB policies and procedures, and the ICH Good Clinical Practice guidelines. Further, you are required to submit a Progress Report before March 1, 2015; it can be reviewed by the IRB without lapse of approval. The approval of this proposal will automatically be suspended on April 1, 2015 pending the acceptance of the Progress Report. You also need to notify the IRB as soon as possible in the case of:

1. Any amendments to the project;
2. Termination of the study;
3. Any serious unexpected adverse events (within two working days);
4. Any event or new information that may affect the benefit/risk ratio of the proposal.

Please observe the following:

1. Personal identifying data should only be collected when necessary for research;
2. The data collected should only be used for this proposal;
3. Data should be stored securely so that a few authorized users are permitted access to the database;
4. Secondary disclosure of personal identifiable data is not allowed;
5. Copy of the Consent Form should be kept in the Research Subject’s Medical Record and the consent process should be documented in the medical record;
Appendix 3: The approval of clinical laboratory to analyse the blood samples

 Saúde da palavra. Fãs de fãs do nuvi.

سلام عليكم ورحمة الله وبركاته

لقد أحال فريقك بنجاح اختبارات الجملة العصبية الكبيرة أعلاه في الاجتماع الذي حضر يوم الخميس 13/03/2014 م برعاية سيدتي إلهام. من خلال ذلك، يمكننا أن نقول:

"The Effect of Vitamin D supplementation on Markers of Glycaemic and Oxidative Stress in Saudi Men with Poorly Controlled Type 2 Diabetes Mellitus."

وقد رأفت اللجنة على التعديل الذي تم من خلاله بشأن عدد المشاركين المشاركين بالنظر إلى أن

يجب بهذا التغيير في النسخة الإصلية المقدمة للجنة.

القرار: الموافقة.

ونتهوا خالص تحياتي وتدمير...

رئيس لجنة اختبارات الجملة العصبية العلمية

 بكلية العلوم

ج. ناصر بن محمد الداغري

P.O. Box 4855, Riyadh 11451 Fax 966(1) 907280

E-mail: cad@ku.edu

151
Appendix 4: Registration of the clinical trial into the Saudi clinical trials registry (SCTR)

<table>
<thead>
<tr>
<th>PART 1: STUDY IDENTIFICATION</th>
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<tbody>
<tr>
<td><strong>1.1 Scientific Title</strong> *</td>
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<td><strong>1.2 Public Title</strong></td>
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<tr>
<td><strong>1.3.1 Protocol Information</strong></td>
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<td>☐ Cohort studies ☐ Randonized, placebo-controlled, double blind trial</td>
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<td><strong>1.5 Therapeutic Area</strong> *</td>
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<tr>
<td><strong>1.6 Disease Name</strong></td>
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</table>

PART 2: TRIAL SUMMARY

| **2.1.1 Involves** * | ☐ Healthy Volunteers |
| ☐ Patients |
| ☐ Both Healthy Volunteers and Patients |
| **2.1.2 Does it Involve Vulnerable Volunteers?** | ☐ Yes ☐ No |
| if yes specify: |
| **2.2 Blinding** * | ☐ Single-Blind |
| ☐ Double-Blind |

Appendix 5: Approval from Saudi Food and Drug Authority (SFDA) of products intended to be cleared or imported from outside Saudi
<table>
<thead>
<tr>
<th>Intended use</th>
<th>Quantity/Pack size</th>
<th>Pharmaceutical Form</th>
<th>Product name (Trade Name)</th>
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</thead>
<tbody>
<tr>
<td>Clinical trials</td>
<td>135</td>
<td>120</td>
<td>Capsules</td>
</tr>
</tbody>
</table>

**Other (please write it):**
- By internet (write the website address)
- Pharmacy from outside the kingdom (please write it)

**School Of Healthcare Science Manchester Metropolitan University**

Because this Clinical trials will be carried out under supervision in cooperation between Manchester Metropolitan University in the United Kingdom and Ministry of Health (King Fahad Medical City) in Saudi Arabia. Participants will be randomly assigned to receive one of three oral treatments: 50 µg/day (2000 IU) of vitamin D₃, 100 µg/day (4000 IU) of vitamin D₂, or a placebo.

**Declaration**

- The products are for my clinical trial use only and I'll take full responsibility if any side effects happen from usage.

**Signature**

ID Number | Date | Signature
---|---|---
| | |
Appendix 6: MMU informed consent form

INFORMATION ABOUT THE PROJECT

Project title: The effect of vitamin D₃ on biomarkers of glycaemia and oxidative stress in Saudi men with poorly controlled type 2 Diabetes

Dear Participant,

This document gives details about and explains a PhD project in Nutrition and Physiology at Manchester Metropolitan University (MMU).

The information collected in the study includes:

- **Pre-screening study questionnaire:**
  You will be given a questionnaire to finish at your convenience. The questionnaire will take between five and ten minutes to complete.

- **Anthropometric Data:**
  Your height, weight and waist circumference will need to be measured at the beginning and at end of the project.

- **Blood samples:**
  A blood sample needs to be taken at the beginning of the project and again 16 weeks later.

- **Intervention:**
  Those taking part in the study will need to take a tablet of vitamin D or placebo once a day for 16 weeks and to write down each day if the tablet was taken or if it was missed.

- **Debriefing questionnaire**
  The debriefing questionnaire will follow the same principles as the medical questionnaire. This questionnaire contains questions about the need for assistance and the presence of questionnaire items which were confusing, difficult to answer or upsetting.

Confidentiality and data protection

You can choose whether to answer any or all of the questions you are asked. If at any point in the study you would like to end your participation, you are free to do so. All information from you will be treated confidentially, but you may be asked if you will allow information to be quoted anonymously. You can choose for the information apart from these quotations to be included simply as part of the study’s background, together with printed material, Internet sources and the information others provide. The Data Protection Act applies to all the
Please initial

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 you will be asked to complete a 24-hour food diary?

Are you aware that two blood samples will be taken?
Are you aware that these blood samples will be analysed?

Are you aware that you need to consume a vitamin D tablet or placebo 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?

Are you willing to take part? If yes, please sign your name below.

I .......................................................... agree/do not 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 Hend Alharbi on this E-mail 10976029@stu.mmu.ac.uk
Appendix 7: King Fahad Medical City informed consent form

King Fahad Medical City

مدينة الملك فهد الطبية

Riyadh, Kingdom of Saudi Arabia

الرياض- المملكة العربية السعودية

CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH

موافقة للمشاركة في البحث

CONSENT BY SUBJECT FOR
PARTICIPATION IN RESEARCH

Protocol Number:

رقم الدراسة

Name of Subject:

اسم المشارك

Medical Record Number:

رقم السجل الطبي

Study Title: The effect of vitamin D3 on biomarkers of glycaemia and oxidative stress in Saudi men with poorly controlled Type 2 Diabetes Mellitus (T2DM).

عنوان الدراسة "تأثير مكملات فيتامين د3 على المؤشرات الحيوية لنسبة السكر بالدم و الأجهاد التأكسدي لدى الرجال المصابون بالسكري النوع الثاني غير مستلم "

Principal Investigator: Dr. Naji Aljohani

الباحث الرئيس د. ناجي الجهني

Hend Alharbi

هند فصل الحربي

Address:

العنوان

Telephone:

رقم الهاتف
A member of the research team will explain what is involved in this study and how it will affect you. This consent form describes the study procedures, the risks and benefits of participation, and how your confidentiality will be maintained. Please take your time to ask questions and feel comfortable making a decision whether to participate or not. This process is called informed consent. If you decide to participate in this study, you will be asked to sign this form and will be given a copy for your records. Throughout this consent form, “you” will refer to you or your child, as appropriate.

WHY IS THIS STUDY BEING DONE?

To determine whether vitamin D3 supplementation can reduce biomarkers of inflammation and oxidative stress in Saudi men aged between 18 to 60 years with type 2 Diabetes Mellitus

Cultural Bureau in London (Qassim University)

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

135 patients will be randomly in 3 groups (45 in each groups)

WHAT WILL HAPPEN IF I TAKE PART IN THIS STUDY?

Your part in the study will confirm if vitamin D will reduce the biomarker of oxidative stress with T2DM poorly controlled.
Study location:

Riyadh

WHAT IS EXPECTED OF ME DURING THE STUDY?

You will complete a medical questionnaire then you will be given randomly vitamin D supplements (2000IU or 4000IU) or placebo to take them and you will have a blood test at baseline and after 4 months. Also, the skin autofluorescence reader will measure AGE.

HOW LONG WILL I BE IN THE STUDY?

4 Months

CAN I STOP BEING IN THE STUDY?

Yes. You can decide to stop at any time. Tell the study doctor if you are thinking about stopping or you’ve decided to stop. He or she will tell you how to stop your participation safely. No one will try to get you to change your mind.

ARE THERE RISKS IF I STOP BEING IN THE STUDY?

There is not any risk.

WHAT SIDE EFFECTS OR RISKS CAN I EXPECT FROM BEING IN THE STUDY?

There are not any side effects.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?
Taking part in this study may or may not make your health better. While doctors hope the procedure/drug/intervention/device will be more effective/have fewer side effects than the standard (usual) treatment, there is no proof of this yet.

WHAT OTHER OPTIONS ARE THERE?

There are no other options

Instead of being in this study, you have these options:

WHAT HAPPENS IF I AM INJURED BECAUSE I TOOK PART IN THIS STUDY?

It is important that you tell Dr. Naji Aljohani and the researcher Hend Alharbi if you feel that you have been injured because of taking part in this study. You can tell the doctor in person or call him at 0556605558. If you are injured as a result of being in this study, treatment will be available. The costs of the treatment may be covered by KFMC or the study sponsor, depending on a number of factors. KFMC and the study sponsor do not normally provide any other form of compensation for injury. For further information about this, you may call the office of the Institutional Review Board (IRB).

WHAT ARE THE COSTS OF TAKING PART IN THE STUDY?

You will not be charged for any study activities.

Funding has been granted through the Saudi Arabian Government Scholarship scheme by the Saudi Arabian culture bureau.

WILL I BE PAID FOR MY TAKING PART IN THIS STUDY?

You will not be paid for your participation in this study. However, funding has been granted through the Saudi Arabian Government Scholarship scheme.
You will not be paid for taking part in this study. If you are employer, we can give you sick leave on a day when you visit.

WILL MY MEDICAL INFORMATION BE KEPT PRIVATE?

We will do our best to make sure that the personal information in your medical record is kept private. However, we cannot guarantee total privacy. Your personal information may be given out if required by law. If information from this study is published or presented at scientific meetings, your name and other personal information will not be used.

WHAT ARE MY RIGHTS IF I TAKE PART IN THIS STUDY?

Taking part in this study is your choice. You may choose either to take part or not to take part in the study. If you decide to take part in this study, you may leave the study at any time. No matter what decision you make, there will be no penalty to you and you will not lose any of your regular benefits. Leaving the study will not affect your medical care. You can still get your medical care from KFMC. Dr. Naji Aljohani and the researcher Hend Alharbi may use information that was collected prior to your leaving the study.

We will tell you about new information or changes in the study that may affect your health or your willingness to continue in the study.

Will my medical information be kept private?

We will do our best to make sure that the personal information in your medical record is kept private. However, we cannot guarantee total privacy. Your personal information may be given out if required by law. If information from this study is published or presented at scientific meetings, your name and other personal information will not be used.

What are my rights if I take part in this study?

Taking part in this study is your choice. You may choose either to take part or not to take part in the study. If you decide to take part in this study, you may leave the study at any time. No matter what decision you make, there will be no penalty to you and you will not lose any of your regular benefits. Leaving the study will not affect your medical care. You can still get your medical care from KFMC. Dr. Naji Aljohani and the researcher Hend Alharbi may use information that was collected prior to your leaving the study.

We will tell you about new information or changes in the study that may affect your health or your willingness to continue in the study.

Taking part in this study is your choice. You may choose either to take part or not to take part in the study. If you decide to take part in this study, you may leave the study at any time. No matter what decision you make, there will be no penalty to you and you will not lose any of your regular benefits. Leaving the study will not affect your medical care. You can still get your medical care from KFMC. Dr. Naji Aljohani and the researcher Hend Alharbi may use information that was collected prior to your leaving the study.
In the case of injury resulting from this study, you do not lose any of your legal rights to seek payment by signing this form.

WHO DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

Before you agree to be in this study, you will talk to a study team member qualified to tell you about this study. You can ask questions about any aspect of the research. If you have further questions about the study, you may ask them at any time. You may call 0556605558.

CONSENT

Subject:

The research and procedures have been explained to me. I have been allowed to ask any questions I have at this time. I can ask any additional questions I may think of later. I may quit being in the study at any time without affecting my health care.

I will receive a signed copy of this consent form.

I agree to participate in this study. My agreement is voluntary. I do not have to sign this form if I do not want to be part of this research study.

Subject Signature

Subject:

The research and procedures have been explained to me. I have been allowed to ask any questions I have at this time. I can ask any additional questions I may think of later. I may quit being in the study at any time without affecting my health care.

I will receive a signed copy of this consent form.

I agree to participate in this study. My agreement is voluntary. I do not have to sign this form if I do not want to be part of this research study.

Subject Signature

Subject:
I have explained the nature and purpose of the study and the risks involved. I have answered and will answer questions to the best of my ability. I will give a signed copy of the consent form to the subject.

Signature of Person Obtaining Consent

Date

Time (AM □ PM □ )

Principal Investigator:

Signature of Principal Investigator

Date

Time (AM □ PM □ )

[STOP! Do not use the following signature lines unless third party consent is being requested and has been.]

AND/OR:

Legally Authorized Representative

Date

Person Obtaining Consent

Date

OR
The person being considered for this study is unable to consent for himself/herself because he/she is a minor. By signing below, you are giving your permission for your child to be included in this study.

Parent or Legal Guardian-------------------

Date: / /
Appendix 8: Pre-study questionnaire

Pre-study Screening Questionnaire

A. Participant Section:

Background

Name:.............................................................. Today's date: ______/_____/_____

Address:..........................................................

Telephone:.................................

• Are you male or female?
  1) Male  2) Female

• How old are you?
  1) Less than 18  2) 18 - 24  3) 25 - 34  4) 35 - 44  5) 45 - 54  6) 5-60  7) 61 and over
  Date of birth: ------/-/--/-------

• Are you currently?
  1) Single  2) married  3) divorced  4) widowed

• How many children do you have?
  1) 1  2) 2  3) 3  4) 4  5) 5  6) more than 5

• Please circle the highest year of school completed:
  1) Illiteracy  2) Primary  3) Secondary school  4) high school  5) diploma  6) university  7) Higher education

• Are you currently?
  1) Student  2) Employed  3) unemployed  4) Retire

• Do you have any health or nutrition related qualifications?
  1) Yes  2) No

• Do you currently smoke cigarettes or any other tobacco products on a daily basis?
  1) Yes  2) No
  if Yes, How often: 1) often  2) average  3) Not often  4) rarely

• Are you on a special diet?
  1) Yes  2) No
  If Yes Please specify: . . . . . .

Medications
• When were you first diagnosed with type 2 diabetes?
  1) 1-2 years  2) 3-5 years  3) 6-8 years  4) over 8 years

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

• Do you have any diabetic complications?
  1) Yes  2) No  3) Don’t know
If yes please choose form following relevant diabetic complications:
  1) hypoglycaemia diabetic ketoacidosis (DKA)  2) retinopathy
  3) Hyperosmolar hyperglycaemic state HHS)  4) cardiovascular disease
  5) Nephropathy nerves and feet (neuropathy)

• 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

• Are you currently taking prescribed medications
   Name of Drug  Dose  How many times a day?
   e.g. Metformin  1 tablet of 10g  3 times with meals

• Do you take daily vitamin supplements?
  1) Yes  2) No
If yes, please specify type and brand …………………………………………………...

• 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
• Waist circumference cm
• Current HbA1C % Date tested
  ____/____/____

Thank you for your time much appreciated
Appendix 9: Debriefing questionnaire

Debriefing Questionnaire

Participant Section:

Background

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

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

.........................................................................................................................................................
4- Have you stopped taking any prescribed medications since you started taking the tablets?
(1) No       (2) Yes
If yes, please explain

.................................................................
.................................................................

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 . . . . . . .................................................................

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

.................................................................
.................................................................
10- Overall, how would you score your diabetes during the last 16 weeks?
(1) Better than before   (2) Worse than before   (3) No change

11- Have you been diagnosed with any other conditions?

Researcher Section - Anthropometric Measurements

Participant’s Height? __________________cm
Participant’s Weight? __________________kg
Calculated BMI __________________m²
Waist circumference __________________cm
Current HbA1C ____________________%
Calcium level ____________________mmol/l
Date tested ____/____/____

Participant number…………………………….

Checklist:

Questionnaire complete □

Tablets returned □

Calendar returned □
Anthropometric measures

Blood sample taken
Appendix 10: Supplement calendar

Supplement recoding calendar

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Researcher Hend Alharbi
## Appendix 11: Block supplement randomisation

Seed: 271161023181735

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</thead>
<tbody>
<tr>
<td>Actual list length: 156</td>
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</tbody>
</table>

block identifier, block size, sequence within block, treatment

<table>
<thead>
<tr>
<th>Group A</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group B</td>
<td>Vitamin D 2000 IU / day</td>
</tr>
<tr>
<td>Group C</td>
<td>Vitamin D 4000 IU / day</td>
</tr>
</tbody>
</table>

### Block 1
- 1. 12. 1, Group B
- 1. 12. 2, Group A
- 1. 12. 3, Group C
- 1. 12. 4, Group C
- 1. 12. 5, Group C
- 1. 12. 6, Group B
- 1. 12. 7, Group C
- 1. 12. 8, Group B
- 1. 12. 9, Group A
- 1. 12. 10, Group A
- 1. 12. 11, Group B
- 1. 12. 12, Group A

### Block 2
- 2. 12. 1, Group B
- 2. 12. 2, Group C
- 2. 12. 3, Group C
- 2. 12. 4, Group B
- 2. 12. 5, Group C
- 2. 12. 6, Group A
- 2. 12. 7, Group B
- 2. 12. 8, Group A
- 2. 12. 9, Group C
- 2. 12. 10, Group B
- 2. 12. 11, Group A
- 2. 12. 12, Group A

### Block 3
- 3. 12. 1, Group C
- 3. 12. 2, Group A
- 3. 12. 3, Group B
- 3. 12. 4, Group C
- 3. 12. 5, Group B
- 3. 12. 6, Group A
- 3. 12. 7, Group A
- 3. 12. 8, Group C
- 3. 12. 9, Group B
- 3. 12. 10, Group B
- 3. 12. 11, Group A
- 3. 12. 12, Group C

### Randomization within Blocks

<table>
<thead>
<tr>
<th>Group B</th>
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<tbody>
<tr>
<td>Group B</td>
<td>4. 12. 2</td>
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</tbody>
</table>
Appendix 12: Measurement of the concentration of vitamin D kits
Appendix 13: Measurement of the concentration of calcium
Appendix 14: Measurement of the concentration of albumin
Appendix 15: Measurement of the concentration of blood glucose kit

GLUCOSE (GOD-POD)

THIS PACKAGE INSERT IS APPLICABLE FOR USE OUTSIDE THE US.

INTENDED USE
For the in vitro diagnostic use in the qualitative and quantitative determination of glucose concentration in human whole blood or serum/plasma. Any determination by the glucose method also includes the following:

Sensitivity:
Glucose is the primary energy source for the human body. In persons on a typical western diet, glucose is provided by the breakdown of starches and sugars in foods. After consumption, glucose is absorbed by the cells of the body and converted to energy. The excess glucose is stored in the liver as glycogen, but is released into the bloodstream as glucose, or as fructose or galactose. The body reacts quickly to ensure that the glucose concentration in the bloodstream remains fairly constant. The level of glucose in the blood is controlled by the liver and by the hormone insulin.

PREVENTION OF THE METHOD

The method is based on the GOD (glucose oxidase) and the modified PO (peroxidase) reaction.

PROCEDURE:
1. Collect a blood sample by venipuncture or capillary puncture. The sample should be collected in a gel separator tube and allowed to clot at room temperature for 30 minutes. The sample should be centrifuged at 2500 rpm for 5 minutes.
2. Place 30 μl of the sample into a reaction cuvette and add 200 μl of the reagent mixture to the reaction cuvette. Mix the contents of the reaction cuvette by gently inverting the cuvette.
3. Place the reaction cuvette into the Thermo TS-16T analyzer for 10 minutes.
4. Record the absorbance at 500 nm. The absorbance value is directly proportional to the glucose concentration in the sample.

CALCULATION OF RESULTS

The result is calculated automatically by the analyzer using a calibration curve.

Calibration Curve (example):

Glucone (mg/dL) | Absorbance
--- | ---
50 | 0.5
100 | 1.0
150 | 1.5
200 | 2.0

For this test, a dilution of the standard 1 mg/mL Glucose was used to make the calibration curve.

Quality Control:
Use quality control (QC) samples at least once a day and after each calibration and every time the analyzer is turned on. QC samples should be tested within 1 hour of the manufacturer’s recommended expiration date. Always follow the local, state and federal regulations pertaining to the use of laboratory equipment and chemicals.

LIMITATIONS OF THE PROCEDURE

The test result should be interpreted in the context of the patient’s medical history, clinical examination and other findings.

Interference:
Common interferents include:
- Uric acid
- Bilirubin
- Hemoglobin
- Inorganic phosphate

For these interferents, the results should be interpreted in the context of the patient’s medical history, clinical examination and other findings.

Thermo
Appendix 16: Measurement of the concentration of glycated haemoglobin

HEMOGLOBIN A1C BY THE DCA VANTAGE

I. PURPOSE AND PRINCIPLE

DCA VANTAGE is intended for the monitoring of hemoglobin A1c levels only. The measurement of hemoglobin A1c concentration is recommended for monitoring the long term care of patients with diabetes. The assay, using whole blood samples, is based on a latex immunogglutination inhibition methodology.

Hemoglobin A1c is formed by the non-enzymatic glycation of the N-terminus of the Beta chain of hemoglobin A. The level of hemoglobin A1c is proportional to the level of glucose in the blood over a 2 month period. Therefore, hemoglobin A1c is an acceptable indicator of the average daily glucose levels over the preceding two months. Recent studies have shown, that the clinical values obtained through regular measurement of the hemoglobin A1c leads to changes in diabetes treatment and improvement of metabolic control by lowering hemoglobin A1c values.

Both the concentration of hemoglobin A1c and the concentration of total hemoglobin are measured, and the ratio reported as percent hemoglobin A1c. All of the reagents for performing both reactions are contained in the DCA Hemoglobin A1c Reagent Cartridge. For the measurement of total hemoglobin, potassium ferricyanide is used to oxidize the hemoglobin present in the sample to methemoglobin. The methemoglobin, then complexes with thiocyanate to form thioctyan-methemoglobin. This colored compound is measured spectrophotometrically in the DCA Vantage instrument at 531nm.

For the measurement of hemoglobin A1c, an inhibition of latex agglutination is used. A synthetic polymer containing multiple copies of the immunoreactive portion of hemoglobin A1c causes agglutination of latex coated with hemoglobin A1c-specific murine monoclonal antibody. This causes an increase of the light scattering which is measured as an increase of absorbance at 531nm. Hemoglobin A1c in whole blood samples competes for a limited number of binding sites causing an inhibition of agglutination and a decrease in light scatter. This decrease in scattering is measured as a decrease in absorbance at 531nm. The hemoglobin A1c concentration is then quantified using a lot-specific calibration curve of absorbance.
Appendix 17: Measurement of the concentration of fasting insulin

Insulin

cobas®

1201T47 122

Appendix 17: Measurement of the concentration of fasting insulin

Introduction

Insulin is a peptide hormone with a molecular weight of approximately 6000 daltons. It is secreted by the beta cells of the pancreas and released into circulation in response to glucose and other stimuli. Insulin is involved in the regulation of glucose metabolism, particularly in the uptake of glucose by cells and the inhibition of glucagon secretion.

Test principle

The test principle for the cobas 188 immunoassay is based on the measurement of insulin levels in serum. The assay uses a sandwich immunoassay format, where antibodies specific to insulin are used to capture the hormone from the sample.

Reagents

- **Antibody-coated wells**
- **Streptavidin-coated microparticles**
- **Monoclonal anti-insulin antibody (mouse) 1 mg/mL**
- **Urea buffer, pH 9.0, 0.5 M**
- **Triton X-100 (0.5% v/v)**
- **1 M NaCl**
- **Plastic 96-well plate**

Precautions and warnings

- The assay is intended for use on human serum only.
- The assay is not suitable for use on hemolyzed or lipemic samples.
- The assay is not recommended for use on urine samples.
- The assay is not suitable for use on samples containing anti-coagulants.
- The assay is not suitable for use on samples containing preservatives.
- The assay is not suitable for use on samples containing any other additives.
- The assay is not suitable for use on samples containing any other analytes.
- The assay is not suitable for use on samples containing any other chemicals.
- The assay is not suitable for use on samples containing any other ingredients.
- The assay is not suitable for use on samples containing any other substances.

Storage and stability

Store the assay reagents at 2-8 °C. Do not freeze.

Use the assay reagents expiring in or before the expiration date.

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Appendix 18: Measurement of the concentration of cholesterol
Appendix 19: Measurement of the concentration of HDL

### HDL-CHESTEROL PLUS

**Sample Types**
- Serum and plasma samples can be used. EDTA plasma causes delayed results.
- Fasting and non-fasting samples can be used. (Fasting samples should preferably be analyzed on the day of collection.)
- Samples containing heparin should be centrifuged before performing the assay.

**Storage**
- The samples can be stored as follows:
  - 3 to 5 days at 4°C
  - Up to 1 month at 2 to 8°C
  - Up to 6 months at -20°C

**Intended Use**
- For in vitro diagnostic use in the quantitative determination of HDL-cholesterol in human serum and plasma on Thermo scientific analyzers.

**SUMMARY**
- Cholesterol is synthesized throughout the body and is also derived from dietary intake. It is an essential component of cell membranes, a precursor of bile acids, vitamin D, and adrenal hormones, all of which are synthesized in the body.
- Most of the HDL cholesterol is present in HDL1, HDL2, and HDL3 subfractions. HDL1 dominates the HDL subpopulations.
- The HDL cholesterol content is regulated by the production of cholesterol transporters, which are responsible for the removal of cholesterol from the liver.
- The measurement of HDL cholesterol is an important factor in the assessment of the risk of cardiovascular disease.

**PRINCIPLE OF THE PROCEDURE**
- This test is a homogeneous enzymatic reaction test, where the presence of cholesterol in the sample reacts with the reagents to form a complex containing HDL cholesterol and a precipitate of cholesterol esters.
- The measurement of HDL cholesterol is performed by determining the amount of cholesterol transported, which is proportional to the HDL cholesterol content.
- The test is performed on a Thermo scientific analyzer.

**REAGENT INFORMATION**

<table>
<thead>
<tr>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A</td>
</tr>
<tr>
<td>Reagent B</td>
</tr>
<tr>
<td>Reagent C</td>
</tr>
</tbody>
</table>

**PROCEDURE**
- For an in vitro diagnostic use only. Each batch of reagent must be handled and stored according to the instructions provided.
- Preparation of reagents and solutions is required.
- Measurement of the concentration of HDL cholesterol is performed using a Thermo scientific analyzer.

**CALIBRATION**
- The calibration curve is essential for accurate measurements.
- The calibration curve is generated using a standard solution containing HDL cholesterol.

**LIMITATIONS OF THE PROCEDURE**
- The test is intended for in vitro diagnostic use only.
- The test is done in conjunction with the patient's medical history, clinical examination, and other tests.
- The test is not suitable for therapeutic drug monitoring.

**REFERENCE**
- A study performed using the GLSI document EP7 as a guideline.

**Citation:**
- Thermo Scientific N屿e of the measurement of HDL cholesterol.
Appendix 20: Measurement of the concentration of triglycerides

TRIGLYCERIDES

INTENDED USE

For an automatic quantitative determination of the triglycerides in serum or other biological samples, according to the methodology described in this section.

SUMMARY (L.H)

Triglycerides are lipids that contain 3 fatty acids. In human nutrition, triglycerides contribute 95% of the energy intake. The triglyceride analysis evaluates the triglyceride levels present in the sample.

The triglyceride analysis is based on a colorimetric method, which uses a combination of potassium permanganate and alcoholic KOH.

PRINCIPLE OF THE PROCEDURE

The triglyceride levels are determined using a colorimetric method. A combination of potassium permanganate and alcoholic KOH is used to oxidize the triglycerides in the sample. The reaction between the peroxidase and the triglycerides produces a colored compound.

The absorbance of the formed color is measured at 550 nm.

Triglycerides

Glycerol + Fatty acids

Glycerol + ATP

Glycerol + Phosphatase + ATP

Reagent Information

Concentrations

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides</td>
<td>0.05 mg/dL</td>
</tr>
</tbody>
</table>

Precautions

- For in vitro diagnostic use only.
- Use the normal procedures for handling all laboratory reagents.
- Avoid contact with skin and mucous membranes.

Preparation

- Keep the reagent ready for use.
- Store the reagent at 2-8°C in the refrigerator or at -20°C in the freezer.

Storage and Stability

- Store the reagent in a cool place at 2-8°C or at the same temperature as the refrigerator.

SPECIMEN COLLECTION

- Ensure that the specimen is collected according to the instructions provided.

Sample Type

- Serum or plasma:
  - Triglyceride levels can be measured in serum or plasma using this kit.
- Whole blood:
  - Whole blood samples can be used for the triglyceride analysis.

Precautions

- Ensure that the sample is collected according to the instructions provided.

Sample Handling

- Samples can be stored at 2-8°C for up to 24 hours.

Troubleshooting

- Refer to the troubleshooting guide provided within the kit.

Materials provided

- Reagents and chemicals as described above.

Materials required but not provided

- Calibration and control substances are not included in the kit.

Calculation

- Use the standard curve provided to calculate the triglyceride concentration.

Toxicity

- Refer to the package insert for specific data.

Quality Control

- Use quality control samples provided to ensure accurate results.

Interference

- Interferences may affect the assay results, which may lead to inaccurate results.

For other questions, please refer to the reference (1).

EXPECTED VALUES

- Normal range: 0.00 - 1.50 mg/dL

PERFORMANCE CHARACTERISTICS

- Precision: 5.00 - 10.00 mg/dL

Limit of Detection

- Sensitivity: 0.00 - 0.10 mg/dL

Variation:

- Within-run: 0.2 - 0.7 %
- Between-run: 0.5 - 1.4 %

Limit of Quantitation

- 0.05 mg/dL

Limit of Detection: 0.00 mg/dL

Limit of Quantitation: 0.05 mg/dL

Thermo
Appendix 21: Measurement of the concentration of total antioxidant capacity

Product Manual

OxiSelect™ Total Antioxidant Capacity (TAC) Assay Kit

Catalog Number
STA-360 200 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures