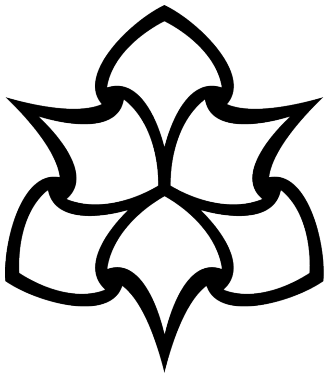

The Effect of Vitamin D_3 Supplementation on Markers of Glycaemia, Lipidaemia and Oxidative Stress in Saudi Men with Poorly-Controlled Type-2 Diabetes Mellitus

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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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 D_3 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 D_3 or 3) 100µg/day vitamin D_3 as capsules matched in shape and size over a 16-week period. Fasting glucose, HbA_{1c}, 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 HbA_{1c} ($p<0.001$) 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 D_3 (100µg/day) without any change in the 50µg/day vitamin D_3 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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Qassim University which supported me in every way during the completion of this project.

List of Abbreviations

AGEs	Advanced glycation end products
Apo-A1	Apolipoprotein A-1
ApoB	Apolipoprotein B
AR	Aldose reductase
BCG	Bromocresol green
BHT	Butylated hydroxytoluene
BMI	Body mass index
BSA	Bovine serum albumin
Calciferol	Vitamin D
Calcitriol	1,25-dihydroxycholecalciferol
CAT	Catalase
CETP	Cholesteryl ester transfer protein
Cholecalciferol	Vitamin D_3
DAG	Diacylglycerol
DBP	vitamin D–binding protein
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
Ergocalciferol	Vitamin D_2
FBS	Fasting blood sugar
FGIR	Fasting glucose/insulin ratio
g/L	grammes per/litre

GLM	General Linear Model
HbA1c	Glycated haemoglobin
HDL	High density lipoprotein
HOMA-β	Homeostasis model assessment of β-cell function
HOMA-IR	Homeostasis model assessment for insulin resistance
HSL	Hormone-sensitive lipase
IM injection	Intramuscular injection
IR	Insulin resistance
IU	International unit
LDL	Low density lipoprotein
MDA	Malondialdehyde
mg/dl	milligrammes per decilitre
mM	millimolar
mmol/L	millimoles per litre
MPO	Myeloperoxidase
NF-κB	Nuclear factor-kappaB
ng/ml	Nanogramme per millilitre
nmoL/L	nanomoles per litre
NPDR	Non-proliferative diabetic retinopathy
O_2	Oxygen
Ox-LDL	Oxidised LDL
PCO	Pro-oxidants protein carbonyl

PDR	Proliferative diabetic retinopathy
PKC	Protein kinase C
PTH	Parathyroid hormone
RAGE	Receptors for advanced glycation end products
RCT	Randomised control trial
ROS	Reactive oxygen species
RXR	Acid X receptor
sdLDL	Small dense low density lipoprotein
T2DM	Type 2 Diabetes Mellitus
TAC	Total antioxidant capacity
TC	Total cholesterol
TG	Triglycerides
µg	microgramme
µU/mL	microunits per millilitre
UV	Ultraviolet
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VLDL	Very-low density lipoprotein
WHO	World Health Organization
1,25(OH)2D3	1,25-Dihydroxycholecalciferol
25(OH)D	25-Hydroxyvitamin-D
25-OHase	Vitamin D-25-hydroxylase

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

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

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-cell s 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 (HbA_{1c}) measurements. HbA_{1c} 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

Diagnosis	**Fasting blood glucose**	**HbA_{1c}**	**References**
High risk of diabetes	6.1–7.0 mmol/L	6–6.4%	(WHO, 2006; WHO, 2011)
Diabetes	≥7.0 mmol/L	6.5–7.5%	
Poorly controlled diabetes	≥10 mmol/L	≥8%	

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).

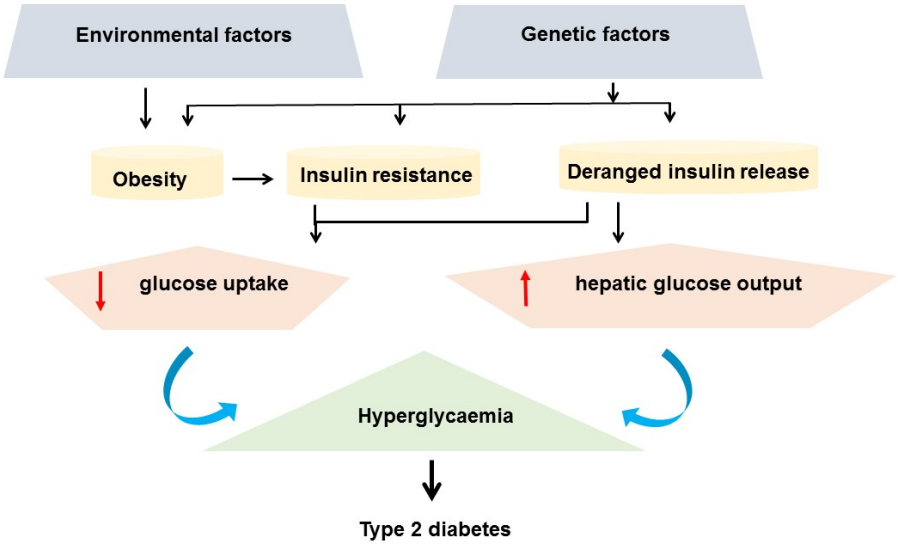

Figure 1.1. Pathogenesis of type 2 diabetes
Adapted from Lowell & Shulmanz, 2005.

1.2. Diabetic complications

Long-term of 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 comlications.

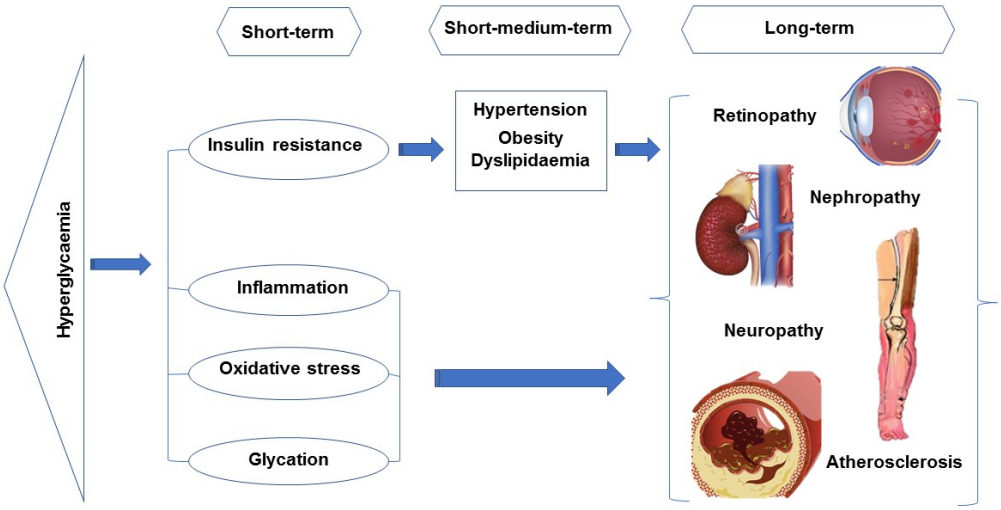

Figure 1.2. Type 2 diabetic complications
Adapted from Giacco & Brownlee, 2010.

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 ooxidative stress and advanced glycation end products (AGEs) pathway (Giacco & Brownlee, 2010) (Figure 1.3). All of these pathways contribute to oxidative stress.

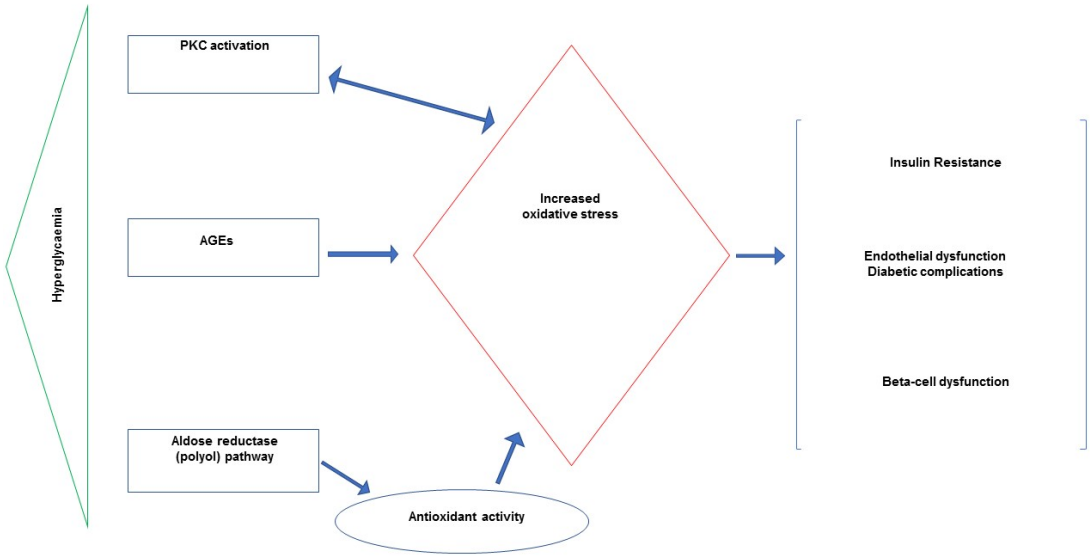

Figure 1.3. Role of hyperglycaemia in diabetic complications
Adapted from Hadi & Suwaidi, 2007.

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 phosphatidylserine (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).

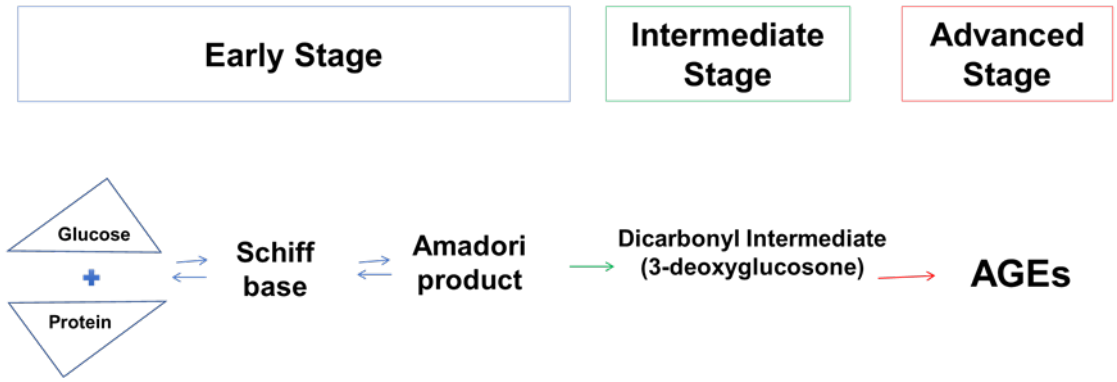

Figure 1.4. The three stages of glycation
Adapted from (Ahmed, 2005)

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 ($O_2^{\bullet}$) 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).

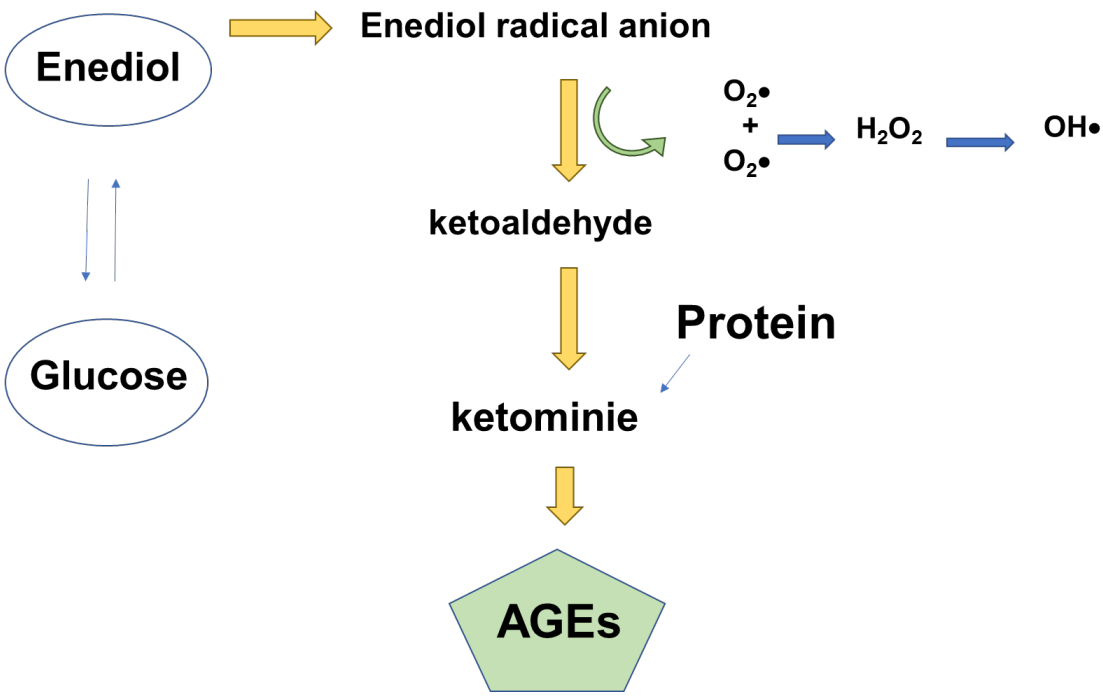

Figure 1.5. Autoxidative glycation pathway
Adapted from (Ahmed, 2005)

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).

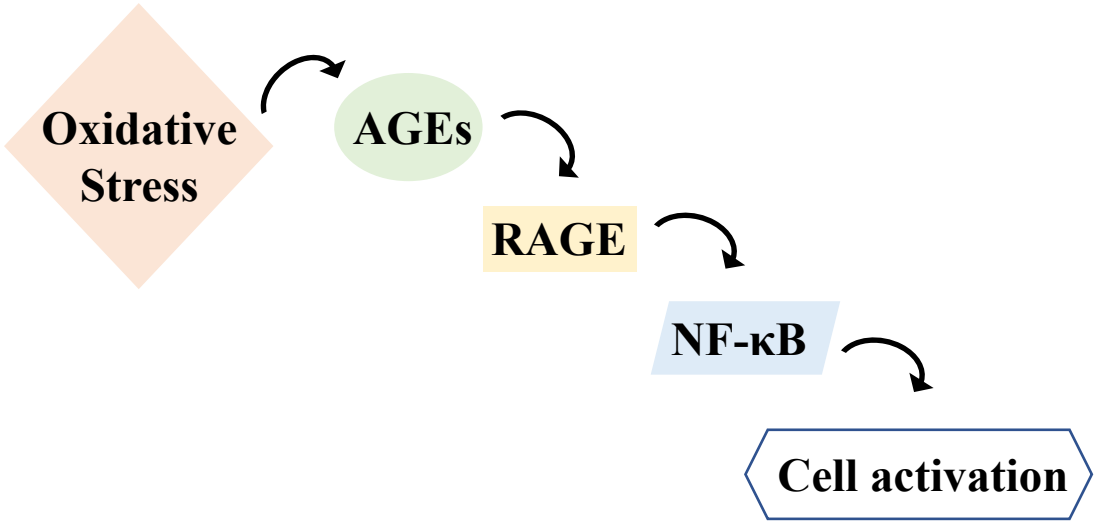

Figure 1.6. Interaction of AGE with RAGE on microphages induces oxidative stress and activation of intracellular signalling causing inflammation
Adapted from (Ahmed, 2005; Yagihashi *et al.,* 2011)

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.

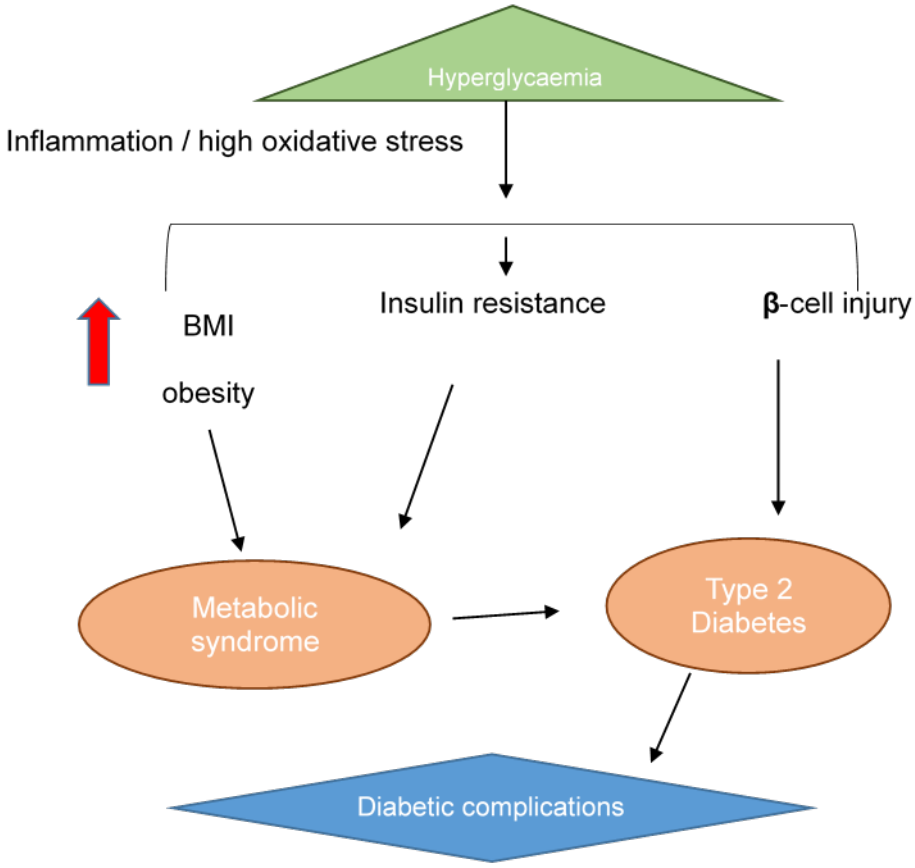

Figure 1.7. Inflammation and the presence of oxidative stress leading to diabetic complications
Adapted from Vlassara & Uribarri, 2014)

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, actions

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 plaquse. Therefore, the development of atherosclerosis is accelerated (Schofield *et al.*, 2016).

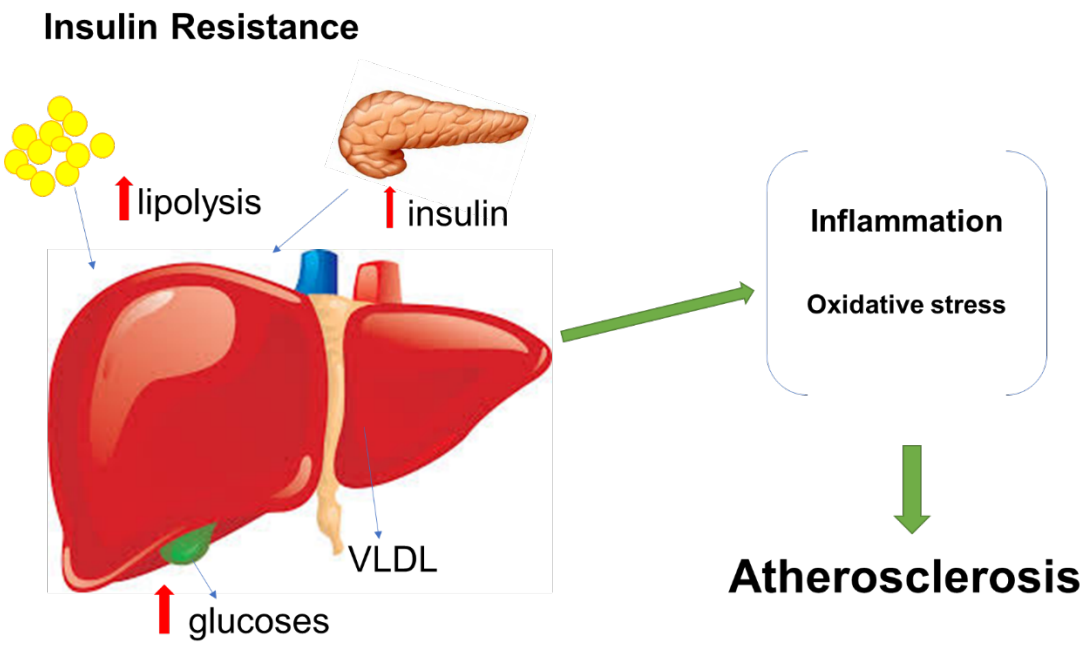

Figure 1.8. The pathogenesis of dyslipidaemia
(Adapted from Gaggini *et al.,* 2013)

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).

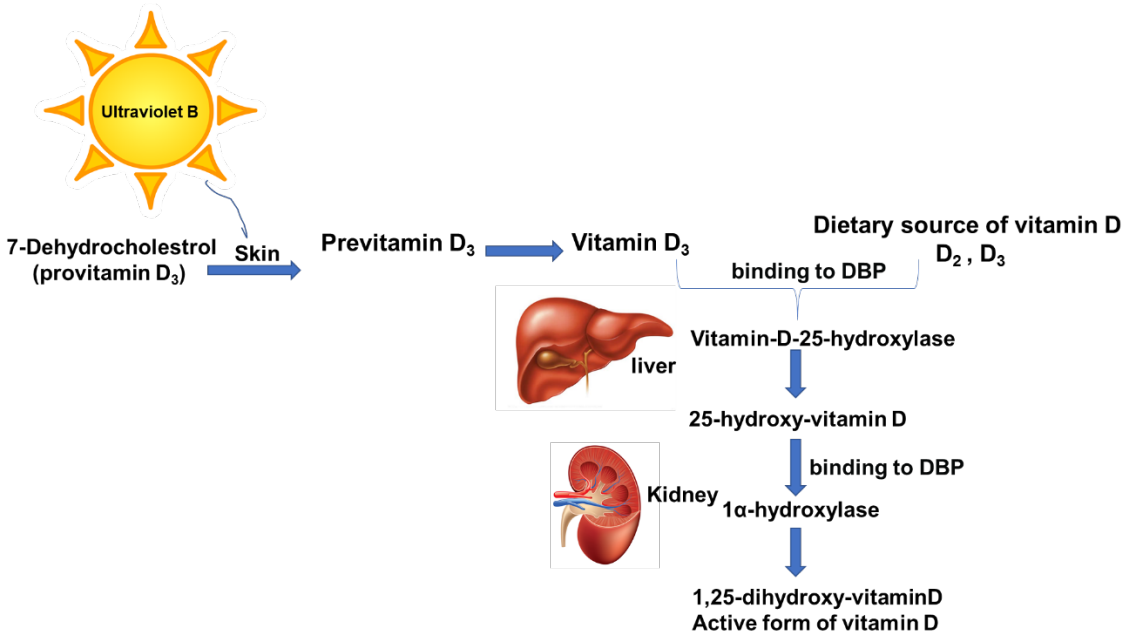

Figure 1.9. Vitamin D synthesis
(Adapted from Bikle, 2012)

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 > 20ng / mL and < 30ng / 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

Vitamin D concentration (ng/mL)	**Vitamin D concentration (nmoL/L)**	**Classification**	**References**
<20	<50	Deficiency	(Hollis, 2005; Alshahrani & Aljohani, 2013)
20-32	50–80	Insufficiency	
32-54	80-135	Normal	
54-90	135–225	Normal in sunny countries	
>100	>250	Excess	
>150	>325	Intoxication	

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 1-α-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 D_3), in the blood, binds to the vitamin D receptor on pancreatic β-cell s and boosts insulin receptor expression, which can enhance insulin sensitivity (Thacher & Clarke, 2011).

Table 1.4. The role of vitamin D

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

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).

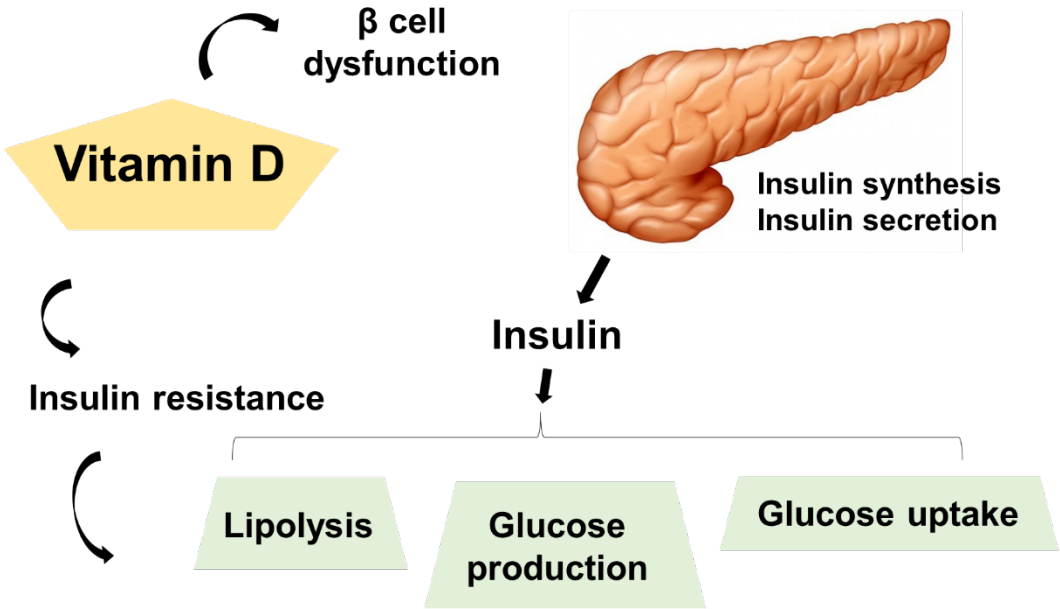

Figure 1.10. The effect of vitamin D on insulin resistance and β-cell function (Adapted from Van Belle *et al.*, 2013)

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 D_3 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 D_3 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 D_3 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 D_3 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 D_3 supplementation improved insulin sensitivity in subjects with impaired fasting glucose. Thus, in relation to public health, it has been recommended that high-dose vitamin D_3 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 D_3 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*

al., (2008). 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

Study	**Intervention**	**sample**	**Outcome**
Sugden *et al.*, (2008)	2500µg/week vitamin D_2 for 8 weeks	34 subjects	Significantly improved
Tai *et al.*, (2008)	250µg vitamin D_3 over 2 weeks	33 subjects	No effect on glycaemia
Jorde *et al., (*2009)	1000µg/week Vitamin D_3 or placebo for 6 months	36 subjects with T2DM	No significant effects on glucose metabolism
Gallagher *et al.*, (2009)	20µg vitamin D 24 to 62 months	5,292 subjects in England and Scotland	No improvement in glycaemia
Nagpal *et al.*, (2009)	3000µg of vitamin D_3 over 6 weeks	41 male subjects	Increased insulin sensitivity
Nazarian *et al.*, (2011)	250µg/day vitamin D_3 4 weeks	8 subjects (3 men, 5 women)	Improved insulin sensitivity in subjects with impaired fasting glucose
Nikooyeh *et al.*, (2011)	Fortified yogurt drink (25 µg /d vitamin D_3), or fortified yogurt drink (25 µg /d and calcium 500 mg/d) or plain yogurt drink for 12 weeks	90 patients with T2DM	Fasting serum glucose, HOMA-IR and HbA_{1c} decreased significantly more in both vitamin D groups than in the plain yogurt group
Mitri *et al.*, (2011)	50µg/day vitamin D_3 For 16 weeks	92 American adults	Slight improvement in β-cell function
Al-Daghri *et al.,* (2012a)	50µg vitamin D for 18 months	92 type 2 Saudi diabetics (34 men, 58 women)	Improved insulin levels more in women than men.
Breslavsky *et al.,* (2013)	25µg/day vitamin D_3 or placebo for 12 months	32 subjects	No improvement in glucose homeostasis parameters
Talaei *et al.*, (2013)	1250µg/week vitamin D_3 8 weeks	100 Iranians (70 women and 30 men)	Significant effect on insulin resistance fasting plasma glucose
Study	**Intervention**	**sample**	**Outcome**
Elkassaby *et al.*, (2014)	150 µg /d vitamin D_3 or placebo 6 months	50 patients with type 2 diabetes	Improvement of fasting plasma glucose after 3 months that not shown after 6 months
Ryu *et al.*, (2014)	25 µg /d vitamin D_3 or placebo after 24 weeks	158 Korean patients with T2DM	No effect on glycaemic control
Jehle *et al.*, (2014)	7500 µg vitamin D_3 or placebo for 6 months	55 patients with T2DM	Insulin sensitivity (HOMA-IR) and HbA_{1c} improved in the vitamin D group
Tabesh *et al.*, (2014)	1250 µg /week vitamin D_3, calcium	118 patients with T2DM	Significant decrease in serum insulin, HbA_{1c},

	1000 mg/d, or placebo for 8 weeks		HOMA-IR, and LDL cholesterol, and an increase in β-cell function
Sadiya *et al.*, (2015)	150 µg /d vitamin D_3 for 3 months followed by 75 µg /d for 3 months or placebo for 6 months	87 vitamin D deficient obese patients with T2DM	No effects on fasting blood glucose, HbA_{1c}
Krul-Poel *et al.*, (2015b)	1250 µg /month vitamin D_3 or placebo for 6 months	261 non-insulin dependent T2DM	Significant decrease of HbA_{1c} after 6 months in the vitamin D group compared with the placebo group

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

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

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 D_3 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 D_3 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 D_3 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

Study	Intervention	sample	Outcome
Salum *et al.* (2013)	12.5µg/day of vitamin D_3 for 10 week	Diabetic rats	Significant improvement in the total antioxidants P<0.001
Tarcin *et al.*, (2009)	250µg/day of vitamin D_3 for 12 weeks	23 Turkish subjects	Significant effect on reducing oxidative stress
Yiu *et al.*, (2013)	125µg/day vitamin D_3 for 12 weeks	100 Hong Kong subjects	No significant effect on serum biomarkers of inflammation and oxidative stress with T2DM
Eftekhari *et al.*, (2014).	0.25 µg of vitamin D twice per day or placebo tablets for 12 weeks	70 T2DM patients	Reduction of malondialdehyde, but not statistically significant
Nikooyeh *et al.*, (2014)	Fortified yogurt drink (25 µg/d vitamin D_3), or (25 µg/d and calcium 500 mg/d) or plain yogurt drink for 12 weeks	90 T2DM subjects aged 30-50 years	Significant decrease in serum AGEs and increased levels of the antioxidant superoxide dismutase
Krul-Poel, Agca, *et al.*, (2015a)	1250µg/month or a placebo for 6 months	245 patients with T2DM	No effect observed on skin AGE accumulation compared to the placebo
Shab-Bidar *et al.*, (2015)	Fortified yogurt containing 25µg of vitamin D_3 and after 12 weeks	100 T2DM patients	Significant improvement in serum total antioxidant capacity and malondialdehyde

1.7. Aims and Objectives

1.7.1. Aims

The purpose of this study is to test whether vitamin D_3 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 D_3 supplementation on glycaemia, including blood glucose levels, insulin levels and glycated haemoglobin (HbA_{1c}).
- To study the effect of vitamin D_3 supplementation on lipidaema, including low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides and total cholesterol.
- To study the effect of vitamin D_3 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 D_3; 100µg vitamin D_3 (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)
- HbA_{1c} 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 – 20C (Philip Kirsch, Germany)
- Laboratory freezer - 80C (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)

- Statistics Package for Social Sciences (SPSS, version 24 for Windows, Chicago, USA)
- Microsoft Excel 2013 (Microsoft, USA)
- Powerful statistical software (Minitab 17) (Minitab Inc, USA)
- Microsoft Paint 2013 (Microsoft, USA)
- BMI calculator (NHS Tools, UK)

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.

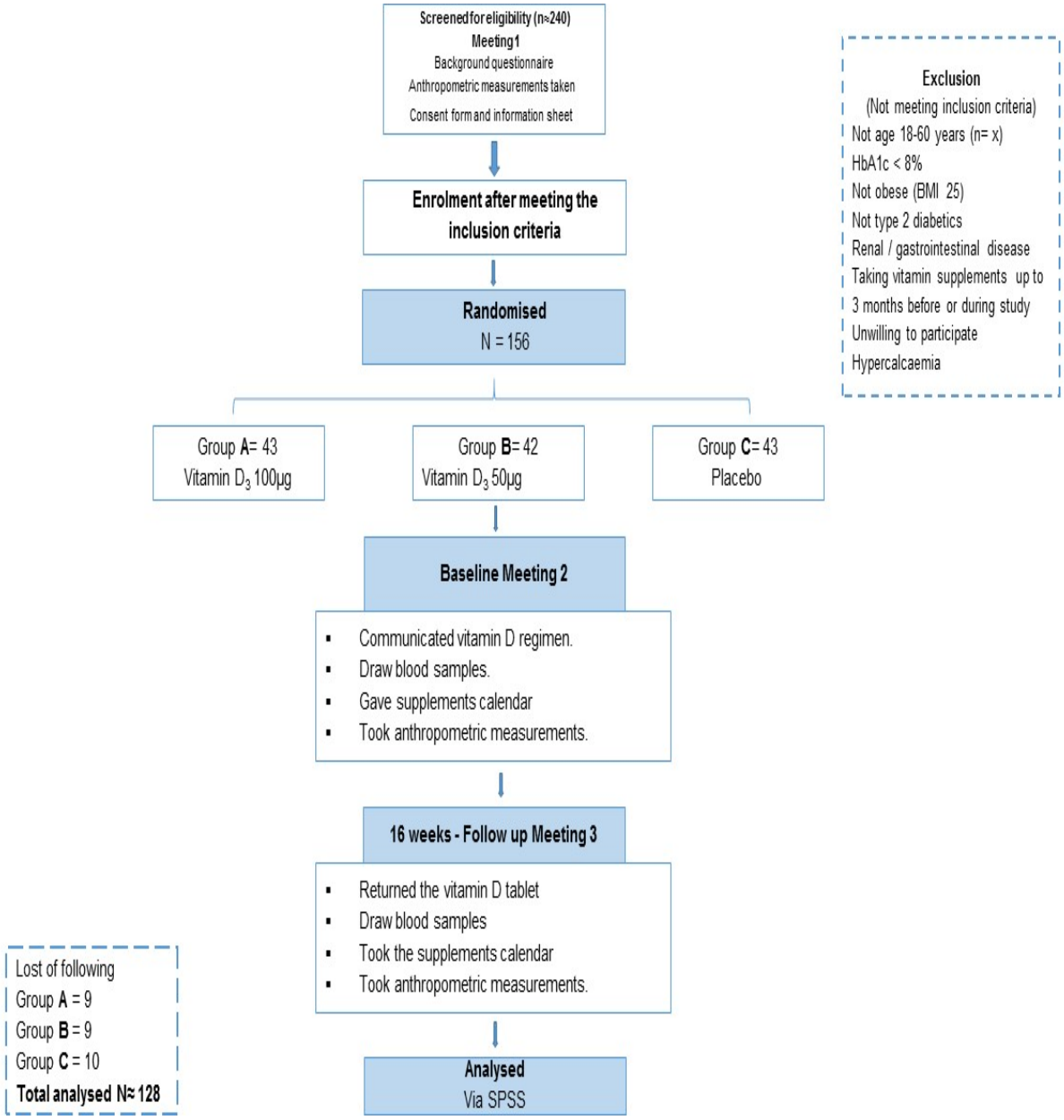

Figure 2.1. Flowchart of the parallel-randomised trial of the three groups
Individuals in the vitamin D groups received 100µg or 50µg vitamin D_3 per day, and those in placebo received a cellulose tablet per day as controls, at study baseline to the 16 weeks of the intervention.

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 D_3 (Group A), 50μg/day of vitamin D_3 (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 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 D_3 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 D_3 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 (HbA_{1c}) > 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

Inclusion criteria	**Exclusion criteria**
18 - 60 years	Major systemic illness such as renal disease
HbA_{1c}> 8%	Diabetes mellitus type 1
BMI > 25	Diabetes requiring medication including vitamin D supplements
Saudi citizen	Participants who may have altered their hypoglycaemic medication during study

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 participates 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/m^2 (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 (HbA_{1c}) 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.

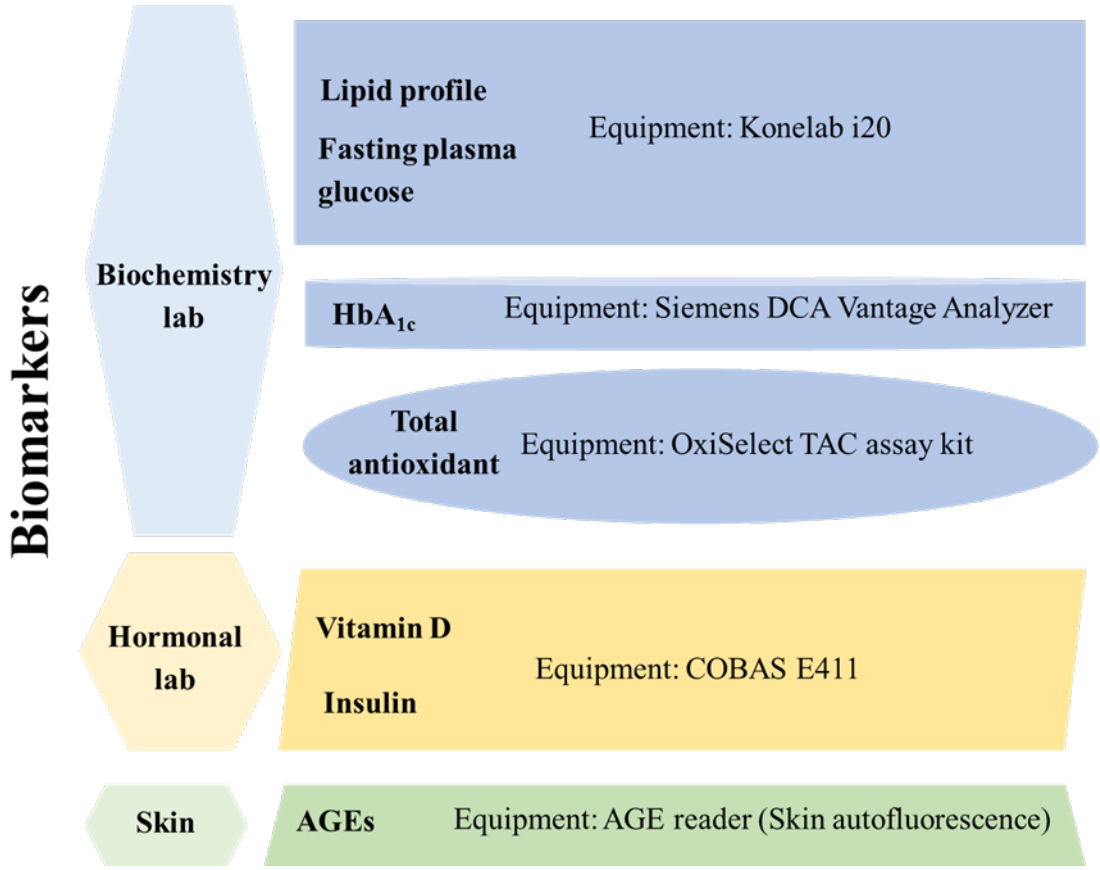

Figure 2.2. Blood analysis flowchart

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):

$$Corrected\ total\ calcium\ (mmol/L) = total\ calcium\ (mmol/L) + 0.01\ [30\ (g/L) - albumin\ (g/L)]$$

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_{1c})

This study measured HbA_{1c} using a Siemens DCA Vantage immunoassay analyser. HbA_{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_{1c}, and results came within 6 minutes (see insert sheet in Appendix 16). Cut-off points for HbA_{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-Daghri *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 electro-chemiluminescent 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

Classification	Cut-off	References
Optimal level	5 to 11 µU/mL	Lee *et al.*, 2006
Standard level	<12 µU/mL	
Insulin resistance levels	12.94 to 17 µU/mL	

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):

$$HOMA-\beta = 20 * [Insulin] / ([Glucose\ mmol/L] - 3.5)$$

$$HOMA-IR = [Glucose\ mmol/l] * [Insulin] / 22.5$$

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

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

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.

2.20. 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

Group		Skewness		Kurtosis	
			Std. Error		Std. Error
A	BMI pre	0.972	0.361	0.282	0.709
	BMI _post	1.316	0.361	0.749	0.709
	Vit D (nmol/l)_pre	0.065	0.361	-0.954	0.709
	Vit D (nmol/l)_post	0.774	0.361	0.467	0.709
	Ca (mmol/l)_pre	-0.105	0.361	-0.672	0.709
	Ca (mmol/l)_post	0.502	0.361	1.289	0.709
	ALB IT (g/L)_ pre	1.175	0.361	0.460	0.709
	ALB IT (g/L)_ post	1.203	0.361	0.791	0.709
	Corrected Ca (mmol/L)_ pre	0.461	0.361	0.788	0.709
	Corrected Ca (mmol/L)_ post	0.551	0.361	1.140	0.709
	glucose (mmol/l)_ pre	0.774	0.361	-0.512	0.709
	glucose (mmol/l)_ post	0.901	0.361	0.706	0.709
	HbA1c (%)_pre	1.562	0.361	2.082	0.709
	HbA1c (%)_post	-0.316	0.361	0.010	0.709
B	BMI pre	2.244	0.365	2.680	0.717
	BMI _ post	2.065	0.365	2.000	0.717
	Vit D (nmol/l)_ pre	0.421	0.365	-0.475	0.717
	Vit D (nmol/l)_ post	0.394	0.365	-0.545	0.717

	Ca (mmol/l)_ pre	-0.317	0.365	-0.651	0.717
	Ca (mmol/l)_ post	0.915	0.365	1.415	0.717
	ALB IT (g/L)_ pre	0.518	0.365	-0.755	0.717
	ALB IT (g/L)_ post	0.823	0.365	-0.043	0.717
	Corrected Ca (mmol/L)_ pre	-0.099	0.365	-0.864	0.717
	Corrected Ca (mmol/L)_ post	0.873	0.365	1.021	0.717
	glucose (mmol/l)_ pre	0.856	0.365	0.138	0.717
	glucose (mmol/l)_ post	1.099	0.365	2.060	0.717
	HbA1c (%)_pre	1.532	0.365	1.745	0.717
	HbA1c (%)_post	1.193	0.365	1.982	0.717
C	BMI pre	0.672	0.361	-0.789	0.709
	BMI _ post	0.611	0.361	-0.988	0.709
	Vit D (nmol/l)_ pre	-0.043	0.361	-0.746	0.709
	Vit D (nmol/l)_ post	-0.039	0.361	-0.463	0.709
	Ca (mmol/l)_ pre	0.321	0.361	0.519	0.709
	Ca (mmol/l)_ post	1.566	0.361	1.006	0.709
	ALB IT (g/L)_ pre	0.285	0.361	-0.906	0.709
	ALB IT (g/L)_ post	0.469	0.361	-0.893	0.709
	Corrected Ca (mmol/L)_ pre	0.831	0.361	0.209	0.709
	Corrected Ca (mmol/L)_ post	1.825	0.361	1.542	0.709
	glucose (mmol/l)_ pre	0.546	0.361	-0.601	0.709
	glucose (mmol/l)_ post	0.893	0.361	0.180	0.709
	HbA1c (%)_pre	2.050	0.361	2.031	0.709
	HbA1c (%)_post	0.342	0.361	-0.209	0.709
	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 D_3) and B (50 µg vitamin D_3), 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 HbA_{1c} 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)

Reason	Group A (100 µg)		Group B (50 µg)		Group C (placebo)	
	n	%	n	%	n	%
Non-compliance with taking vitamin D supplements	9	17.3	9	17.6	7	13.2
Contracted gastrointestinal malabsorption during the intervention	2	3.8	2	3.9	2	3.8
Changed diabetic medication during the intervention	4	7.7	3	5.9	4	7.5
Took additional vitamin D during the study	3	5.8	5	9.8	5	9.4

Table 3.2. Distribution of sample number of the three groups

Group		Number of participants	Percentage
A (100 µg vitamin D_3)	Included	43	82.7%
	Excluded	9	17.3%
	Total	52	100.0%
B (50 µg vitamin D_3)	Included	42	82.4%
	Excluded	9	17.6%
	Total	51	100.0%
C (placebo)	Included	43	81.1%
	Excluded	10	18.9%
	Total	53	100.0%

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)

Characteristic	**Category**	**Group**					
		A (100 µg)		**B (50 µg)**		**C (placebo)**	
		n	%	n	%	n	%
Nationality	Saudi	43	100	41	97.6	43	100
Age	18-24 years	1	2.3	2	4.8	1	2.3
	25-34 years	1	2.3	2	4.8	2	4.7
	35-44 years	4	9.3	12	28.6	9	20.9
	45-55	18	41.9	25	59.5	6	13.9
	55-60 years	19	44.2	1	2.4	25	58.1
	Mean+/-SD	51.67+/-7.48		52.29+/-7.127		50.33+/-9.54	
Marital Status	Single	1	2.3	3	7.1	2	4.7
	Married	42	97.7	38	90.5	41	95.3
Education	No schooling	5	11.6	1	2.4	5	11.6
	Primary school	4	9.3	2	4.8	3	7
	Secondary school	4	9.3	4	9.5	6	14
	High school	10	23.3	16	38.1	8	18.6
	Diploma	3	7	6	14.3	7	16.3
	Bachelor's degree	12	27.9	12	28.6	11	25.6
	Postgraduate degree	5	11.6	1	2.4	3	7
Employment	Student	1	2.3	1	2.4	2	4.7
	Employed	26	60.5	25	59.5	19	44.2
	Unemployed	2	4.7	1	2.4	2	4.7
	Retired	14	32.6	15	35.7	20	46.5

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

Characteristic	**Category**	**Groups**					
		A (100µg)		**B (50µg)**		**C (placebo)**	
		n	%	n	%	n	%
Smoking	No	5	11.6	3	7.14	2	4.65
Diet	No special diet	43	100	42	100	43	100
Duration of T2DM	1-2 years	3	7.0	1	2.4	2	4.7
	3-5 years	7	16.3	9	21.4	3	7.0
	6-8 years	6	14.0	2	4.8	3	7.0
	> 8 years	27	62.8	30	71.4	34	79.1
Family history of diabetes	Yes	36	83.7	34	81.0	37	86.0
Health Complications	Yes	43	100	42	100	43	100
	Nephropathy	1	2.3	0	0	0	0
	Neuropathy	13	30	16	38.1	25	58.1
	Diabetic Cataract	14	32.6	11	26.2	13	32.2
	Cardiovascular disease	17	39.5	19	45.2	18	41.9
	Dyslipidaemia	26	60.5	26	61.9	29	67.4
	Retinopathy	6	14	6	14.3	18	18.6
Medical condition	Thyroid and parathyroid disease	4	9.3	6	14.3	5	11.6
Multi-vitamin supplement doesn't contain vitamin D	Yes	28	65.1	30	71.4	30	69.8
Taking vitamin D supplements three months before study date	No	43	100	42	100	43	100

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 (Al Saif & 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 glycated haemoglobin (HbA_{1c}) 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 HbA_{1c} (Bastos *et al.*, 2016). In a further study, there was a significant relationship between the duration of diabetes and 500 patients who had HbA_{1c} 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 D_3 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 D_3 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 D_3 are more potent than vitamin D_2 (Binkley *et al.*, 2007). A daily 50 µg of vitamin D_3 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 cubilinmegalin 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 D_3 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 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 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

Source of difference	F-test	P-value
Between times (pre- and post-test)	93.53**	<0.001
Interaction between different doses, intervention period and groups	71.59**	<0.001
Between groups in per-test	0.810	0.447
Between groups in post-test	14.02**	<0.001

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)

Group	Baseline	After 16 weeks	Mean difference	p-value[b]
A	77.85±5.27	115.14±5.082	37.28**	<0.0001
B	85.04±5.26	94.58±4.66	9.53**	<0.001
C	86.03±5.83	81.50±5.70	-4.51	0.074

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

Time	**Group**	**Group**	**Mean difference**	**p-value[b]**
16 weeks	A	B	20.56**	0.002
	A	C	33.56**	<0.001
	B	C	13.08*	0.044

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 $p < 0.05$; [b] 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).

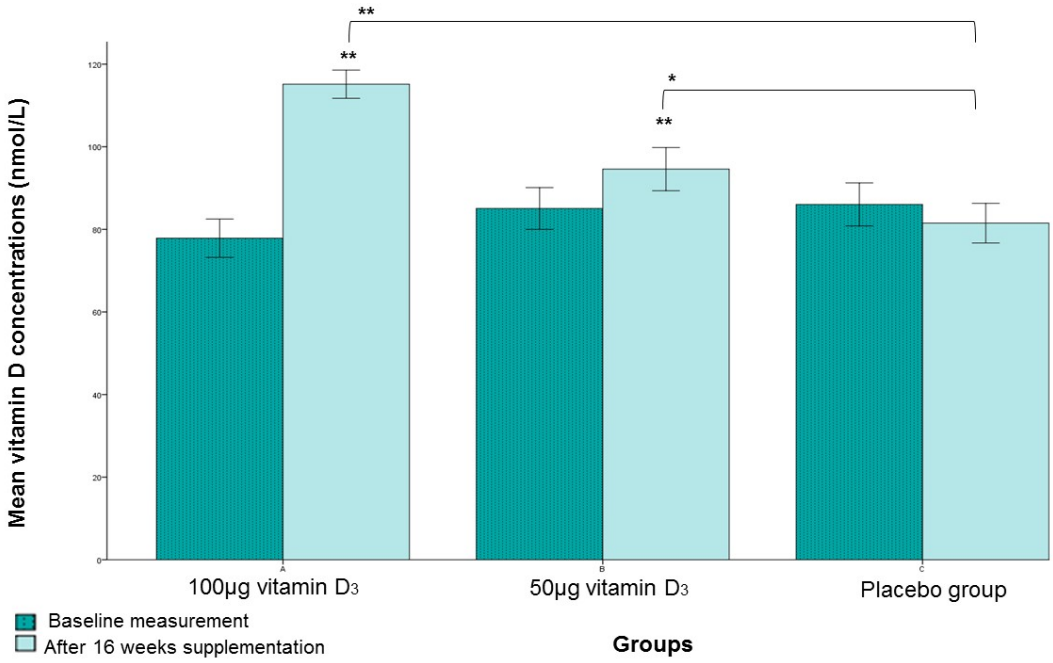

Figure 4.1. Changes in the mean vitamin D status concentrations (nmol/L) in intervention and control groups between the pre-test and post-test measurements
The bar chart shows intervention groups (group A - 100µg vitamin D_3 (n=43) and group B, 50µg vitamin D_3 (n=42) compared with the placebo group (C) as control (n=43) between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences 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 D_3), 42 participants in group B (supplementation with 50 µg vitamin D_3) 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

Source of difference	F-test	P-value
Between times (pre- and post-test)	0.052	0.821
Interaction between different doses, intervention period and groups	0.403	0.669
Between groups in per-test	1.123	0.329
Between groups in post-test	1.482	0.231

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

Group	Baseline	After 16 weeks	Mean difference	p-value[b]
A	2.24±.17	2.24±.15	0.01	0.820
B	2.24±.14	2.31±.26	0.03	0.481
C	2.25±.15	2.26±.24	-0.02	0.584

Intervention groups A, 100µg vitamin D_3 supplementation and B, 50µg vitamin D_3 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).

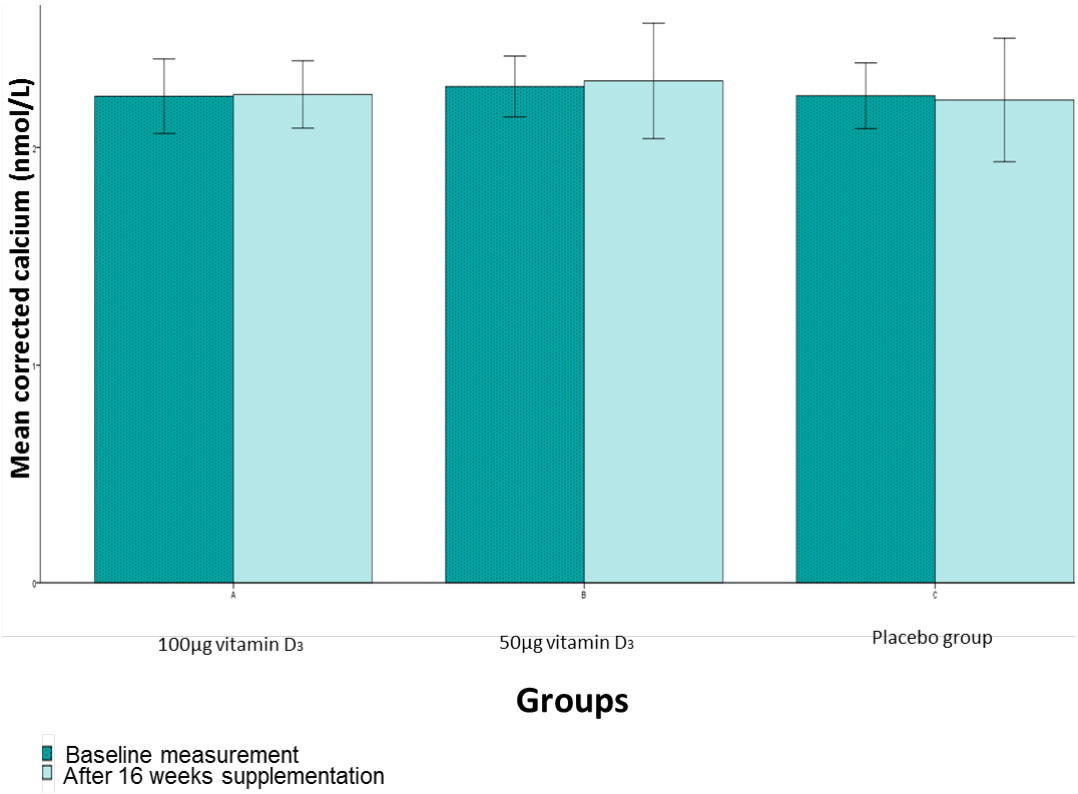

Figure 4.2. Corrected calcium (mmol/L) before and after vitamin D_3 supplementation
The bar chart shows intervention groups A, 100µg vitamin D_3, (n=43) and B, 50µg vitamin D_3, (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements. Corrected calcium remained unchanged after vitamin D_3 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 D_3), 42 participants in group B (supplementation with 50 µg vitamin D_3), 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

Source of difference	F-test	P-value
Between times (pre- and post-test)	30.34**	<0.001
Interaction between different doses, intervention period and groups	6.19**	<0.001
Between groups in per-test	0.021	0.979
Between groups in post-test	0.76	0.467

**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/m^2) among groups (intervention and control) at baseline and after the intervention study

Group	Baseline	After 16 weeks	Mean difference	p-value[b]
A	30.42±5.27	29.63±5.08	-0.794**	0.001
B	30.28±5.26	29.00±4.66	-1.27**	<0.001
C	30.53±6.53	29.69±6.28	0.22	0.690

Intervention groups: group A, 100µg vitamin D_3 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/m^2) between the intervention groups and control after 16 weeks

Time	Group	Group	Mean difference	p-value[b]
16 weeks	A	B	0.62	0.594
	A	C	-0.816	0.484
	B	C	-1.44	0.220

Intervention groups A.100µg vitamin D_3 supplementation, and B, 50µg vitamin D_3 compared with control (placebo) group C after 16 weeks of intervention. [b] indicates adjustment for multiple comparisons.

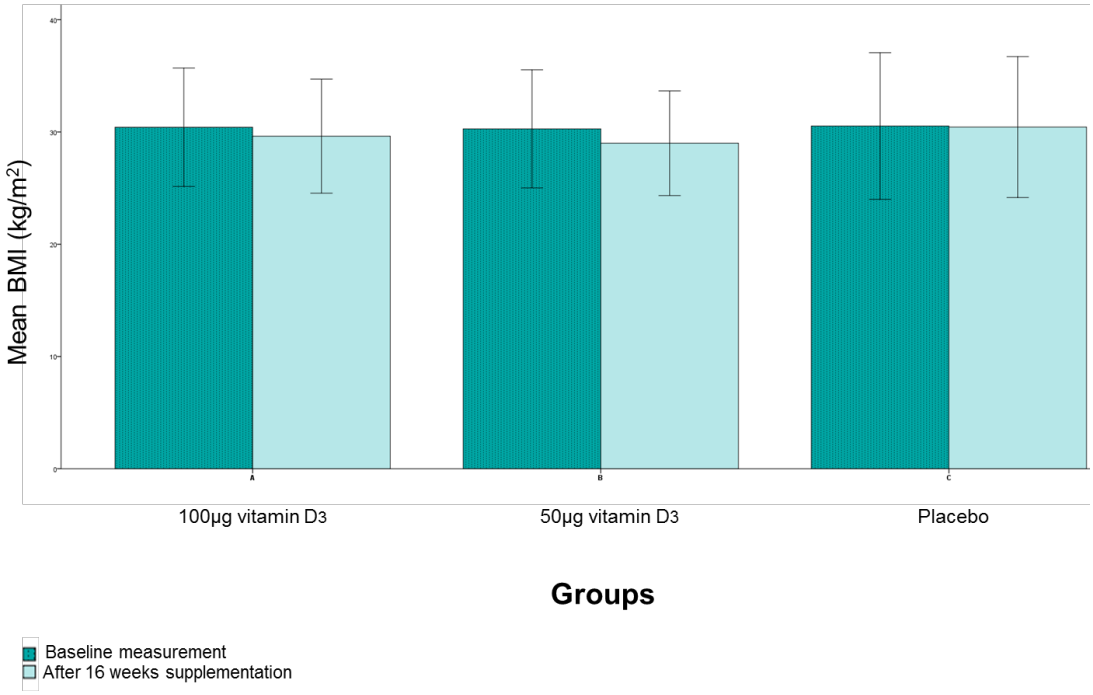

Figure 4.3. The comparisons of mean BMI (kg/m^2) after vitamin D_3 treatment
The bar chart shows intervention groups A, 100µg vitamin D_3 (n=43), and B, 50µg vitamin D_3 (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements. Results are presented as mean ± SD (n=128).

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_3), 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 D_3 supplementation (Dawson-Hughes *et al.*, 2010). A daily dose of 25 µg of vitamin D_3 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 D_3, 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 D_2 is absorbed as vitamin D_3, however, the vitamin D_2 concentration dropped after 3 days (Alshahrani & Aljohani, 2013).

A study of hip-fracture patients with vitamin D insufficiency who were given 25µg of vitamin D_3, 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 D_3 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 D_3 supplementation increased by 37.28 nmol/L (p <0 .001) for 100 µg/day vitamin D_3 supplementation, and by 9.53 nmol/L (p <0.001) after 50 µg/day vitamin D_3 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 D_3, 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 D_3 for 6–8 weeks, thereafter 20-25 µg/per day of vitamin D_3 (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 D_3. 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 D_3) or B (50 µg vitamin D_3). 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/m^2) 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 D_3) and B (50 µg vitamin D_3) from 30.42 ± 5.27 kg/m^2 to 29.63 ± 5.08 kg/m^2 and from 30.28 ± 5.26 kg/m^2 to 29 ± 4.66 kg/m^2, 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 D_3 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 D_3). 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 retuned 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 D_3 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 D_3 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 HbA_{1c}, which is the most important laboratory parameter (Kohnert *et al.*, 2015). The general target of HbA_{1c} 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 D_3 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

Source of difference	F-test	P-value
Between baseline and 16-wk intervention	131.51**	<0.001
Interaction time and groups	34.45**	<0.001
Between groups in pre-test	2.25	0.110
Between groups in post-test	14.47**	<0.001

The comparisons included: the difference in mean HbA_{1c} 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

Group	Baseline	After 16 weeks	Mean difference	p-value[b]
A	9.77±.1.67	7.67±0.98	-2.10**	<0.001
B	9.20±1.10	8.24±1.02	-0.95**	<0.001
C	9.21±1.44	9.01±1.42	-0.20	0.222

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 $p < 0.001$; [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 HbA_{1c} decreased significantly after 16 weeks (mean difference =-2.10, p-value<0.001, see Table 5.2). For group B, the HbA_{1c} also decreased significantly after 16 weeks (mean difference=-0.95, p-value=<0.001). The results of measurements of the HbA_{1c} 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 HbA_{1c} 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 HbA_{1c} 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 HbA_{1c} in group A was significantly lower than in group C (mean difference=-1.34, p <0.001) and the post-intervention mean HbA_{1c} 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 HbA_{1c} concentrations among the intervention and the control groups after 16 weeks

Time	Group	Group	Mean difference	p-value[b]
After 16 weeks	A	B	-0.57	0.023
	A	C	-1.34**	0.001
	B	C	-0.75*	0.003

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 $p < 0.05$.

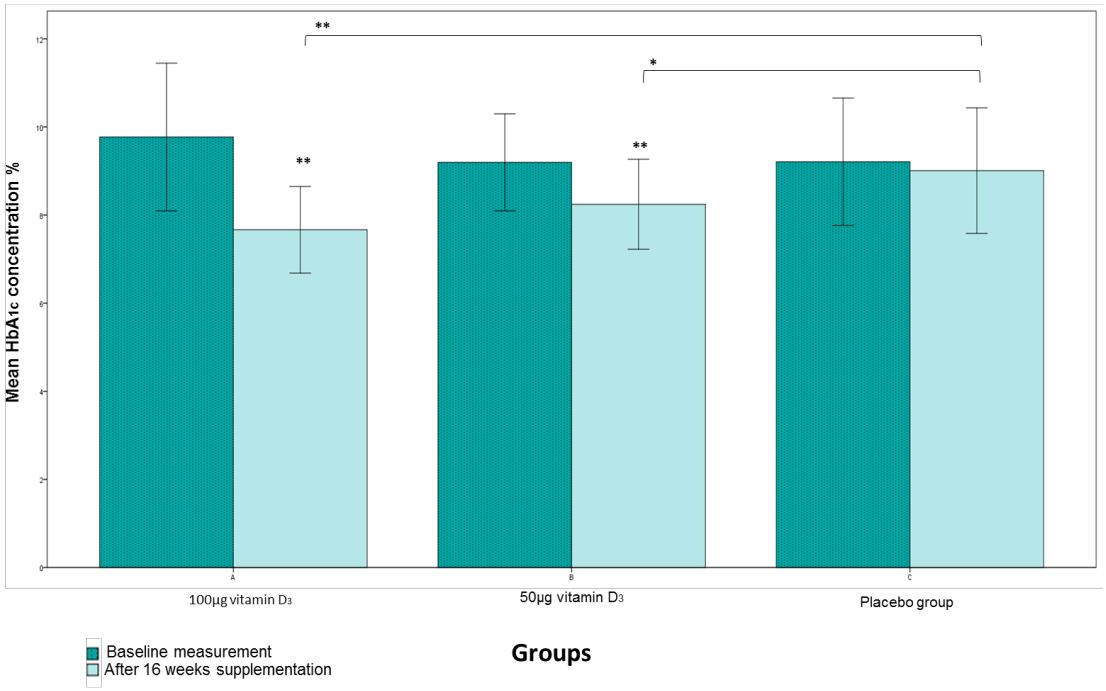

Figure 5.1. Mean changes of HbA_{1c} concentration with vitamin D treatment
The bar chart shows intervention groups A, 100µg vitamin D_3 (n=43) and B, 50µg vitamin D_3 (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences of $p < 0.001$ are marked with (**) 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_3), 42 participants in group B (supplementation with 50 µg vitamin D_3) 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

Source of difference	**F-test**	**P-value**
Between times (pre- and post-test)	67.46**	<0.001
Interaction time and groups	28.07**	<0.001
Between groups in pre-test	1.47	0.232
Between groups in post-test	10.95**	<0.001

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

Group	**Baseline**	**After 16 weeks**	**Mean difference**	**p-value[b]**
A	11.80±.4.31	8.22±2.24	-3.58**	<0.001
B	10.46±.3.30	9.78±2.77	-0.67*	0.044
C	11.51±3.64	11.07±3.64	-0.44	0.179

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

Time	**Group**	**Group**	**Mean difference**	**p-value[b]**
After 16 weeks	A	B	-1.56*	0.012
	A	C	-2.85**	<0.001
	B	C	-1.28*	0.038

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.

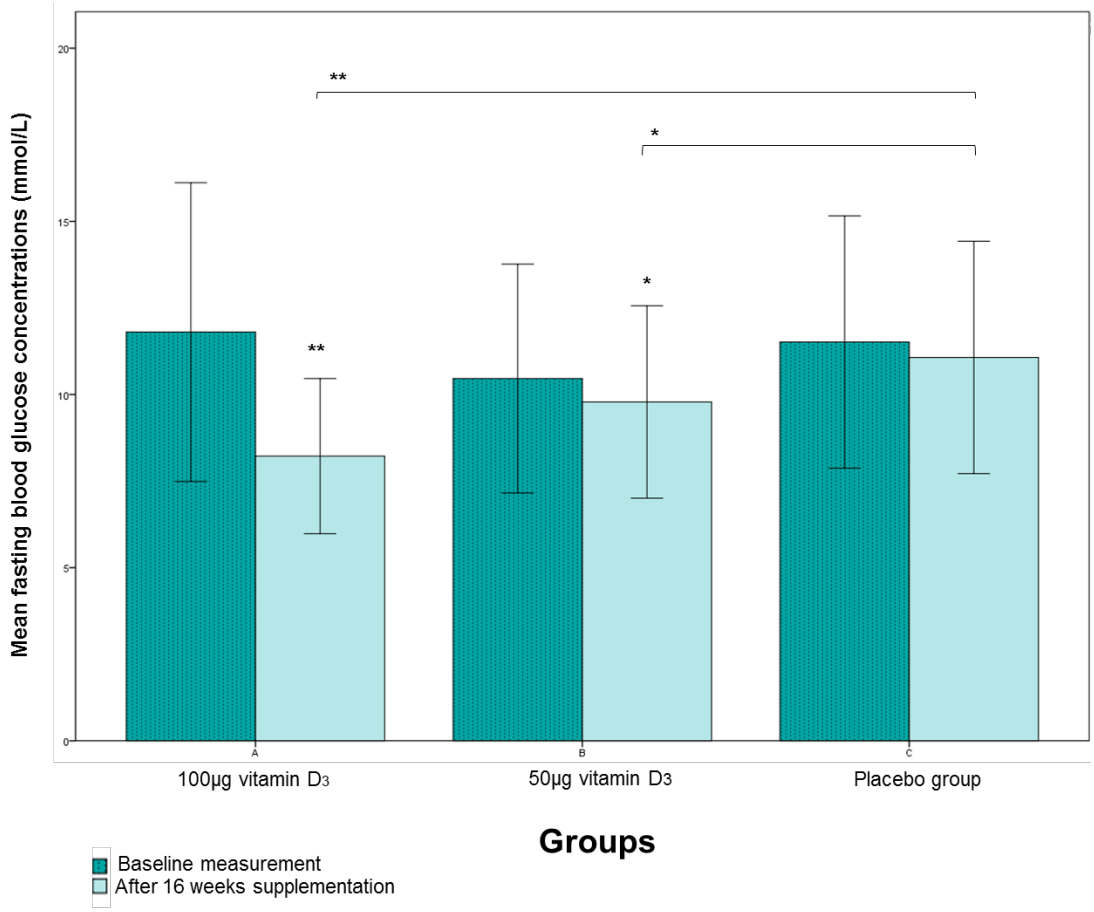

Figure 5.2. Changes in the mean fasting blood glucose concentrations (mmol/L) in intervention and control groups between pre-test and post-test measurements
The bar chart shows intervention groups A, 100µg vitamin D_3 (n=43) and B, 50µg vitamin D_3 (n=42) compared with the placebo group (C) as control (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 (**) 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 D_3), 42 participants in group B (supplementation with 50 µg vitamin D_3) 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 $p \leq 0.05$) 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

Source of difference	F-test	P-value
Between times	0.26	0.610
Interaction time and groups	0.06	0.941
Between groups in pre-test	0.01	0.985
Between groups in post-test	0.04	0.961

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

Group	Baseline	After 16 weeks	Mean difference	p-value[b]
A	16.23±2.81	17.79±2.64	1.57	0.581
B	16.73±2.85	16.88±2.67	0.15	0.956
C	16.06±2.71	16.86±2.6	0.79	0.779

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D and control (C) - placebo group. [b] 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 $p \leq 0.05$) 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.

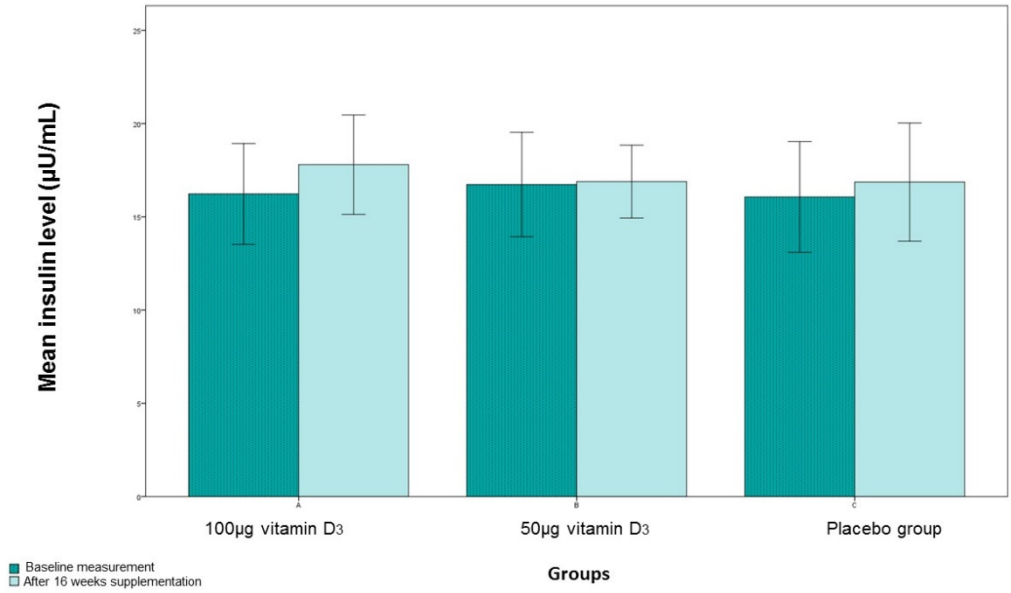

Figure 5.3. Mean changes in insulin level (µU/mL) in the intervention groups A and B, and the control group C (placebo)
The bar chart shows intervention groups A, 100µg vitamin D_3 (n=43) and B, 50µg vitamin D_3 (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements 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 D_3), 42 participants in group B (supplementation with 50 µg vitamin D_3) 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

Source of difference	F-test	P-value
Between times	0.590	0.444
Interaction time and the three groups	0.199	0.820
Between the three groups in pre-test	0.105	0.900
Between the three groups in post-test	0.349	0.706

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

Group	Baseline	After 16 weeks	Mean difference	p-value[b]
A	8.18±1.48	6.62±1.35	-1.56	0.339
B	7.50±1.50	16.88±1.37	-0.35	0.828
C	8.44±1.49	16.86±1.35	0.25	0.876

Intervention groups A, 100µg vitamin D supplementation and B, 50µg vitamin D; control group (C) placebo. [b] 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).

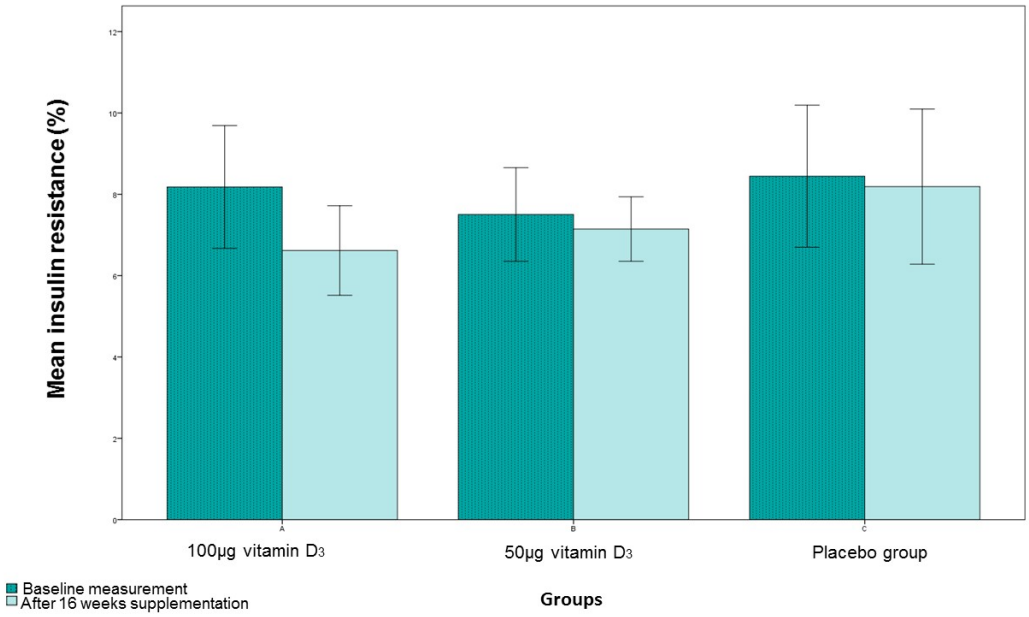

Figure 5.4. Mean changes in insulin resistance (HOMA-IR) in the intervention groups A and B, and the control group C (placebo)
The bar chart shows intervention groups A, 100µg vitamin D_3 (n=43), and B, 50µg vitamin D_3 (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-

test measurements 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 D_3), 42 participants in group B (supplementation with 50 µg vitamin D_3) 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

Source of difference	F-test	P-value
Between times	8.03**	0.005
Interaction time and the three groups	3.85*	0.024
Between the three groups in per-test	0.533	0.588
Between the three groups in post-test	2.66	0.073

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

Group	Baseline	After 16 weeks	Mean difference	p-value[b]
A	50±9.89	96.96±13.72	46.45**	<0.001
B	60.50±9.99	65.54±13.92	5.04	0.675
C	46.38±9.87	53.37±13.76	6.99	0.557

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.

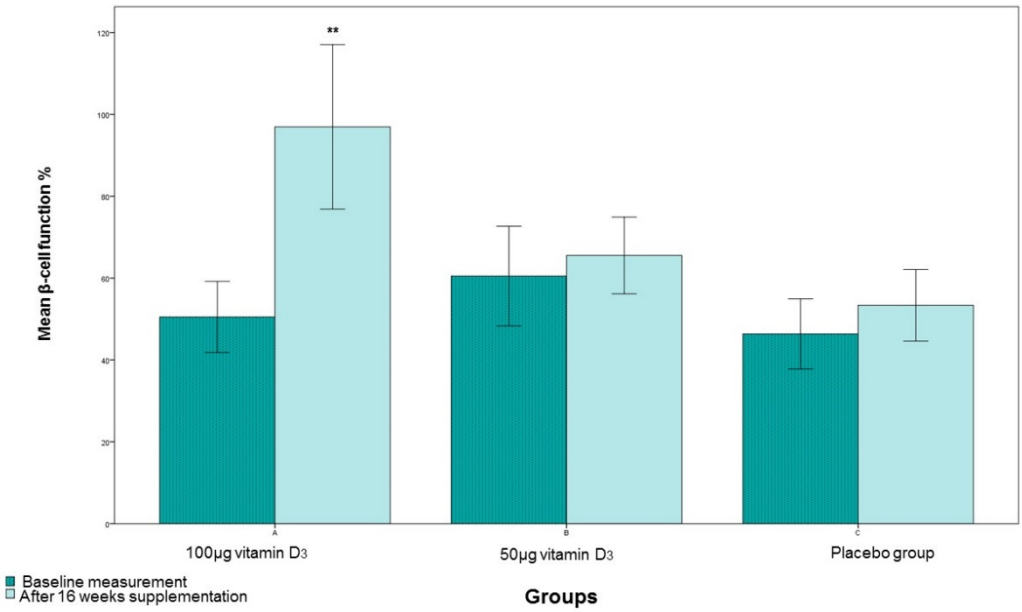

Figure 5.5. Mean changes in β-cell function (HOMA-β) in the intervention groups A and B, and the control group C (placebo)
The bar chart shows intervention groups A, 100µg vitamin D_3 (n=43) and B, 50µg vitamin D_3 (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences of $p < 0.001$ are marked with **

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 HbA_{1c} 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_{1c} 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_{1c} 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 D_3) 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 D_3 for 16 weeks, did not find an improvement in HOMA-IR and HbA_{1c} (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 D_3 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 D_3 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 concertation 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 D_3 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 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 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

Source of difference	**F-test**	**P-value**
Between baseline and 16-wk intervention	19.038**	<0.001
Interaction time and the three groups	7.45**	0.001
Between the three groups per-test	0.951	0.389
Between the three groups post-test	5.555*	0.005

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

Group	**Baseline**	**After 16 weeks**	**Mean difference**	**p-value[b]**
A	4.74±1.38	3.77±0.96	-0.965**	<0.001
B	4.40±1.22	3.89±1.00	-0.513**	0.008
C	4.42±1.17	4.48±1.17	0.056	0.765

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 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

Time	**Group**	**Group**	**Mean difference**	**p-value[b]**
After 16 weeks	A	B	-0.12	0.612
	A	C	-0.71**	0.002
	B	C	-0.59**	0.01

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.

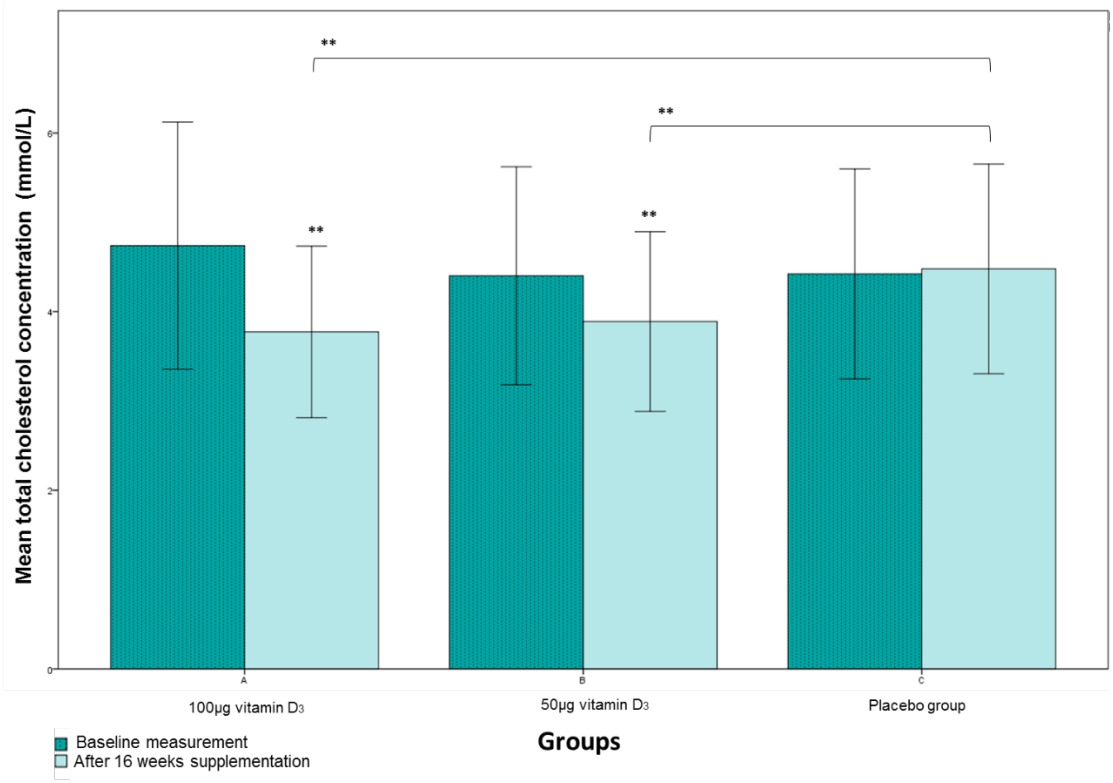

Figure 6.1. Mean changes of total cholesterol concentration in vitamin D treatment
The bar chart shows intervention groups A, 100µg vitamin D_3 (n=43) and group B, 50µg vitamin D_3 (n=42) compared with the placebo group (C) as control (n=43), between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences of $p < 0.001$ are marked with (**).

6.3.2. 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 D_3), 42 participants in group B (supplementation with 50 µg vitamin D_3) 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.

Table 6.4. Two-way mixed ANOVA analysis of differences in the mean triglycerides concentrations (mmol/L) upon vitamin D supplementation

Source of difference	F-test	P-value
Between times (pre- and post-test)	28.19**	<0.001
Interaction time and the three groups	20.02**	<0.001
Between the three groups in per-test	0.100	0.91
Between the three groups in post-test	8.86**	<0.001

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

Group	Baseline	After 16 weeks	Mean difference	p-value[b]
A	2.14±0.85	1.3±0.43	-0.833**	<0.001
B	2.07±.1.16	1.97±.1.07	-0.09	0.337
C	2.16±1.01	2.16±1.24	-0.004	0.972

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

Time	**Group**	**Group**	**Mean difference**	**p-value[b]**
After 16 week	A	B	-0.66**	0.002
	A	C	-0.85**	<0.001
	B	C	-0.19	0.377

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.

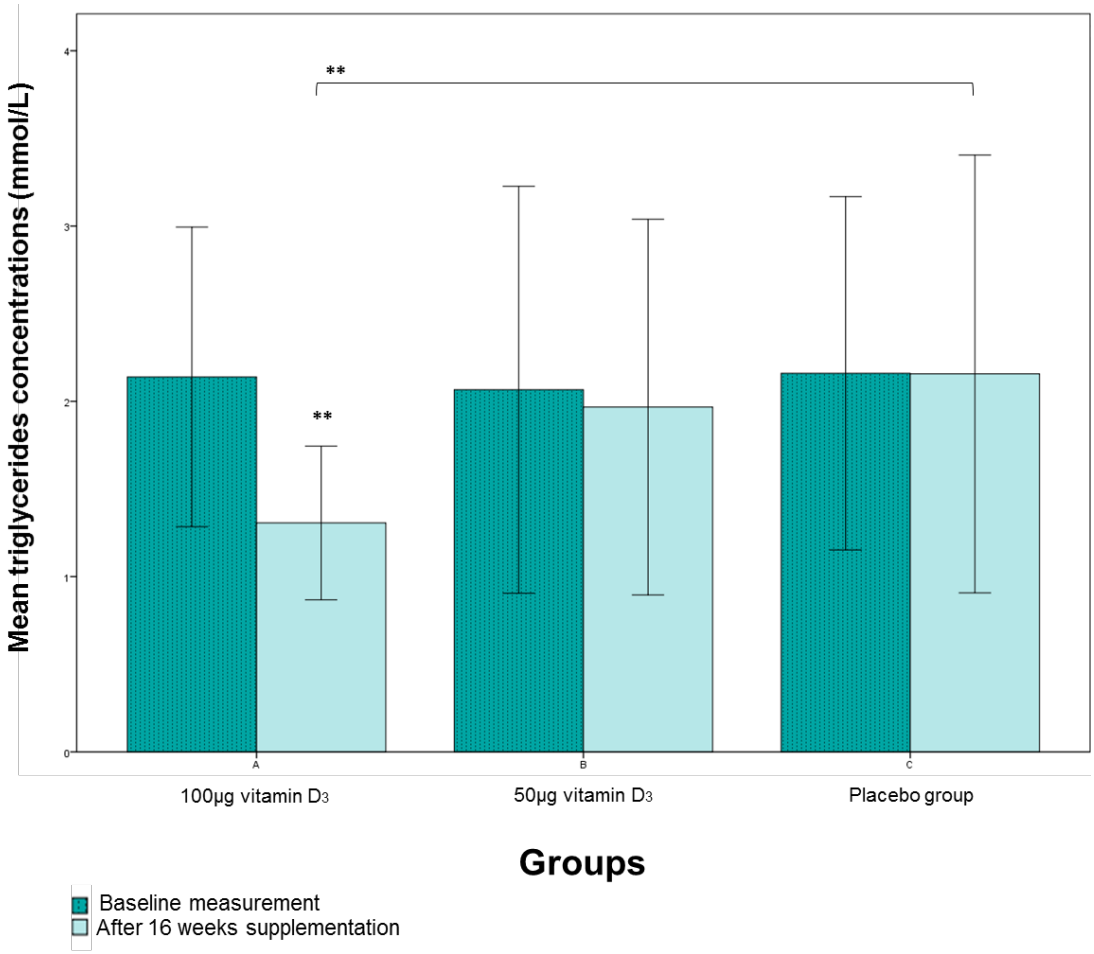

Figure 6.2. Changes in the mean triglyceride concentrations (mmol/L) in the intervention and control groups pre-test and post-test
The bar chart shows intervention groups A, 100µg vitamin D_3 (n=43) and B, 50µg vitamin D_3 (n=42) compared with the placebo group (C) as control (n=43) between pre-test 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

Source of difference	F-test	P-value
Between baseline and 16-wk intervention	79.52**	<0.001
Interaction time and the three groups	39.61**	<0.001
Between the three groups in per-test	0.008	0.992
Between the three groups in post-test	43.61**	<0.001

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

Group	Baseline	After 16 weeks	Mean difference	p-value[b]
A	0.90±.338	1.60±.461	0.70**	<0.001
B	0.89±.311	1.08±.347	0.19**	0.002
C	0.90±.335	0.90±.247	0.004	0.951

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

Time	Group	Group	Mean difference	p-value[b]
After 16 weeks	A	B	0.53**	<0.001
	A	C	0.70**	<0.001
	B	C	0.18**	0.03

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; [b] indicates adjustment for multiple comparisons.

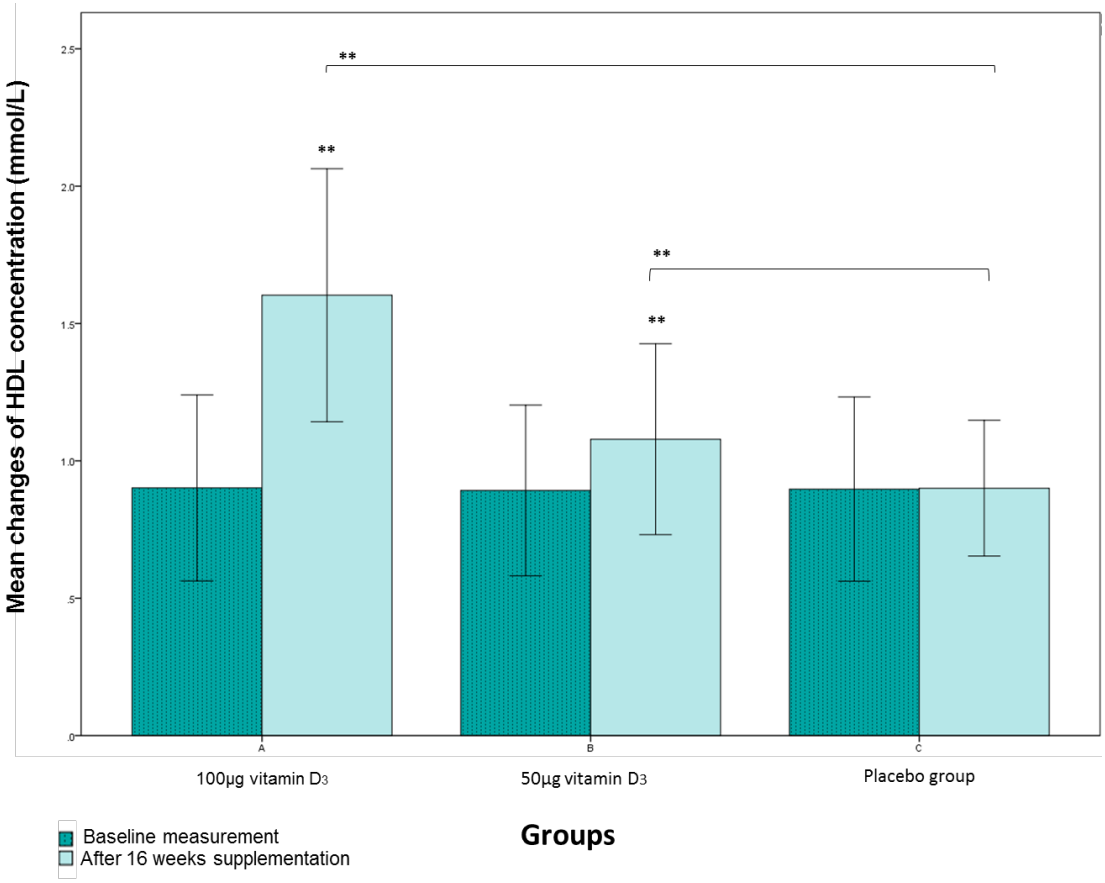

Figure 6.3. Mean changes of HDL concentration in vitamin D treatment
The bar chart shows intervention groups A, 100µg vitamin D_3 (n=43) and B,50µg vitamin D_3 (n=42) compared with the placebo group (C) as control (n=43) between pre-test and post-test measurements. Results are presented as mean ± SD (n=128). Statistically significant differences of p < 0.001) are marked with **.

6.3.4. 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 D_3), 42 participants in group B (supplementation with 50 µg vitamin D_3) 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

Source of difference	**F-test**	**P-value**
Between times (pre- and post-test)	103.22**	<.001
Interaction between different doses, intervention period and groups	26.65**	<.001
Between the three groups in per-test	1.88	.157
Between the three groups in post-test	13.97**	<.001

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

Group	**Baseline**	**After 16 weeks**	**Mean difference**	**p-value[b]**
A	3.56±1.29	1.88±0.98	-1.68**	<0.001
B	3.16±.1.01	2.49±0.97	-0.68**	<0.001
C	3.29±1.11	2.43±1.03	-0.21	0.149

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 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

Time	**Group**	**Group**	**Mean difference**	**p-value[b]**
After 16 week	A	B	-0.61**	0.003
	A	C	-1.06**	<0.001
	B	C	-0.453*	0.03

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; [b] indicates adjustment for multiple comparisons.

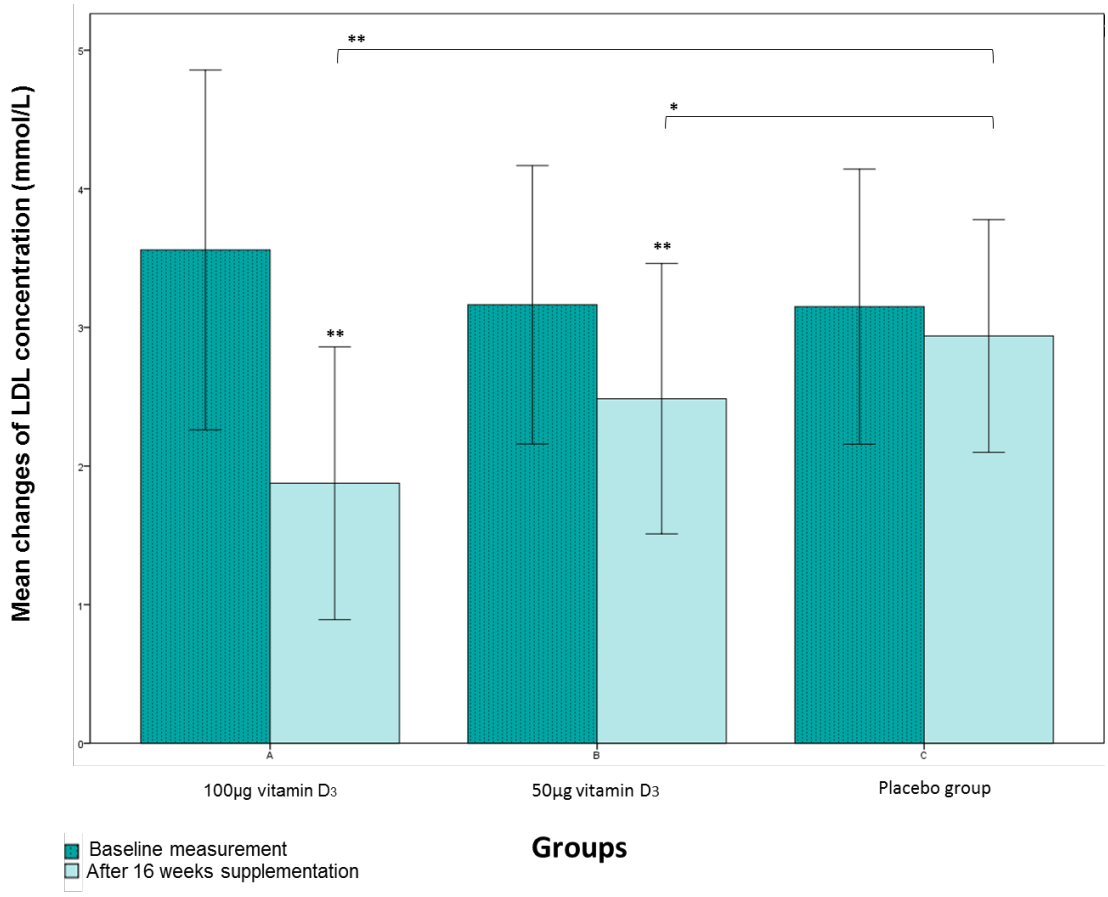

Figure 6.4. Changes in the mean fasting LDL concentrations (mmol/L) in the intervention and control groups between the pre-test and post-test measurements
Interventions groups A, 100µg vitamin D_3 and group B, 50µg vitamin D_3 and group C control (placebo). Results are presented as mean ± SD (n=128). 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 D_3 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 D_3 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 D_3), 42 participants in group B (supplementation with 50 µg vitamin D_3), 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

Source of difference	**F-test**	***p*-value**
Between times	3.882	0.051
Interaction between different doses, intervention period and groups	1.17	0.365
Between the three groups pre-test	0.637	0.530
Between the three groups post-test	1.02	0.361

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

Group	**Baseline**	**After 16 weeks**	**Mean difference**	***p*-value[b]**
A	0.61±0.14	0.66±0.27	0.054	0.077
B	0.65±0.23	0.71±0.28	0.050	0.103
C	0.63±0.18	0.63±0.16	-0.001	0.982

Intervention groups A, 100µg vitamin D supplementation and group B, 50µg vitamin D and control (C)-placebo group; [b] 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.

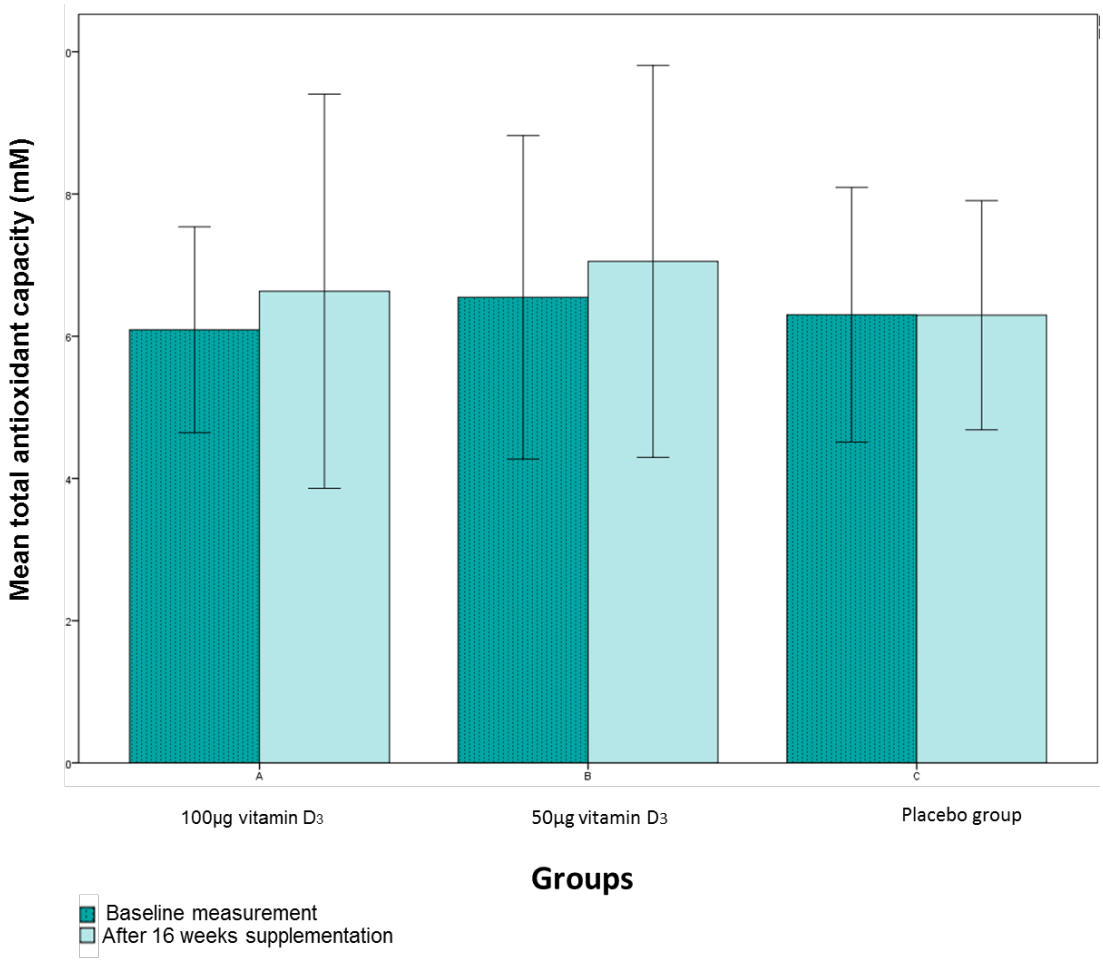

Figure 7.1. Mean changes in plasma total antioxidant capacity (mM) in the intervention groups A and B, and the control group C (placebo)
Interventions groups A, 100µg vitamin D_3 and group B, 50µg vitamin D_3, and group C, control (placebo). Mean total antioxidant capacity measured pre- and post-test; 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 D_3). 42 participants in group B (supplementation with 50 µg vitamin D_3), 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=.0.32, 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

Source of difference	**F-test**	***p*-value**
Between times	4.24	0.050
Interaction between different doses, intervention period and groups	0.57	0.571
Between the three groups in pre-test	0.327	0.722
Between the three groups in post-test	0.258	0.773

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

Group	**Baseline**	**After 16 weeks**	**Mean difference**	***p*-value[b]**
A	2.35±0.49	2.34±0.39	-0.001	0.745
B	2.36±0.55	2..32±0.52	-0.038	0.113
C	2.43±0.50	2.39±0.50	-0.039	0.104

Intervention groups A, 100µg vitamin D supplementation, and group B 50µg vitamin D; control group (C) – placebo; [b] 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).

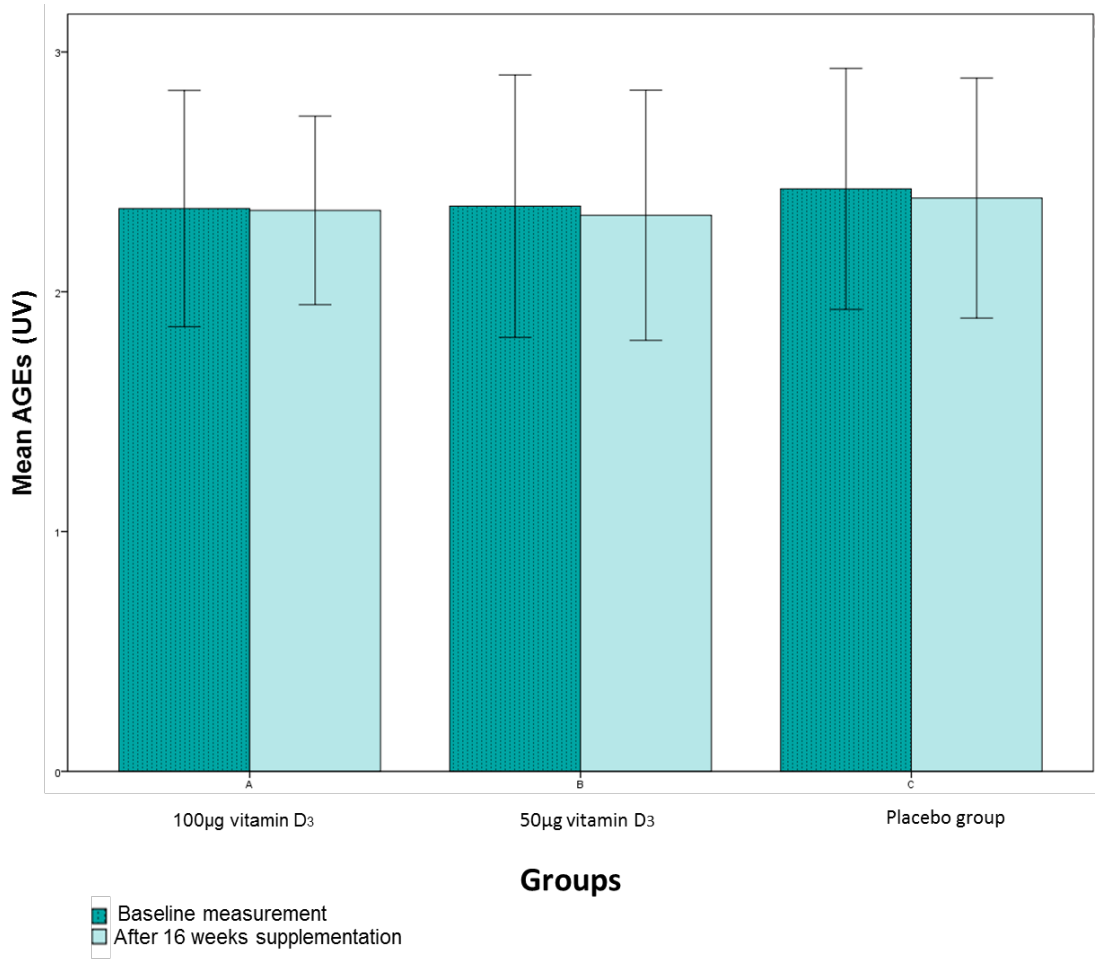

Figure 7.2. Mean changes in AGEs (UV) in the intervention groups A and B, and the control group C (placebo)
Interventions groups A: 100µg vitamin D_3 and B: 50µg vitamin D_3 and group C: control (placebo). Mean AGEs measured pre- and post-test; Results are presented as mean ± SD (n=128). The calculated 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 D_3 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 D_3, showed a significant improvement in serum total antioxidant capacity ($p \leq 0.03$) and malondialdehyde ($p \leq 0.002$) 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 D_3 or a placebo for one month found a significantly increased total antioxidant capacity ($p \leq 0.03$) (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 ($p \leq 0.05$) compared to a healthy group (Saedisomeolia *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 D_3. After 12 weeks, significantly increased levels of the antioxidant superoxide dismutase ($p \leq 0.025$) were found (Nikooyeh *et al.*, 2014). In addition, a Turkish study of 23 subjects deficient in vitamin D who took 250µg/day of vitamin D_3 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 D_3 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 D_3 compared with diabetic rats without treatment ($p \leq 0.001$). 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 D_3 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. H, 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 ($1,25(OH)_2D$) 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

(Wimalawansa, 2016). 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).

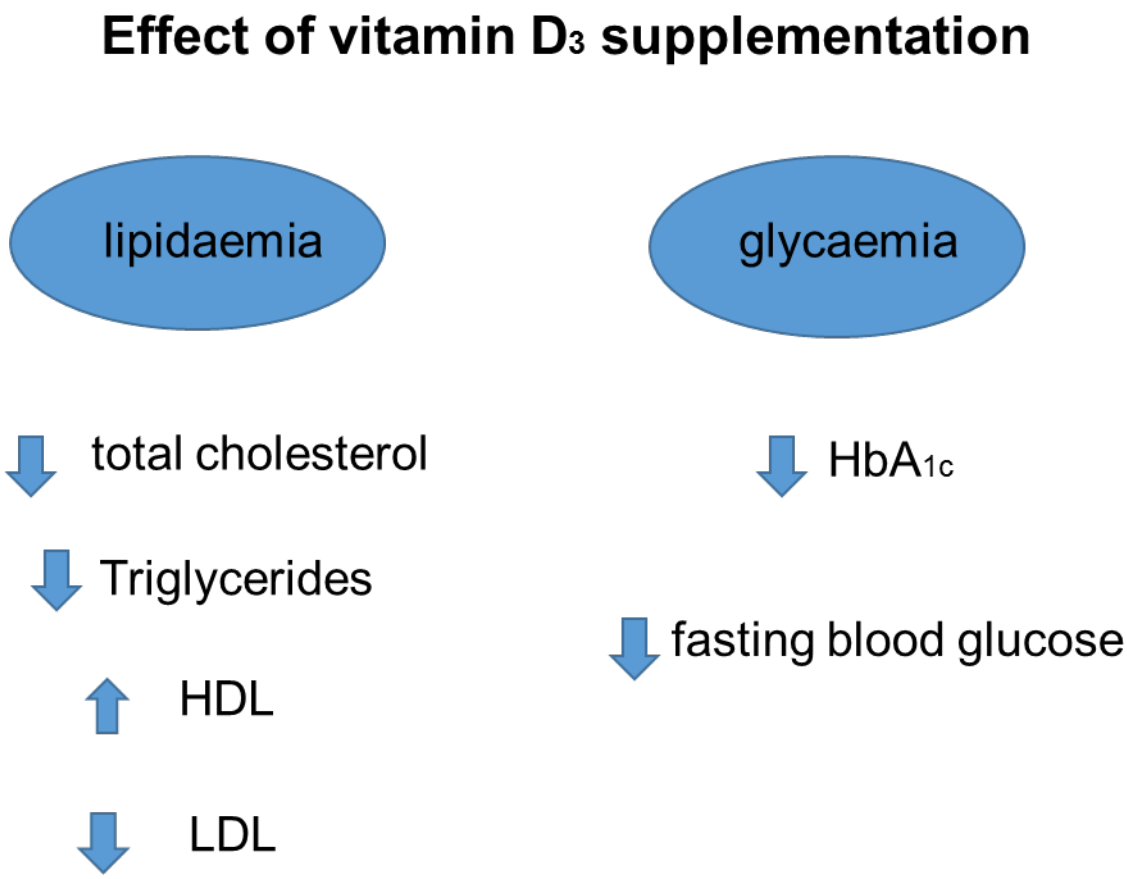

Figure 8.1: The beneficial changes observed in the biomarkers after vitamin D supplementation in this intervention study. Vitamin D supplementation in this study improved the biomarkers for glycaemia and lipidaemia in both groups compared to control.

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 D_3 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

Manchester Metropolitan University

M E M O R A N D U M

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).

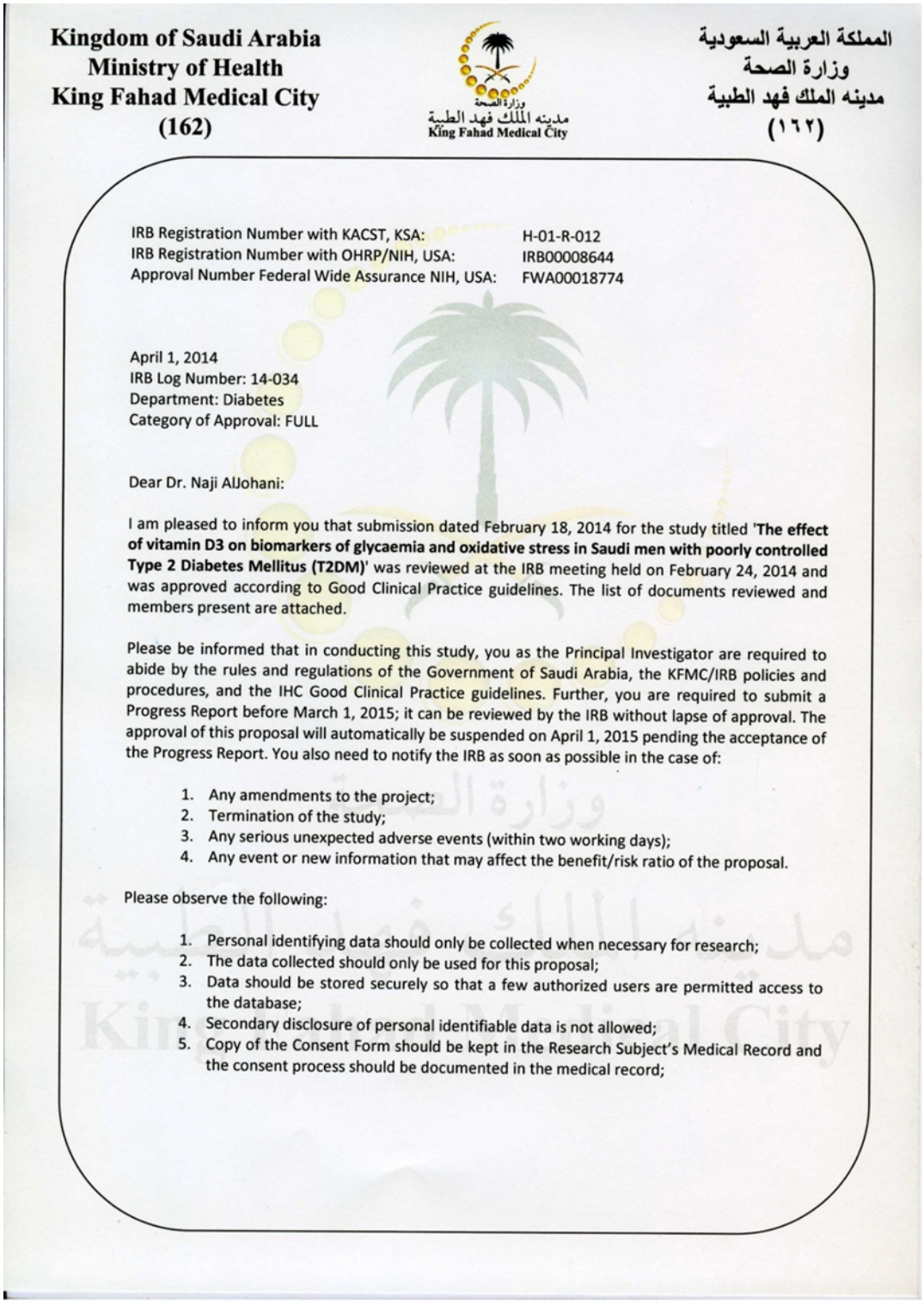
If you make any changes to the approved protocol these must be approved by the Faculty Head of Ethics. If amendments are required you should complete the attached form and submit it to the Administrator.

Regards

Elanor Henry
Assistant Research Administrator
All Saints North

Page **1** of **1**

Appendix 2: King Fahad Medical City ethical approval



Kingdom of Saudi Arabia
Ministry of Health
King Fahad Medical City
(162)

وزارة الصحة
مدينه الملك فهد الطبية
King Fahad Medical City

المملكة العربية السعودية
وزارة الصحة
مدينه الملك فهد الطبية
(١٦٢)

IRB Registration Number with KACST, KSA: H-01-R-012
IRB Registration Number with OHRP/NIH, USA: IRB00008644
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 (T2DM)**' 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 IHC 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

المملكة العربية السعودية
وزارة التعليم العالي
جامعة الملك سعود
رمزها ٠٣٤
كلية العلوم

بسم الله الرحمن الرحيم

الرقم: ٤/٦٧/٤٤٩٨٦
التاريخ: ١٩/ ١٢ /١٤٣٥هـ
المرفقات:
الموضوع:

سعادة الباحثة/ هند فيصل الحربي حفظها الله

السلام عليكم ورحمة الله وبركاته

تفيد سعادتكم بأن لجنة أخلاقيات البحوث العلمية الحيوية بكلية العلوم ناقشت في الاجتماع الرابع يوم الخميس 1435/07/16هـ، الموافق 2014/05/15 م البحث المرسل من سعادتكم تحت عنوان:

" The Effect of Vitamin D_3 supplementation on Markers of Glycaemia and Oxidative Stress in Saudi Men with Poorly-Controlled Type 2 Diabetes Mellitus"

وقد وافقت اللجنة على التعديل الذي تم من قبلكم بشأن عدد المرضى المشاركين بالبحث على أن يعمل بهذا التغيير في النسخة الأصلية المقدمة للجنة.

القرار: الموافقة

وتقبلوا خالص تحياتي وتقديري،،،

رئيس لجنة أخلاقيات البحوث الحيوية العلمية
بكلية العلوم
أ.د. ناصر بن محمد الداغري

P. O. Box 2455, Riyadh 11451 Tel 4674447 – Fax: 4674253
ص.ب ٢٤٥٥ الرياض ١١٤٥١ هاتف: ٤٦٧٤٤٤٧ – فاكس: ٤٦٧٤٢٥٣
E-mail: cscience@ksu.edu.sa

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

Application #14062303

PART 1: STUDY IDENTIFICATION

1.1	Scientific Title *		**The Effect of Vitamin D3 on Biomarkers of Glycaemia and Oxidative Stress in Saudi Men with Poorly-Controlled Type 2 Diabetes Mellitus**
1.2	Public Title		
		Arabic *	تأثيرمكملات فيتامين دال على المؤشرات الحيويه لنسبه السكر بالدم و الاجهاد التاكسدي لدي الرجال المصابون بالسكري النوع الثاني الغير منتظم
		English *	**The Effect of Vitamin D3 on Biomarkers of Glycaemia and Oxidative Stress in Saudi Men with Poorly-Controlled Type 2 Diabetes Mellitus**
1.3.1	Protocol Information		
		Protocol Number *	**H-01-R-012**
		Protocol Date *	**01/04/2014 Tuesday, 1 April, 2014**
1.3.2	Other Identification		
		Number	**SE121327A1**
		Date	**01/05/2014 Thursday, 1 May, 2014**
1.4.1	Type of the study *		(•) Interventional ( ) Non-Interventional
1.4.2	Study Design		( ) Case report ( ) Case control ( ) Cohort studies (•) Other, Specify : **A randomized, placebo-controlled, double blind trial** ( ) Cross sectional ( ) Case series ( ) Survey
1.5	Therapeutic Area *		**Endocrinology and metabolism**
1.6	Disease Name		**Type 2 Diabetes Mellitus**

Next

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

Kingdom of Saudi Arabia
King Saud bin Abdulaziz University
for Health Sciences
King Fahad Medical City
Faculty of Medicine

المملكة العربية السعودية
جامعة الملك سعود بن عبدالعزيز
للعلوم الصحية
كلية الطب مدينة الملك فهد الطبية

سعادة الأستاذ الدكتور / نائب رئيس هيئة الغذاء و الدواء لقطاع الدواء حفظه الله

السلام عليكم ورحمة الله وبركاتة

بناء على موافقة مدينة الملك فهد الطبية لدعم بحث بعنوان " تأثيرمكملات فيتامين دال على المؤشرات الحيويه لنسبه السكر بالدم و الاجهاد التاكسدي لدي الرجال المصابون بالسكري النوع الثاني الغير منتظم " و موافقة لجنة أخلاقيات البحوث الطبية بمدينة الملك فهد الطبية برقم (H-01-R-012) باسم الدكتور ناجي الجهني ، ولحاجة البحث البحث إلى أستيراد أقراص فيتامين د (Vitamin D3).

اسم العينة	عدد العبوات / حجم العبوة
Vitamin D3 trial	١٢٠ / ١٣٥

لذا نرجو من سعادتكم التكرم بتوجيه من يلزم لتسهيل أمر فسح المنتج من شركة (metabolics) ولتشير بريطانيا المتحدة عن طريق منفذ مطار الملك خالد بالرياض.

الباحث الرئيس للدراسة

الدكتور / ناجي الجهني
أستاذ مساعد واستشاري للغدد الصماء والسكر
كلية الطب بمدينة الملك فهد الطبية

الرقم : التاريخ : المرفقات :

هاتف : ٢٨٩٩٩٩ فاكس : ٢٨٨٩٩٩٩ تحويلة ٨٠٤٥ البريد الإلكتروني : fom.college@kfmc.med.sa
Tel: (2889999) Fax: (2889999 Ext. 8045) E-mail: fom.college@kfmc.med.sa

المملكة العربية السعودية
الهيئة العامة للغذاء والدواء

قطاع الدواء
الإدارة التنفيذية للتفتيش وإنفاذ الانظمة
إدارة المنافذ

		Dr Naji Aljohani د. ناجي الجهني	الاسم (Name)
9726655433	رقم بوليصة الشحن (Bill of lading No.)	00 966 59 597 9602	رقم الجوال (Mobile No.)
United Kingdom	بلد الشحن (Source of shipment)	DHL	الشركة الناقلة (Carrier)

المستحضرات المراد استيرادها أو تصديرها
Products intended to be cleared or imported

الغرض من الاستخدام Intended use	عدد العبوات/ حجم العبوة (الكمية داخل العبوة) Quantity/Pack size		الشكل الصيدلاني (كبسول،قرص، ....) Pharmaceutical Form (tablet, capsule,...)	اسم المستحضر (باللغة الانجليزية) Product name (Trade Name)
Clinical trials	135	120	Capsules	Vitamin D3 Trial

طريقة الشراء
Buying method

أخرى (اذكرها) Other (please write it)	عن طريق الانترنت (يكتب موقع الشراء باللغة الانجليزية) By internet (write the website address)	صيدلية من خارج المملكة (اذكرها) Pharmacy from outside the kingdom (please write it)
☑	☐	☐
School Of Healthcare Science Manchester Metropolitan University	http//:	

مبررات الشراء من خارج المملكة
justification for Purchase from outside the kingdom

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 D_3, 100 µg/day (4000 IU) of vitamin D_3, or a placebo.

التعهد
Declaration

- أتعهد بأن المستحضرات المذكورة أعلاه للاستخدام في دراسه سريرية وليست للاستخدام التجاري، مع تحملي كامل المسؤولية في حال نتج عن استخدام هذه المستحضرات أي أثار جانبية .
- **These products are for my clinical trail use only and I'll take full responsibility if any side effects happen from usage.**

التوقيع
Signature

I.D Number رقم إثبات الهوية	Date التاريخ	Signature التوقيع

Appendix 6: MMU informed consent form

INFORMATION ABOUT THE PROJECT

Project title: The effect of vitamin D_3 on biomarkers of glycaemia and

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

information you give. Your information will not be used for any purpose other than this PhD research project.

No information that could be used to identify you, such as your name, will be published in the research paper based on this study. There will also be no information that could lead to your address being known. The data provided will only be available to the study group, except if the information includes evidence of criminal activities. No possible risks have been identified for participants because of their involvement with the study.

If you would like to know more or have questions about the research project, please write to me at the email address below. Your participation in this project is highly appreciated.

Thank you.

Hend Faisal H Alharbi

E-mail **10976029@stu.mmu.ac.uk**

Consent form

Project title: The effect of vitamin D_3 on biomarkers of glycaemia and oxidative stress in Saudi men with poorly-controlled type 2 Diabetes Mellitus

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

أقرار بالموافقة علي المشاركة في دراسة بحثية

CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH

موافقة للمشاركة في البحث

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 " تأثيرمكملات فيتامين دال على المؤشرات الحيويه لنسبه السكر بالدم و الاجهاد التاكسدي لدي الرجال المصابون بالسكري النوع الثاني الغير منتظم"

Principal Investigator: Dr. Naji Aljohani

الباحث الرئيس د. ناجي الجهني

Hend Alharbi

هند فيصل 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.

سيشرح لك عضو من فريق البحث محتويات هذه الدراسة وتأثيرها عليك. و يصف هذا الإقرار إجراءات الدراسة ، والمخاطر والفوائد من المشاركة ، وكيفية الحفاظ على سرية المعلومات. الرجاء اخذ الوقت الكافي في طرح الأسئلة لكي تتخذ قرارك ما إذا كنت ستشارك أم لا. وهذه الموافقة تسمى الموافقة المستنيرة. إذا قررت المشاركة في هذه الدراسة ، سيطلب منك التوقيع على هذا الإقرار وستعطي نسخة لسجلاتك. وطوال هذا الإقرار اللفظ، "أنت" سوف يشير إليك أو إلى طفلك ، حسب الاقتضاء.

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

لماذا تجري هذه الدراسة؟ تحديد ما اذا فيتامين دال ممكن ان يقلل من الموشرات الحيويه للاجهاد التاكسدي في الرجال السعوديين المصابون بمرض السكري النوع الثاني الغير منتظم الذين تتراوح اعمارهم من ١٨-الي ٦٠ عاما

Cultural Bureau in London (Qassim University)

الملحقيه الثقافيه السعوديه بلندن (جامعه القصيم)

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

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

وكم عدد المشاركين في هذه الدراسة ؟

135

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.

ماذا سيحدث إذا شاركت في هذه الدراسة ؟

مشاركتك ونتائج التحاليل سوف تثبت ما ان فيتامين دال يقلل من الموشرات الحيويه للاجهاد التأكسدي.

Study location:

. موقع الدراسة

Riyadh

الرياض

WHAT IS EXPECTED OF ME DURING THE STUDY?

ما هو متوقع من خلال دراسة لي؟

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.

سوف تقوم بتعئبه الاستبيان الخاص بالدراسه اولا ومن ثم سوف نختار عشوائيا لاعطاء حبوب فيتامين (2000IU or 4000IU)دال بجرعتين مختلفتين ، و سوف placebo اودواء وهمي نقوم

عمليه سحب دم لاجراء التحاليل الازمه في بدايه الدراسه و بعد ٤ اشهر. و قياس AGE بقارئي ضوئي من سطح الجلد.

HOW LONG WILL I BE IN THE STUDY?

4 Months

ما هي مدة المشاركة في هذه الدراسة؟

٤ اشهر

CAN I STOP BEING IN THE STUDY?

هل أستطيع إنهاء المشاركة ؟

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.

نعم. يمكنك أن تقرر التوقف في أي وقت. فقط اخبر الطبيب إذا قررت التوقف. ليوضح لك كيفية إنهاء مشاركتك بأمان. لا أحد سيحملك علي تغيير رأيك.

ARE THERE RISKS IF I STOP BEING IN THE STUDY?

هل هناك مخاطر متوقعة إذا أنهيت المشاركة في الدراسة ؟

There is not any risk.

ليس هناك اي ضرر.

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

ما هي المخاطر أو الآثار الجانبية التي يمكن حدوثها من جراء المشاركة في الدراسة؟

There are not any side effects.

لا يوجد هناك اي مخاطر جانبية

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

هل هناك فوائد من المشاركة في الدراسة ؟

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.

مشاركتك في هذه الدراسة قد لا تؤدي إلي تحسن حالتك.ولكن يأمل الأطباء أن يكون الاجراء / الدواء / التدخل / الجهاز اكثر فعالية / لها اقل اثار جانبية من العلاج القياسي او المعتاده، ولا يوجد دليل على ذلك حتى الآن.

WHAT OTHER OPTIONS ARE THERE?

ما هي الخيارات الأخرى ؟ لا يوجد خيارات اخرى

There are no other options

لديك خيارات أخري بدلا عن المشاركة في الدراسة:

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

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

ماذا يحدث لو أنني تعرضت للإصابة بسبب المشاركة في هذه الدراسة ؟

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) at .

من المهم أن تبلغ دكتور ناجي الجهني وباحثه الدكتوراه هند الحربي إذا كنت تظن انك قد تعرضت للإصابة بسبب مشاركتك في هذه الدراسة. يمكنك أن تبلغ الطبيب شخصيا أو الاتصال به علي 0556605558 في حال تعرضك للإصابة . سيكون والعلاج متاحا. ستقدم لك مدينة الملك فهد الطبية تكاليف العلاج، ويتوقف ذلك على عدد من العوامل. عادة لا تقدم مدينة الملك فهد الطبية أو ممول الدراسة أي شكل آخر من أشكال التعويض عن الضرر. وللحصول على مزيد من المعلومات عن هذا الموضوع، يمكنك الاتصال بمكتب المؤسسي استعراض المجلس (IRB) علي الرقم) .

WHAT ARE THE COSTS OF TAKING PART IN THE STUDY?

وما هي تكاليف المشاركة في الدراسة ؟

You will not be charged for any study activities.

لن تتحمل تكاليف أي من أنشطة الدراسة.

هذه الدراسه مدعومه من البعثات الحكوميه من قبل الملحقيه الثقافيه.

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?

هل سأتقاضى اجر نظير المشاركة في هذه الدراسة ؟

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.

لن يكون هناك اجر. ولكن اذا كنت موظف سوف نوفر لك اجازه مرضيه لليوم الذي تراجع به للمستشفى.

WILL MY MEDICAL INFORMATION BE KEPT PRIVATE?

هل سيتم الحفاظ علي المعلومات الطبية الخاصة بي بسرية ؟

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.

سنبذل قصارى جهدنا للتأكد من أن المعلومات الشخصية في سجلك الطبي تحظى بالسرية. ومع ذلك ، لا يمكننا أن نضمن الخصوصية التامة. يمكن أن يفصح عن معلوماتك الشخصية إذا اقتضى الأمر وذلك بموجب القانون. لن يتم الإفصاح عن اسمك أو المعلومات الشخصية إذا تم نشر نتائج هذه الدراسة نشرت أو عرضت في الاجتماعات العلمية.

ما هي حقوقي إذا وافقت على المشاركة في هذه الدراسة ؟

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.

قرار المشاركة في هذه الدراسة من اختيارك. لك حرية اختيار المشاركة في هذه الدراسة أو لا .كما يمكنك إنهاء المشاركة في أي وقت. مهما كان قرارك ، لن يكون هناك أي عقوبة و لن تفقد أي من الفوائد العادية الخاصة بك. ترك الدراسة لن يؤثر علي الرعاية الطبية المقدمة لك من مدينة الملك فهد ناجي الجهني وباحثه الدكتوراه هند الطبية. د. الحربي قد يستخدم المعلومات التي تم جمعها قبل أن تترك لدراسة.

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.

ونحن سوف نبلغك بكل المعلومات والمستجدات أو التغييرات في الدراسة التي يمكن أن تؤثر على صحتك أو على استعدادك لمواصلة الدراسة .

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.

وفي حالة الإصابة الناتجة عن هذه الدراسة ،بتوقيع هذا الإقرار, لن تفقد أيا من الحقوق القانونية في طلب التعويض.

WHO DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

بمن يمكنني الاتصال إذا كانت لدي أي أسئلة أو مشاكل؟

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.

قبل أن توافق على المشاركة هذه الدراسة ،ستتحدث إلى احد أعضاء فريق دراسة المؤهلين ليخبرك عن هذه الدراسة . يمكنك أن تطرح الأسئلة حول أي جانب من جوانب البحث. إذا كان لديك المزيد من الأسئلة عن الدراسة ،يمكنك السؤال في أي وقت. يمكنك الاتصال الباحث الرئيس علي الرقم

0556605558

CONSENT

إقرار بالموافقة

Subject:

المشارك

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.

لقد تم شرح البحث والإجراءات لي. وسمح ي بأن اسأل أي سؤال لدي في هذا الوقت. ويمكنني أن اسأل أي أسئلة إضافية في وقت لاحق. ويمكنني إنهاء المشاركة في الدراسة في أي وقت دون يؤثر ذلك علي الرعاية الصحية المقدمة لي.

I will receive a signed copy of this consent form.

سأحصل علي نسخة موقعة من هذا الإقرار بالموافقة.

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.

إنا اقر بالموافقة على المشاركة في هذه الدراسة. موافقتي طوعية, ولست بحاجة إلى التوقيع على هذا الإقرار إذا كنت لا أريد المشاركة في هذه الدراسة البحثية.

Subject Signature

توقيع المشارك:

Date

التاريخ

Time (AM ☐ PM ☐)

الوقت ص ☐ م ☐

Person Obtaining Consent:

الشخص الحاصل على الموافقة:

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.

لقد شرحت طبيعة الدراسة والغرض منها وما تنطوي عليه من مخاطر. وقد أجبت وسأجيب علي الأسئلة على أفضل قدر من استطاعتي. سأعطي نسخة موقعة من الإقرار بالموافقة إلي المشارك المذكور أعلاه.

Signature of Person Obtaining Consent

توقيع الشخص الحاصل على الموافقة

Date

التاريخ

Time (AM ☐ PM ☐)

الوقت ص ☐ م ☐

Principal Investigator:

الباحث الرئيس :

Signature of Principal Investigator

توقيع الباحث الرئيسي

Time (AM ☐ PM ☐)

الوقت ص ☐ م ☐

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

[قف! لا تستخدم خطوط التوقيع التالية إلا إذا طلبت موافقة طرف ثالث]

AND/OR:

و / أو :

Legally Authorized Representative

الممثل المخول قانونا

Date

التاريخ

Person Obtaining Consent

الشخص الحاصل على الموافقة

Date

التاريخ

OR

أو

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.

الشخص المعني بالدراسة غير قادر على الموافقة بنفسه لأنه / إنها قاصر. من خلال التوقيع أدناه، أنت تعطي إذنك لطفلك بان يضمن في هذه الدراسة

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

----------الأبوين أو الوصي قانونا

Date: / /

/ /التاريخ:

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 _____m^2
- 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 _____m^2

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

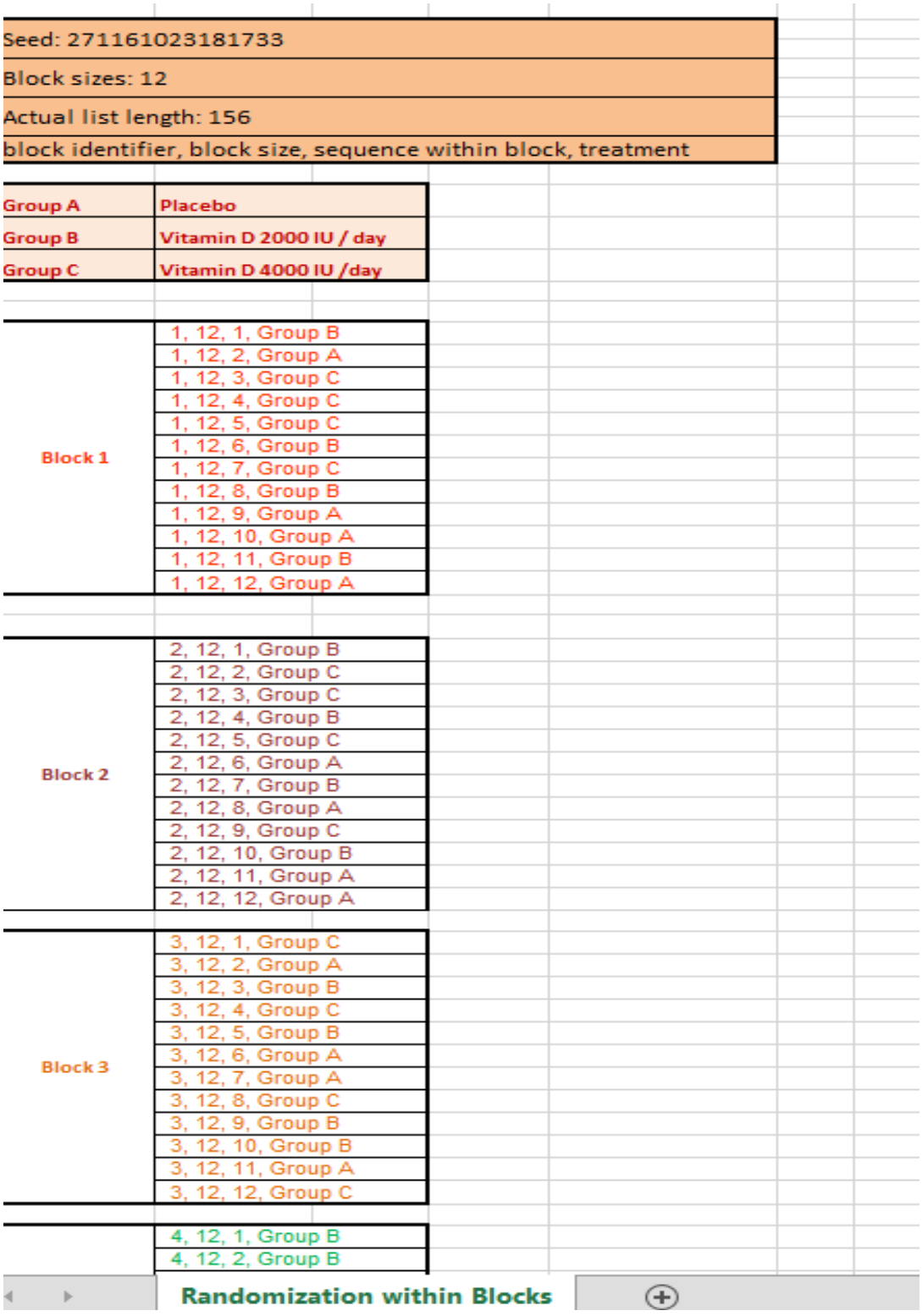
Participant NO:…… رقم المشارك

.....

Day / week	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							

Researcher Hend Alharbi

Appendix 11: Block supplement randomisation



Seed: 271161023181733	
Block sizes: 12	
Actual list length: 156	
block identifier, block size, sequence within block, treatment	
Group A	Placebo
Group B	Vitamin D 2000 IU / day
Group C	Vitamin D 4000 IU /day
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
	4, 12, 1, Group B
	4, 12, 2, Group B

Randomization within Blocks

Appendix 12: Measurement of the concentration of vitamin D kits

Vitamin D total

cobas®

25-Hydroxyvitamin D

REF	Σ	SYSTEM
05894913 190	100	Elecsys 2010 MODULAR ANALYTICS E170 **cobas e** 411 **cobas e** 601 **cobas e** 602

English

Intended use

This assay is intended for the quantitative determination of total 25-hydroxyvitamin D in human serum and plasma. This assay is to be used as an aid in the assessment of vitamin D sufficiency.

The electrochemiluminescence binding assay is intended for use on Elecsys and **cobas e** immunoassay analyzers.

Summary

Vitamin D is a fat-soluble steroid hormone precursor that is mainly produced in the skin by exposure to sunlight. Vitamin D is biologically inert and must undergo two successive hydroxylations in the liver and kidney to become the biologically active 1,25-dihydroxyvitamin D.[1]

The two most important forms of vitamin D are vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). In contrast to vitamin D_3, the human body cannot produce vitamin D_2 which is taken up with fortified food or given by supplements. In human plasma vitamin D_3 and D_2 are bound to the vitamin D binding protein and transported to the liver where both are hydroxylated to form vitamin D (25-OH), i.e. 25-hydroxyvitamin D. It is commonly agreed that vitamin D (25-OH) is the metabolite to determine the overall vitamin D status as it is the major storage form of vitamin D in the human body. This primary circulating form of vitamin D is biologically inactive with levels approximately 1000-fold greater than the circulating 1,25-dihydroxyvitamin D. The half-life of circulating vitamin D (25-OH) is 2-3 weeks.

Most of the vitamin D (25-OH), measurable in serum, is vitamin D_3 (25-OH) whereas vitamin D_2 (25-OH) reaches measurable levels only in patients taking vitamin D_2 supplements.[2,3,4] Vitamin D_2 is considered to be less effective.[5]

Vitamin D is essential for bone health. In children, severe deficiency leads to bone-malformation, known as rickets. Milder degrees of insufficiency are believed to cause reduced efficiency in the utilization of dietary calcium.[6] Vitamin D deficiency causes muscle weakness; in elderly, the risk of falling has been attributed to the effect of vitamin D on muscle function.[7] Vitamin D deficiency is a common cause of secondary hyperparathyroidism.[8,9] Elevations of PTH levels, especially in elderly vitamin D deficient adults can result in osteomalacia, increased bone turnover, reduced bone mass and risk of bone fractures.[10] Low vitamin D (25-OH) concentrations are also associated with lower bone mineral density.[11] In conjunction with other clinical data, the results may be used as an aid in the assessment of bone metabolism.

So far, vitamin D has been shown to affect expression of over 200 different genes. Insufficiency has been linked to diabetes, different forms of cancer, cardiovascular disease, autoimmune diseases and innate immunity.[2]

The Elecsys Vitamin D total assay employs a vitamin D binding protein (VDBP) as capture protein to bind vitamin D_3 (25-OH) and vitamin D_2 (25-OH).

Test principle

Competition principle. Total duration of assay: 27 minutes.

- 1st incubation: By incubating the sample (15 µL) with pretreatment reagent 1 and 2, bound vitamin D (25-OH) is released from the vitamin D binding protein.
- 2nd incubation: By incubating the pretreated sample with the ruthenium labeled vitamin D binding protein, a complex between the vitamin D (25-OH) and the ruthenylated vitamin D binding protein is formed.
- 3rd incubation: After addition of streptavidin-coated microparticles and vitamin D (25-OH) labeled with biotin, unbound ruthenium labeled vitamin D binding proteins become occupied. A complex consisting of the ruthenylated vitamin D binding protein and the biotinylated vitamin D (25-OH) is formed and becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Reagents - working solutions

The reagent rackpack (M, R1, R2) and the pretreatment reagents (PT1, PT2) are labeled as VITD-T.

PT1 Pretreatment reagent 1 (white cap), 1 bottle, 4 mL:
Dithiothreitol 1 g/L, pH 5.5.

PT2 Pretreatment reagent 2 (gray cap), 1 bottle, 4 mL:
Sodium hydroxide 55 g/L.

M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL:
Streptavidin-coated microparticles 0.72 mg/mL; preservative.

R1 Vitamin D binding protein-BPRu (gray cap), 1 bottle, 9 mL:
Ruthenium labeled vitamin D binding protein 150 µg/L; bis-tris propane buffer 200 mmol/L; albumin (human) 25 g/L; pH 7.5; preservative.

R2 25-hydroxyvitamin D–biotin (black cap), 1 bottle, 8.5 mL:
Biotinylated vitamin D (25-OH) 14 µg/L; bis-tris propane buffer 200 mmol/L; pH 8.6; preservative.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

This kit contains components classified as follows in accordance with the Regulation (EC) No. 1272/2008:

Danger

H290 May be corrosive to metals.

H314 Causes severe skin burns and eye damage.

Prevention:

P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.

Response:

Appendix 13: Measurement of the concentration of calcium

EN

CALCIUM

981367 6 x 60 ml (Konelab)
981772 8 x 20 ml (Konelab, Indiko)

THIS PACKAGE INSERT IS APPLICABLE FOR USE OUTSIDE THE US.

INTENDED USE
For *in vitro* diagnostic use in the quantitative determination of the calcium concentration in human serum, plasma or urine on Konelab and Indiko analyzers.
Any reference to the Konelab systems also refers to the T Series.

SUMMARY (1, 2)
Calcium is found mainly in the skeleton, with small amounts in soft tissues and extracellular fluid. In blood, approximately 50% of the plasma calcium is free, 40% is protein-bound, and 10% is complexed. About 80% of protein-bound calcium is associated with albumin and the remaining 20% is associated with globulins.
Extracellular calcium provides calcium ion for maintenance of intracellular calcium, bone mineralization, blood coagulation, and maintenance of plasma membrane potential. A decrease in the serum free calcium concentration causes increased neuromuscular excitability. Calcium is also important in muscle contraction and a second messenger affecting enzyme activity and hormone secretion.
High serum values of calcium (hypercalcemia) are due to an increased flux of calcium into the extracellular fluid compartment from the skeleton, intestine, or kidney. Primary hyperparathyroidism is the most common cause in outpatient clinics, whereas malignancy is the most common cause in hospitalized patients.
Low total serum calcium (hypocalcemia) may be due to either a reduction in the albumin-bound or free fraction of calcium. Hypoalbuminemia is the most common cause of hypocalcemia. The serum calcium level may be also low due to magnesium or vitamin D deficiency, hypoparathyroidism or acute pancreatitis.
The amount of calcium excreted into the urine reflects intestinal absorption, skeletal resorption, and renal tubular filtration and reabsorption.

PRINCIPLE OF THE PROCEDURE
Calcium ions form a highly coloured complex with Arsenazo III at neutral pH. The amount of the complex is measured at 660 nm (3).

REAGENT INFORMATION

R2 code 981367 contains	R2 code 981772 contains
6 vials of 60 ml Reagent	8 vials of 20 ml Reagent

Concentrations

Arsenazo III	0.2 mmol/l
Imidazole Buffer	100 mmol/l
Surfactant	
Stabilizers	
pH 6.75	

Precautions
For *in vitro* diagnostic use only. Exercise the normal precautions required for handling all laboratory reagents.
Arsenazo III is a toxic compound. Avoid contact with skin, eyes or mucous membranes.

Preparation
The reagent is ready for use.
Note: Check that there are no bubbles in the bottleneck or on the surface of the reagent when you insert the reagent vials or vessels in the analyzer.

Storage and Stability
Reagent in unopened vials is stable at 2…25 °C until the expiration date printed on the label.
Keep away from sunlight.
Refer to the Application Notes of your analyzer for the on board stability of reagents.

SPECIMEN COLLECTION
Note: When processing samples in sample collection tubes, follow the instructions of the tube manufacturer carefully to avoid erroneous results. Pay special attention to the preanalytical variables such as mixing, standing time before centrifugation and centrifuge settings.

Sample Type
Use serum, Li-heparin plasma or urine.
Plasma gives approximately 2% lower values. Do not use citrate, oxalate or EDTA because they remove calcium by complex formation. Any substance which either chelates or contains calcium will interfere with the test.
Urine samples should be acidified to prevent calcium salt precipitation. 24-hour specimens may be collected in 20 to 50 mL of 6 N HCl. Random specimens may be acidified with 1 – 2 mL of 6 N HCl (2).

Precautions
Human samples should be handled and disposed of as if they were potentially infectious.

Storage (4)
The samples can be stored as shown below.

Serum and plasma	7 days at 20…25 °C, 3 weeks at 4…8 °C, or 6 months at -20 °C
Urine	2 days at 20…25 °C, 4 days at 4…8 °C, or 3 weeks at -20 °C

TEST PROCEDURE
Refer to the Application Notes for an automated procedure on your analyzer. Any application which has not been validated by Thermo Fisher Scientific Oy cannot be performance guaranteed and therefore must be evaluated by the user.
Note: Because of the need to obtain highly accurate calcium results, it is recommended to perform assay in duplicate and with manual acceptance.

Indiko Instrument settings
Enter the application parameters via barcode found in the Indiko application sheet or via electronical file as appropriate.

Materials provided
Reagent as described above.

Materials required but not provided
Calibrators and controls as indicated below.

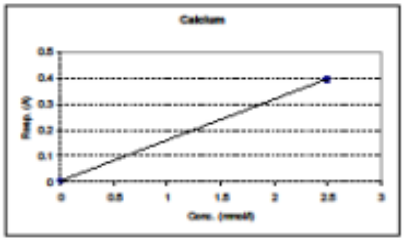
Calibration
Use sCal, code 981831, according to the instructions provided for your analyzer.

Traceability
Refer to the package insert of sCal.

Quality Control
Use quality control (QC) samples at least once a day, after each calibration and every time a new bottle of reagent is used. It is recommended to use at least two levels (low and high) of controls or sample pools. Always follow the local, state and federal regulations in performing QC.
Available serum/plasma controls:
Nortrol, code 981043
Abtrol, code 981044
Available urine controls:
u1Trol, code: 981821
u1Trol High, code: 981822
The control intervals and limits must be adapted to the individual laboratory requirements.
The results of the quality control samples should fall within the limits pre-set by the laboratory.

CALCULATION OF RESULTS
The results are calculated automatically by the analyzer using a calibration curve.

Calibration Curve (example)



The calibration curve is lot-dependent.

LIMITATIONS OF THE PROCEDURE (7)
For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Interference
For Konelab and Indiko, a study was performed using the CLSI Document EP7-A2 as a guideline.

Criterion: Difference within ± 10% or ± 0.2 mmol/l of initial values.

Konelab: serum/plasma

Interfering Substance	Interferent Concentration	Target Ca (mmol/l)	Difference from Target
Conjugated Bilirubin	1000 µmol/l (58.5 mg/dl)	1.19	-0.5 %
	1000 µmol/l (58.5 mg/dl)	1.90	-2.3 %
	1000 µmol/l (58.5 mg/dl)	2.80	+2.5 %
Unconjugated Bilirubin	1000 µmol/l (58.5 mg/dl)	1.18	-0.18 mmol/l
	1000 µmol/l (58.5 mg/dl)	1.84	-0.5 %
	1000 µmol/l (58.5 mg/dl)	2.82	+1.8 %
Hemoglobin in hemolysate	10 g/l	1.22	-0.6 %
	10 g/l	1.94	+0.2 %
	10 g/l	2.88	+0.7 %
Lipemic Intralipid® (trademark of Fresenius Kabi AB)	10 g/l	1.21	+0.18 mmol/l
	10 g/l	1.96	+0.2 %
	10 g/l	2.96	+4.8 %

Indiko: serum/plasma

Interfering Substance	Interferent Concentration	Target Ca (mmol/l)	Difference from Target
Conjugated Bilirubin	1010 µmol/l (59 mg/dl)	1.65	+0.6%
	1030 µmol/l (60 mg/dl)	3.13	+1.3%
Unconjugated Bilirubin	1100 µmol/l (64 mg/dl)	1.68	-0.2%
	1110 µmol/l (65 mg/dl)	3.21	+1.3%
Hemoglobin in hemolysate	10.9 g/l	1.66	-0.1%
	11.3 g/l	3.01	+0.4%
Lipemic Intralipid® (trademark of Fresenius Kabi AB)	10 g/l	1.73	+0.19 mmol/l
	10 g/l	3.29	+4.2%

Urine

Interfering Substance	Interferent Concentration	Target Ca (mmol/l)	Difference from Target (%)
Ascorbic Acid	200 mg/dl	1.69	-2.5
	400 mg/dl	2.97	-1.3
Glucose	1000 mg/dl	1.70	-0.2
	1000 mg/dl	3.03	-1.2
Salicylate	500 mg/dl	1.68	-1.3
	500 mg/dl	2.96	-2.9
Acetaminophen	20 mg/dl	1.69	-0.3
	20 mg/dl	3.01	-0.9

Copper may interfere with this method (3). Therefore, care should be taken to avoid contamination with reagents that contain high concentration of copper such as Biuret reagent (Total Protein reagent).
There is a poor correlation between turbidity and concentration of triglycerides.
In very rare cases gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results (6). Not tested at Thermo Fisher Scientific Oy.
For other interfering substances, please refer to the reference (5).

EXPECTED VALUES (1)
Serum/Plasma Adults: 2.15 - 2.57 mmol/l (8.6 - 10.3 mg/dl)
Urine 2.50 - 7.50 mmol/24h (100 - 300 mg/24h) with normal food intake

The quoted values should serve as a guide only. It is recommended that each laboratory verify this range or derives a reference interval for the population that it serves.

Thermo

Appendix 14: Measurement of the concentration of albumin

EN

Konelab™ / T Series
ALBUMIN (BCP)

REF 981357 5 x 60 ml
981766 7 x 20 ml

THIS PACKAGE INSERT IS APPLICABLE FOR USE OUTSIDE THE US. ANY REFERENCE TO THE KONELAB SYSTEMS ALSO REFERS TO THE T SERIES.

INTENDED USE
For *in vitro* diagnostic use in the quantitative determination of the albumin in human serum or plasma on Konelab analyzers. All test results must be interpreted with regard to the clinical context.

SUMMARY (1)
Normally, albumin is the most abundant protein found in plasma from midgestation until death, accounting for approximately one-half the plasma protein mass. It has no carbohydrate side chains but is highly soluble in water due to its high net negative charge at physiological pH.
Albumin is synthesized by the hepatic parenchymal cells. Its synthetic rate is controlled primarily by colloidal osmotic pressure (COP) and secondarily by protein intake. The normal plasma half-life of albumin is 15 to 19 days. Its primary function is the maintenance of COP in both the vascular and extravascular spaces. It also binds and transports a large number of compounds.
Increased levels of albumin are present only in acute dehydration and have no clinical significance. Decreased levels are seen in a multitude of clinical conditions, like analbuminemia, inflammation, hepatic disease, urinary loss, gastrointestinal loss, protein energy malnutrition, edema and ascites.

PRINCIPLE OF THE PROCEDURE
When albumin reacts with a specific dye, bromocresol purple (BCP), a coloured product is formed. This test is based on the measurement of the formed colour intensity at 600 nm. (2, 3)
The method is suited for human samples only.

REAGENT INFORMATION
Kit code 981357 contains
5 vials of 60 ml Reagent

Kit code 981766 contains
7 vials of 20 ml Reagent

Concentration

Bromocresol purple (BCP)	60 µmol/l
Acetate buffer, pH 5.2	100 mmol/l
NaN_3	< 0.1 %

Precautions
For *in vitro* diagnostic use only. Exercise the normal precautions required for handling all laboratory reagents.
The reagent contains sodium azide as preservative. Do not swallow. Avoid contact with skin and mucous membranes.

Preparation
The reagent is ready for use.
Note: Check that there are no bubbles in the bottleneck or on the surface of the reagent when you insert the reagent vials or vessels in the Konelab analyzer.

Storage and Stability
Reagents in unopened vials are stable at 2…8 °C until the expiration date printed on the label. Keep away from sunlight.
Refer to the Application Notes of your Konelab analyzer for the on board stability of reagents.

SPECIMEN COLLECTION

Sample Type
Serum or heparin plasma can be used.

Precautions
Human samples should be handled and disposed of as if they were potentially infectious.

Storage (4)
The sample can be stored at least for 7 days at 4…8 °C or for 3 months at –20 °C.

TEST PROCEDURE
Refer to the Reference Manual and Application Notes for an automated procedure on your Konelab analyzer. Any application which has not been validated by Thermo Fisher Scientific Oy cannot be performance guaranteed and therefore must be evaluated by the user.

Materials provided
Reagent as described above.

Materials required but not provided
Controls as indicated below.

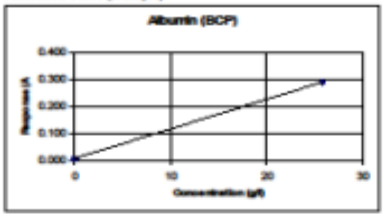
Calibration
Use eCal (981830) according to the instructions given to your Konelab analyzer.

Traceability:
Refer to the package insert of eCal.

Quality Control
Use quality control samples at least once a day and after each calibration and every time a new bottle of reagent is used. It is recommended to use at least two levels (low and high) of controls or sample pools. Always follow the local, state and federal regulations in performing QC.
Available controls:
Normal, code 981043
Abnol, code 981044
The Control intervals and limits must be adapted to the individual laboratory requirements. The results of the quality control samples should fall within the limits pre-set by the laboratory.

CALCULATION OF RESULTS
The results are calculated automatically by the Konelab analyzer using a calibration curve.

Calibration Curve (example)



Konelab 20/30/60. The calibration curve is lot dependent.

LIMITATIONS OF THE PROCEDURE
For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Interference
Criterion: Recovery within ± 10% of initial values.
Bilirubin: No interference found up to 1000 µmol/l (58 mg/dl).
Hemolysate: No interference found up to 10 g/l of hemoglobin.
Lipemia: No interference found up to 6 g/l of Intralipid® (trademark of Fresenius Kabi AB). There is a poor correlation between turbidity and triglycerides concentration.
For other interfering substances, please refer to the reference 5.

In very rare cases gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results. (5). Not tested in Thermo Fisher Scientific Oy.

EXPECTED VALUES (1)
35 – 52 g/l (3.5 – 5.2 g/dl)
The quoted values should serve as a guide only. It is recommended that each laboratory verify this range or derives a reference interval for the population that it serves.

MEASURING RANGE
4 - 60 g/l (0.4 – 6.0 g/dl)
Extended measuring range after secondary dilution:
4 – 180 g/l (0.4 – 18.0 g/dl).

PERFORMANCE CHARACTERISTICS
The results obtained in individual laboratories may differ from the given performance data.

Detection limit
2 g/l (0.2 g/dl)
The detection limit represents the lowest measurable concentration/activity that can be distinguished from zero. It is calculated as the concentration of zero sample + 3 SD (within run, n=24).

Imprecision (result unit g/l)

	Mean 22 g/l		Mean 40 g/l	
	SD	CV%	SD	CV%
Within run	0.4	1.9	0.4	1.0
Between day	0.7	3.0	0.9	2.3

A precision study was performed using Konelab 60 during 17 days, with the number of measurements being at least n = 100.

Method comparison
A comparison study was performed using the CLSI Document EP9-A as a guideline and using commercially available immunoturbidimetric method as a reference.
Linear regression (result unit g/l):

$y = 0.99 x + 3$
$r = 0.987$
$n = 110$

The sample concentrations were between 20 and 81 g/l.

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Date of revision (yyyy-mm-dd)
2009-11-03

Changes from previous version
The line beside the text indicates changes.

CE

Thermo

Appendix 15: Measurement of the concentration of blood glucose kit

EN

GLUCOSE (GOD-POD)

981379 10 x 60 ml (Konelab)
981780 12 x 20 ml (Konelab, Indiko)

THIS PACKAGE INSERT IS APPLICABLE FOR USE OUTSIDE THE US.

INTENDED USE
For *in vitro* diagnostic use in the quantitative/qualitative determination of glucose concentration in human serum or plasma on Konelab and Indiko analyzers.
Any reference to the Konelab systems also refers to the T series.

SUMMARY (1, 2)
Glucose is the primary energy source for the human body. It is derived from the breakdown of carbohydrates in the diet and in body stores. When energy intake exceeds expenditure, the excess is converted to fat and glycogen for storage in adipose tissue and liver or muscle. When energy expenditure exceeds calorie intake, endogenous glucose formation occurs from the breakdown of carbohydrate stores and from noncarbohydrate sources.
The glucose level in the blood is maintained within a narrow range under diverse condition by regulatory hormones such as insulin, glucagon or epinephrine. The most frequently encountered disorder of carbohydrate metabolism is high blood glucose due to diabetes mellitus. Other applications are the detection of hypoglycemia in the neonate, in childhood, and due to extrapancreatic tumors.

PRINCIPLE OF THE PROCEDURE
This method employs glucose oxidase (GOD) and a modified Trinder colour reaction, catalysed by the enzyme peroxidase (POD). Glucose is oxidised to D-gluconate by glucose oxidase with the formation of an equimolar amount of hydrogen peroxide. In the presence of peroxidase, 4-aminoantipyrine and phenol are oxidatively coupled by hydrogen peroxide to form a quinoneimine dye, coloured in red. The intensity of colour in the reaction is measured at 510 nm and it is proportional to the glucose concentration in the sample.

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$2\,H_2O_2 + \text{4-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + 4\,H_2O$$

REAGENT INFORMATION

Kit code 981379 contains	Kit code 981780 contains
10 vials of 60 ml	12 vials of 20 ml

Concentrations

Phosphate buffer (pH 7.5)	250 mmol/l
Phenol	5 mmol/l
4-aminoantipyrine	0.5 mmol/l
Glucose oxidase	≥ 15 kU/l
Peroxidase	≥ 1 kU/l
NaN_3	< 0.1%

Precautions
For *in vitro* diagnostic use only. Exercise the normal precautions required for handling all laboratory reagents.
The reagent contains sodium azide as preservative. Do not swallow. Avoid contact with skin and mucous membranes.

Preparation
The reagent is ready for use.
Note: Check that there are no bubbles in the bottleneck or on the surface of the reagent when you insert the reagent vials or vessels in the analyzer.

Storage and Stability
Reagents in unopened vials are stable at 2…8 °C until the expiration date printed on the label.
Keep away from sunlight.
Refer to the Application Notes of your analyzer for the on board stability of reagents.

SPECIMEN COLLECTION
Note: When processing samples in sample collection tubes, follow the instructions of the tube manufacturer carefully to avoid erroneous results. Pay special attention to the preanalytical variables such as mixing, standing time before centrifugation and centrifuge settings.

Sample Type
Konelab Unhemolyzed serum, heparin or EDTA plasma can be used.
Indiko Unhemolyzed serum or heparin plasma can be used.
The sample should be separated from the cells as soon as possible after collection in order to avoid glycolysis. If the sample is not separated or analyzed without delay a glycolytic inhibitor should be used (3).

Precautions
Human samples should be handled and disposed of as if they were potentially infectious.

Storage
The samples can be stored as shown below.

Separated, nonhemolyzed sterile sample	8 hours at 25 °C or 3 days at 4 °C (1)
Stabilized sample	1 day at 20…25 °C or 7 days at 4…8 °C (4)

TEST PROCEDURE
Refer to the Application Notes for an automated procedure on your analyzer. Any application which has not been validated by Thermo Fisher Scientific Oy cannot be performance guaranteed and therefore must be evaluated by the user.

Indiko Instrument settings
Enter the application parameters via barcode found in the Indiko application sheet or via electronical file as appropriate.

Materials provided
Reagent as described above.

Materials required but not provided
Calibrator and controls as indicated below.

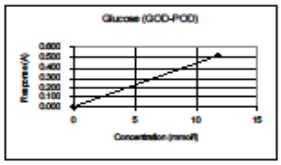
Calibration
Use sCal, code 981831, according to the instructions provided for your analyzer.

Traceability
Refer to the package insert of sCal.

Quality Control
Use quality control (QC) samples at least once a day and after each calibration and every time a new bottle of reagent is used. It is recommended to use at least two levels (low and high) of controls or sample pools. Always follow the local, state and federal regulations in performing QC.
Available serum/plasma controls:
Nortrol, code: 981043
Abtrol, code: 981044
The Control intervals and limits must be adapted to the individual laboratory requirements. The results of the quality control samples should fall within the limits pre-set by the laboratory.

CALCULATION OF RESULTS
The results are calculated automatically by the analyzer using a calibration curve.

Calibration Curve (example)



The calibration curve is lot-dependent.

LIMITATIONS OF THE PROCEDURE (7)
For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Interference
Criterion: Difference ± 5% of initial values.

Konelab

Interfering Substance	Interferent Concentration	Target Glucose (mmol/l)	Difference from Target (%)
Unconjugated Bilirubin	104 µmol/l 120 µmol/l 245 µmol/l	4.17 6.42 14.50	- 5.0 - 5.0 - 5.0
Hemoglobin in hemolysate	12 g/l	15.4	+ 1.9
Lipemia: Intralipid® (trademark of Fresenius Kabi AB)	3 g/l	15.5	+ 4.1

For Indiko, a study was performed using the CLSI document EP7-A2 as a guideline.

Indiko

Interfering Substance	Interferent Concentration	Target Glucose (mmol/l)	Difference from Target (%)
Conjugated Bilirubin	80 µmol/l (5 mg/dl) 120 µmol/l (7 mg/dl) 250 µmol/l (15 mg/dl)	4.3 6.3 15.3	- 5.0 - 5.0 - 5.0
Unconjugated Bilirubin	120 µmol/l (7 mg/dl) 230 µmol/l (13 mg/dl) 190 µmol/l (11 mg/dl)	4.3 6.1 15.4	- 5.0 - 5.0 - 5.0
Hemoglobin in hemolysate	4 g/l 9 g/l 11 g/l	4.2 6.1 14.7	+ 5.0 + 5.0 + 3.7
Lipemia: Intralipid® (trademark of Fresenius Kabi AB)	1 g/l 1 g/l 3 g/l	4.3 6.0 14.9	+ 5.0 + 5.0 + 5.0

There is a poor correlation between turbidity and triglycerides concentration.
In very rare cases gammopathy, in particular type IgM (Waldenström's macro-globulinemia), may cause unreliable results (5). Not tested at Thermo Fisher Scientific Oy.
For other interfering substances, please refer to the reference (5).

EXPECTED VALUES (1)
Serum (fasting) Adults 4.1 - 5.9 mmol/l (74 - 106 mg/dl)
The quoted values should serve as a guide only. It is recommended that each laboratory verify this range or derives a reference interval for the population that it serves.

Conversion factor
mmol/l x 18.01 → mg/dl

MEASURING RANGE
0.3 – 20.0 mmol/l (5 – 360 mg/dl)
Extended measuring range after secondary dilution: 0.3 - 60.0 mmol/l (5 - 1080 mg/dl).

PERFORMANCE CHARACTERISTICS (7)
The results obtained in individual laboratories may differ from the given performance data.

Limit of Detection
Konelab 0.1 mmol/l (2 mg/dl)
The detection limit represents the lowest measurable concentration/activity that can be distinguished from zero. It is calculated as the concentration of zero sample + 3 SD (within run, n = 24).
Indiko LoB: 0.01 mmol/l (0.18 mg/dl)
Limit of Blank (LoB) represents the highest measurement result that is likely to be observed for an analyte-free sample (n = 96).
LoD: 0.02 mmol/l (0.36 mg/dl)
Limit of Detection (LoD) represents the lowest amount of analyte in sample that can be detected (n = 60).
LoQ: 0.02 mmol/l (0.36 mg/dl)
Limit of Quantitation (LoQ) represents the lowest actual concentration in a sample that can be quantitatively determined (n = 60).
Note: For Indiko, a study was performed using the CLSI Document EP17-A as a guideline.

Imprecision
Imprecision of the glucose assay is ≤ 5.0% Total CV.

Thermo

Appendix 16: Measurement of the concentration of glycated haemoglobin

Director Approval _____ Date _____

UCSF Medical Center UCSF Benioff Children's Hospital **Clinical Laboratories**	**Point of Care Testing**

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 immunoagglutination 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 thiocyan-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

re_12017547122V14.0

Insulin

cobas®

Insulin

REF	Σ	SYSTEM
		Elecsys 2010
		MODULAR ANALYTICS E170
12017547 122	100	**cobas e** 411
		cobas e 601
		cobas e 602

English

Intended use

Immunoassay for the in vitro quantitative determination of human insulin in human serum and plasma. The determination of insulin is utilized in the diagnosis and therapy of various disorders of carbohydrate metabolism, including diabetes mellitus and hypoglycemia.

The electrochemiluminescence immunoassay "ECLIA" is intended for use on Elecsys and **cobas e** immunoassay analyzers.

Summary

Insulin is a peptide hormone with a molecular weight of approximately 6000 daltons. It is secreted by the B-cells of the pancreas and passes into circulation via the portal vein and the liver. Insulin is generally released in pulses, with the parallel glucose cycle normally about 2 minutes ahead of the insulin cycle.[1]

The insulin molecule consists of two polypeptide chains, the α-chain with 21 and the β-chain with 30 amino acids. Biosynthesis of the hormone takes place in the β-cells of the islets of Langerhans in the form of single-chain preproinsulin, which is immediately cleaved to give proinsulin. Specific proteases cleave proinsulin to insulin and C-peptide which pass into the bloodstream simultaneously. About half of the insulin, but virtually none of the C-peptide, is retained in the liver. Circulating insulin has a half-life of 3-5 minutes and is preferentially degraded in the liver, whereas inactivation or excretion of proinsulin and C-peptide mainly takes place in the kidneys.

The amino acid sequence of insulin has remained surprisingly constant during evolution, with the result that prior to the development of genetically engineered human insulin it was possible to successfully use porcine or bovine insulin in the therapy of diabetes mellitus.[2]

The action of insulin is mediated by specific receptors and primarily consists of facilitation of the uptake of sugar by the cells of the liver, fatty tissue and musculature; this is the basis of its hypoglycemic action.

Serum insulin determinations are mainly performed on patients with symptoms of hypoglycemia. They are used to ascertain the glucose/insulin quotients and for clarification of questions concerning insulin secretion, e.g. in the tolbutamide test and glucagon test or in the evaluation of oral glucose tolerance tests or hunger provocation tests.

Although the adequacy of pancreatic insulin synthesis is frequently assessed via the determination of C-peptide, it is still generally necessary to determine insulin. For example, therapeutic administration of insulins of non-human origin can lead to the formation of anti-insulin antibodies. In this case, measurement of the concentration of serum insulin shows the quantity of free - and hence biologically active - hormone, whereas the determination of C-peptide provides a measure of the patient's total endogenous insulin secretion.[3,4,5]

A disorder in insulin metabolism leads to massive influencing of a number of metabolic processes. A too low concentration of free, biologically active insulin can lead to the development of diabetes mellitus. Possible causes of this include destruction of the β-cells (type I diabetes), reduced activity of the insulin or reduced pancreatic synthesis (type II), circulating antibodies to insulin, delayed release of insulin or the absence (or inadequacy) of insulin receptors.

On the other hand, autonomous, non-regulated insulin secretion is generally the cause of hypoglycemia. This condition is brought about by inhibition of gluconeogenesis, e.g. as a result of severe hepatic or renal failure, islet cell adenoma, or carcinoma. Hypoglycemia can, however, also be facilitated intentionally or unintentionally (factitious hypoglycemia).

In 3 % of persons with reduced glucose tolerance, the metabolic state deteriorates towards diabetes mellitus over a period of time. Reduced glucose tolerance during pregnancy always requires treatment. The clearly elevated risk of mortality for the fetus necessitates intensive monitoring.

The Elecsys insulin assay employs two monoclonal antibodies which together are specific for human insulin.

Test principle

Sandwich principle. Total duration of assay: 18 minutes.

- 1st incubation: Insulin from 20 µL sample, a biotinylated monoclonal insulin-specific antibody, and a monoclonal insulin-specific antibody labeled with a ruthenium complex[a)] form a sandwich complex.
- 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

a) Tris(2,2'-bipyridyl)ruthenium(II)-complex ($Ru(bpy)_3^{2+}$)

Reagents - working solutions

The reagent rackpack is labeled as INSULIN.

M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL:
Streptavidin-coated microparticles 0.72 mg/mL; preservative.

R1 Anti-insulin-Ab–biotin (gray cap), 1 bottle, 10 mL:
Biotinylated monoclonal anti-insulin antibody (mouse) 1 mg/L; MES[b)] buffer 50 mmol/L, pH 6.0; preservative.

R2 Anti-insulin-Ab–$Ru(bpy)_3^{2+}$ (black cap), 1 bottle, 10 mL:
Monoclonal anti-insulin antibody (mouse) labeled with ruthenium complex 1.75 mg/L; MES buffer 50 mmol/L, pH 6.0; preservative.

b) MES = 2-morpholino-ethane sulfonic acid

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

Avoid foam formation in all reagents and sample types (specimens, calibrators and controls).

Reagent handling

The reagents in the kit have been assembled into a ready-for-use unit that cannot be separated.

All information required for correct operation is read in from the respective reagent barcodes.

Storage and stability

Store at 2-8 °C.

Do not freeze.

Store the Elecsys reagent kit **upright** in order to ensure complete availability of the microparticles during automatic mixing prior to use.

Stability:	
unopened at 2-8 °C	up to the stated expiration date
after opening at 2-8 °C	12 weeks
on the analyzers	4 weeks

Appendix 18: Measurement of the concentration of cholesterol

EN

CHOLESTEROL

981812 10 x 60 ml (Konelab)
981813 12 x 20 ml (Konelab, Indiko)

THIS PACKAGE INSERT IS APPLICABLE FOR USE OUTSIDE THE US.

INTENDED USE
For *in vitro* diagnostic use in the quantitative determination of cholesterol concentration in human serum or plasma on Konelab and Indiko analyzers.
Any reference to the Konelab systems also refers to the T Series.

SUMMARY (1, 2)
Cholesterol is found in virtually all cells and body fluids. It is the starting point in many metabolic pathways including vitamin D synthesis, steroid hormone synthesis and bile acid metabolism. Although a portion of the body's cholesterol is derived from dietary intake, most is synthesized by the liver and other tissues from simpler molecules, particularly acetate. Almost 90 % of synthesis occurs in the liver and gut; peripheral cells and other organs therefore depend largely on cholesterol delivery from the circulation. Approximately one third of the daily production of cholesterol is catabolized into bile acids. The total cholesterol concentration provides a baseline value, which indicates whether further laboratory investigations of lipoprotein metabolism should be carried out.

PRINCIPLE OF THE PROCEDURE
Cholesterol esters are enzymatically hydrolysed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol, including that originally present, is then oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide combines with HBA and 4-aminoantipyrine to form a chromophore (quinoneimine dye) which may be quantitated at 500-550 nm (3).

$$\text{Cholesterol esters} \xrightarrow{\text{CE}} \text{Cholesterol} + \text{Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CO}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{HBA} + 4\text{AAP} \xrightarrow{\text{POD}} \text{Quinoneimine Dye} + 4\,H_2O$$

CE Cholesterol Esterase
CO Cholesterol Oxidase
HBA Hydroxybenzoic Acid
4AAP 4-aminoantipyrine
POD Peroxidase

REAGENT INFORMATION

Kit code 981812 contains	Kit code 981813 contains
10 vials of 60 ml Reagent	12 vials of 20 ml Reagent

Concentrations

Cholesterol oxidase (microbial)	> 200 U/l
Cholesterol esterase (microbial)	> 500 U/l
Peroxidase (horseradish)	> 300 U/l
4-Aminoantipyrine	0.75 mmol/l
HBA	19 mmol/l
Norgo (= 3-(N-Morpholino) propanesulfonic acid) Buffer	50 mmol/l
Surfactants	
pH 6.7	

Precautions
For *in vitro* diagnostic use only.
Exercise the normal precautions required for handling all laboratory reagents.

Preparation
Reagent is ready for use.
Note: Check that there are no bubbles in the bottleneck or on the surface of the reagent when you insert the reagent vials or vessels in the analyzer.

Storage and Stability
Reagents in unopened vials are stable at 2…8 °C until the expiration date printed on the label. Refer to the Application Notes of your analyzer for the on board stability of reagents.

SPECIMEN COLLECTION
Note: When processing samples in sample collection tubes, follow the instructions of the tube manufacturer carefully to avoid erroneous results. Pay special attention to the preanalytical variables such as mixing, standing time before centrifugation and centrifuge settings.

Sample Type
Serum or heparin plasma can be used.

Precautions
Human samples should be handled and disposed of as if they were potentially infectious.

Storage (4)
The samples can be stored as shown below.

Sample	7 days at 20…25 °C or at 4…8 °C, or 3 months at -20 °C

TEST PROCEDURE
Refer to the Application Notes for an automated procedure on your analyzer. Any application which has not been validated by Thermo Fisher Scientific Oy cannot be performance guaranteed and therefore must be evaluated by the user.

Indiko instrument settings
Enter the application parameters via barcode found in the Indiko application sheet or via electronical file as appropriate.

Materials provided
Reagents as described above.

Materials required but not provided
Calibrator and controls as indicated below.

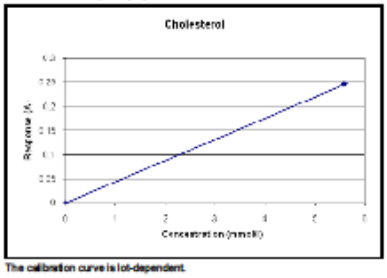
Calibration
Use sCal, code 981831, according to the instructions provided for your analyzer.

Traceability
Refer to the package insert of sCal.

Quality Control
Use quality control (QC) samples at least once a day and after each calibration and every time a new bottle of reagent is used. It is recommended to use at least two levels (low and high) of controls or sample pools. Always follow the local, state and federal regulations in performing QC.
Available controls:
Lipotrol, code: 981653
Nortrol, code 981043
Abtrol, code 981044
The control intervals and limits must be adapted to the individual laboratory requirements.
The results of the quality control samples should fall within the limits pre-set by the laboratory.

CALCULATION OF RESULTS
The results are calculated automatically by the analyzer using a calibration curve.

Calibration Curve (example)



The calibration curve is lot-dependent.

LIMITATIONS OF THE PROCEDURE (8)
For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Interference
Criterion: Difference within ± 10% of initial values.

Konelab

Interfering Substance	Interferent Concentration	Target Cholesterol (mmol/l)	Difference from Target (%)
Unconjugated Bilirubin	100 µmol/l (5.8 mg/dl)	4.7	- 8.5
Hemoglobin in hemolysate	10 g/l	4.8	+ 8.5

No interference found up to 20 mmol/l (1770 mg/dl) of triglycerides

For Indiko, a study was performed using the CLSI document EP7-A as a guideline.

Indiko

Interfering Substance	Interferent Concentration	Target Cholesterol (mmol/l)	Difference from Target (%)
Conjugated Bilirubin	90 µmol/l (5 mg/dl) 130 µmol/l (8 mg/dl)	4.1 6.2	- 10.0 - 10.0
Unconjugated Bilirubin	100 µmol/l (6 mg/dl) 180 µmol/l (11 mg/dl)	4.2 6.3	- 10.0 - 10.0
Hemoglobin in hemolysate	9 g/l 11 g/l	4.0 6.0	+ 10.0 + 7.2
Lipemia Intralipid® (trademark of Fresenius Kabi AB)	10 g/l 10 g/l	4.0 6.0	+ 1.7 + 0.7

For other interfering substances, please refer to the reference (5).
In very rare cases gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results (7). Not tested at Thermo Fisher Scientific Oy.

EXPECTED VALUES (6)
Desirable < 5.2 mmol/l (< 200 mg/dl)
Borderline high 5.2 - 6.2 mmol/l (200 - 239 mg/dl)
High ≥ 6.2 mmol/l (≥ 240 mg/dl)
The quoted values should serve as a guide only. It is recommended that each laboratory verify this range or derives a reference interval for the population that it serves.

Conversion factor
mmol/l x 38.67 → mg/dl

MEASURING RANGE
0.2 - 15.0 mmol/l (8 - 579 mg/dl)
Extended measuring range after secondary dilution: 0.2 - 45.0 mmol/l (8 - 1737 mg/dl)

PERFORMANCE CHARACTERISTICS (8)
The results obtained in individual laboratories may differ from the given performance data.

Limit of Detection
Konelab 0.1 mmol/l (4 mg/dl)
The detection limit represents the lowest measurable concentration/activity that can be distinguished from zero. It is calculated as the concentration of zero sample + 3 SD (within run, n=24).
Indiko LoB: 0.003 mmol/l (0.1 mg/dl)
The Limit of Blank (LoB) represents the highest measurement result that is likely to be observed for an analyte-free sample (n = 60).
LoD: 0.01 mmol/l (0.4 mg/dl)
The Limit of Detection (LoD) represents the lowest amount of analyte in sample that can be detected (n = 60).
LoQ: 0.01 mmol/l (0.4 mg/dl)
The Limit of Quantitation (LoQ) represents the lowest actual concentration in a sample that can be quantitatively determined (n = 60).
Note: For Indiko, a study was performed using the CLSI Document EP17-A as a guideline.

Thermo

Appendix 19: Measurement of the concentration of HDL

EN

HDL-CHOLESTEROL Plus

981823 6 x 24 ml (Konelab, Indiko)
981824 4 x 80 ml (Konelab)

THIS PACKAGE INSERT IS APPLICABLE FOR USE OUTSIDE THE US.

INTENDED USE
For *in vitro* diagnostic use in the quantitative determination of HDL-cholesterol in human serum and Li-heparin plasma on Konelab and Indiko analyzers.
Any reference to the Konelab systems also refers to the T Series.

SUMMARY (1, 2, 3)
Cholesterol is synthesized throughout the body and is also derived from dietary intake. It is an essential component of cell membranes and lipoproteins and a precursor for the synthesis of steroid hormones and bile acids. About 25% of total serum cholesterol is transported in the high density lipoproteins (HDL) fraction.
About 50% of HDL mass is protein, 30% is phospholipid and 20 % is cholesterol. Both the liver and the intestine are involved in the production of HDL, which appears to play an important part in cholesterol efflux from tissues, by reducing the amount of cholesterol stored there.
HDL also has a role in returning cholesterol from the periphery to the liver for removal as bile acids, a process known as reverse cholesterol transport. HDL concentration is inversely associated with the incidence of coronary heart disease, and therefore the determination of HDL-cholesterol is essential for assessing coronary heart disease risk.
Several techniques are available to determine HDL-cholesterol, including ultracentrifugation, electrophoresis, HPLC and precipitation-based methods. Of these, the precipitation methods have been used routinely, which are time-consuming and are not amenable to automated analysis. Thus there is a great clinical need for developing a convenient and reliable method for measuring HDL-cholesterol in serum without any pretreatment.
This automated method uses PEG-modified enzymes and dextran sulfate. When cholesterol esterase and cholesterol oxidase enzymes are modified with PEG, they show selective catalytic activities toward lipoprotein fractions, with the reactivity increasing in the order: low-density-lipoproteins < very-low-density-lipoproteins·· chylomicron < HDL.

PRINCIPLE OF THE PROCEDURE
This test is homogeneous enzymatic colorimetric test, where in the presence of magnesium sulfate, dextran sulfate selectively forms water- soluble complexes with LDL, VLDL and chylomicrons, which are resistant to PEG modified enzymes. The cholesterol concentration of HDL-cholesterol is determined enzymatically by cholesterol oxidase coupled with PEG to the amino groups (approx. 40%).

$$\text{HDL-cholesterol esters} + H_2O \xrightarrow[\text{esterase}]{\text{PEG cholesterol}} \text{HDL- cholesterol} + \text{RCOOH}$$

$$\text{HDL-cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{PEG cholesterol}} \text{4-cholestenone} + H_2O_2$$

$$2\,H_2O_2 + \text{4-amino-antipyrine} + \text{HSDA} + H^+ + H_2O \xrightarrow[\text{pigment}]{\text{peroxidase}} \text{purple blue} + 5\,H_2O$$

REAGENT INFORMATION

Kit code 981823 contains	Kit code 981824 contains
6 vials of 15 ml Reagent A	4 vials of 60 ml Reagent A
6 vials of 5 ml Reagent B	4 vials of 20 ml Reagent B

Concentrations

Reagent A	
HEPES* buffer, pH 7.4	10.07 mmol/l
CHES**, pH 7.4	98.5 mmol/l
Dextran sulfate	1.5 g/l
Magnesium nitrate hexahydrate	≥ 11.7 mmol/l
HSDA***	0.98 mmol/l
Ascorbate oxidase (Eupenicillium sp.)	3.0 kU/l
Peroxidase	≥ 1.0 kU/l
Preservative	

Reagent B	
HEPES* buffer, pH 7.0	10.07 mmol/l
PEG cholesterol esterase	0.2 kU/l
PEG cholesterol oxidase	≥ 7.6 kU/l
Peroxidase	20 kU/l
4-Amino-antipyrine	2.48 mmol/l
Preservative	

* HEPES = 4-(2-hydroxyethyl)-1 piperazine-ethanesulfonic acid
** CHES = 2-(N-cyclohexylamino)-ethanesulfonic acid
*** HSDA = N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline

Precautions
For *in vitro* diagnostic use only. Exercise the normal precautions required for handling all laboratory reagents.

Preparation
Reagent A and B are ready for use.
The pink intrinsinc color of the reagent does not interfere with the test.
Note: Check that there are no bubbles in the bottleneck or on the surface of the reagent when you insert the reagent vials or vessels in the analyzer.

Storage and Stability
Reagents in unopened vials are stable at 2…8 °C until the expiration date printed on the label.
Refer to the Application Notes of your analyzer for the on board stability of reagents.

SPECIMEN COLLECTION
Note: When processing samples in sample collection tubes, follow the instructions of the tube manufacturer carefully to avoid erroneous results. Pay special attention to the preanalytical variables such as mixing, standing time before centrifugation and centrifuge settings.

Sample Type
Serum and Li-heparin plasma can be used. EDTA plasma causes decreased results. (4)
Fasting and non-fasting samples can be used. (4). Specimens should preferably be analyzed on the day of collection.
Samples containing precipitate should be centrifuged before performing the assay.

Precautions
Human serum and plasma samples should be handled and disposed of as if they were potentially infectious.

Storage
The samples can be stored as shown below.

Sample	4 days at 2…8 °C or 30 days at -70 °C (4)

TEST PROCEDURE
Refer to the Application Notes for an automated procedure on your analyzer. Any application which has not been validated by Thermo Fisher Scientific Oy cannot be performance guaranteed and therefore must be evaluated by the user.

Indiko instrument settings
Enter the application parameters via barcode found in the Indiko application sheet or via electronical file as appropriate.

Materials provided
Reagents as described above.

Materials required but not provided
Calibrator and controls as indicated below.

Calibration
Use HDL/LDL Calibrator, code 981857, according to the instructions given to your analyzer. For recommended calibration frequency refer to the Application Note.
Recalibration is required when reagent lots are changed and when quality control results fall outside the range used in the individual laboratory.

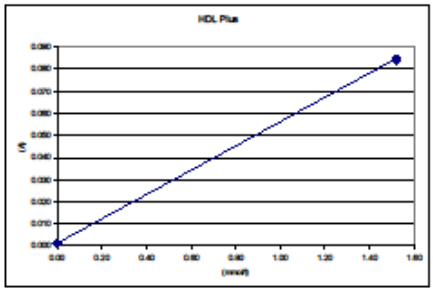
Traceability
CDC Designated Comparison method.

Quality Control
Use quality control (QC) samples at least once a day and after each calibration and every time a new bottle of reagent is used. It is recommended to use at least two levels (low and high) of controls or sample pools. Always follow the local, state and federal regulations in performing QC.
Recommended controls:
Liquitrol, code 981853
Liquitrol HDL/LDL Abnormal, code 981907

The control intervals and limits must be adapted to the individual laboratory requirements. The values should fall within the limits preset by the laboratory.

CALCULATION OF RESULTS
The results are calculated automatically by the analyzer using a calibration curve.

Calibration Curve (example)



The calibration curve is lot-dependent.

LIMITATIONS OF THE PROCEDURE (8)
For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Interference
A study was performed using the CLSI document EP7-A as a guideline.

Criterion: Difference within ± 10% of initial values.

Konelab

Interfering Substance	Interferent Concentration	Target HDL (mmol/l)	Difference from Target (%)
Conjugated Bilirubin	400 µmol/l (23 mg/dl)	0.73	- 10.0
	400 µmol/l (23 mg/dl)	1.61	- 7.4
Unconjugated Bilirubin	1000 µmol/l (58 mg/dl)	0.72	- 5.8
	1000 µmol/l (58 mg/dl)	1.54	- 1.9
Hemoglobin in hemolysate	11 g/l	0.82	+ 7.2
	11 g/l	1.61	+ 1.3
Lipemia: Intralipid® (trademark of Fresenius Kabi AB)	10 g/l	0.79	- 7.9
	10 g/l	1.66	- 6.5

Thermo

Appendix 20: Measurement of the concentration of triglycerides

EN

TRIGLYCERIDES

981301 6 x 60 ml (Konelab)
981786 12 x 20 ml (Konelab, Indiko)

THIS PACKAGE INSERT IS APPLICABLE FOR USE OUTSIDE THE US.

INTENDED USE
For *in vitro* diagnostic use in the quantitative determination of the triglycerides concentration in human serum and plasma on Konelab and Indiko analyzers.
Any reference to the Konelab systems also refers to the T Series.

SUMMARY (1, 2)
Triglycerides are esters of glycerol with 3 fatty acid esters. In human nutrition, triglycerides constitute 95 % of tissue storage fat and are predominant form of glycerol esters found in plasma. Triglycerides are digested in the duodenum and proximal ileum. Through action of lipases and bile acids, triglycerides are hydrolyzed into glycerol and fatty acids. They are trasported in the plasma bound to apolipoproteins. Triglyceride-rich lipoproteins are the chylomicrons and the very low density lipoproteins (VLDL).
The combination of raised LDL cholesterol and elevated triglyceride levels constitutes an especially high risk for coronary heart disease. Extreme hypertriglyceridemia occur mainly in chylomicronemia. In such cases values of 10 g/l (11.4 mmol/l) are measured.
Hypertriglyceridemia is a disease of adulthood, and it is often associated with diabetes mellitus, insulin resistance, obesity and hyperinsulinemia. Secondary hypertriglyceridemia occurs in diseases of many organs such as hepatopathy, nephropathy, hypothyroidism and pancreatitis.

PRINCIPLE OF THE PROCEDURE
Triglycerides are hydrolysed by lipase to glycerol and fatty-acids. The glycerol is phosphorylated to glycerol-3-phosphate, which then is oxidized to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and 4-chlorophenol forming a quinoneimine dye. The absorbance of the formed colour is measured at 510 nm.

$$\text{Triglycerides} \xrightarrow{\text{LPL}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyaceton phosphate} + H_2O_2$$

$$2\ H_2O_2 + \text{Aminoantipyrine} + \text{4-Chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + \text{HCl} + 4\ H_2O$$

REAGENT INFORMATION

Kit code 981301 contains	Kit code 981786 contains
6 vials of 60 ml Reagent	12 vials of 20 ml Reagent

Concentrations

Good's buffer, pH 7.2	50 mmol/l
4-Chlorophenol	4 mmol/l
ATP	2 mmol/l
Mg^{2+}	15 mmol/l
Glycerolkinase (GK)	≥ 400 U/l
Peroxidase (POD)	≥ 2000 U/l
Lipoprotein lipase (LPL)	≥ 2000 U/l
4-Aminoantipyrine	0.5 mmol/l
Glycerol-3-phosphateoxidase (GPO)	≥ 500 U/l
NaN$_3$	< 0.1 %

Precautions
For *in vitro* diagnostic use only. Exercise the normal precautions required for handling all laboratory reagents.
The reagent contains sodium azide as preservative. Do not swallow. Avoid contact with skin and mucous membranes.

Preparation
The reagent is ready for use.
Note: Check that there are no bubbles in the bottleneck or on the surface of the reagent when you insert the reagent vials or vessels in the analyzer.

Storage and Stability
Reagent in unopened vial is stable at 2…8 °C until the expiration date printed on the label when protected from light.
Refer to the Application Notes of your analyzer for the on board stability of reagents.

SPECIMEN COLLECTION
Note: When processing samples in sample collection tubes, follow the instructions of the tube manufacturer carefully to avoid erroneous results. Pay special attention to the preanalytical variables such as mixing, standing time before centrifugation and centrifuge settings.

Sample Type
Konelab Serum or heparin or EDTA plasma can be used.
Indiko Serum or heparin plasma can be used.
The specimen should be collected after a 10 - 14 hour fast.

Precautions
Human samples should be handled and disposed of as if they were potentially infectious.

Storage (3)
The samples can be stored as shown below.

Sample	2 days at 20…25 °C, 7 days at 4…8 °C, or years at -20 °C

TEST PROCEDURE
Refer to the Application Notes for an automated procedure on your analyzer. Any application which has not been validated by Thermo Fisher Scientific Oy cannot be performance guaranteed and therefore must be evaluated by the user.

Indiko Instrument settings
Enter the application parameters via barcode found in the Indiko application sheet or via electronical file as appropriate.

Materials provided
Reagent as described above.

Materials required but not provided
Calibrator and controls as indicated below.

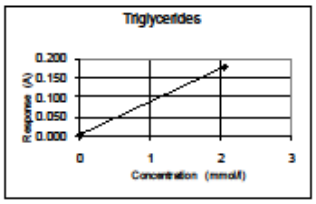
Calibration
Use sCal, code 981831, according to the instructions provided for your analyzer.

Traceability
Refer to the package insert of sCal.

Quality Control
Use quality control samples at least once a day and after each calibration and every time a new bottle of reagent is used. It is recommended to use at least two levels (low and high) of controls or sample pools. Always follow the local, state and federal regulations in performing QC.
Available controls:
Liquitrol, code: 981653
Normtrol, code 981043
Abtrol, code 981044
The control intervals and limits must be adapted to the individual laboratory requirements.
The results of the quality control samples should fall within the limits pre-set by the laboratory.

CALCULATION OF RESULTS
The results are calculated automatically by the analyzer using a calibration curve.

Calibration Curve (example)



The calibration curve is lot-dependent.

LIMITATIONS OF THE PROCEDURE (7)
For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Interference
Criterion: Difference within ± 10% of initial values.

Konelab

Interfering Substance	Interferent Concentration	Target Triglycerides (mmol/l)	Difference from Target (%)
Unconjugated Bilirubin	75 µmol/l (4.4 mg/dl)	1.17	+ 6.9
Hemoglobin in hemolysate	5 g/l	1.50	+ 4.7

For Indiko, a study was performed using the CLSI document EP7-A as a guideline.

Indiko

Interfering Substance	Interferent Concentration	Target Triglycerides (mmol/l)	Difference from Target (%)
Conjugated Bilirubin	260 µmol/l (15 mg/dl)	1.36	- 10.0
	180 µmol/l (10 mg/dl)	5.34	- 10.0
Unconjugated Bilirubin	120 µmol/l (7 mg/dl)	1.36	+ 10.0
	680 µmol/l (39 mg/dl)	5.20	+ 7.9
Hemoglobin in hemolysate	7 g/l	1.57	+ 6.8
	8 g/l	4.71	+ 1.4

For other interfering substances, please refer to the reference (4).
In very rare cases gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results (5). Not tested at Thermo Fisher Scientific Oy.

EXPECTED VALUES (5)

Normal	< 1.70 mmol/l (< 150 mg/dl)
Borderline high	1.70 - 2.25 mmol/l (150 - 199 mg/dl)
High	2.26 - 5.64 mmol/l (200 - 499 mg/dl)
Very high	≥ 5.65 mmol/l (≥ 500 mg/dl)

The quoted values should serve as a guide only. It is recommended that each laboratory verify this range or derives a reference interval for the population that it serves.

Conversion factor
mmol/l x 88.57 → mg/dl

MEASURING RANGE
0.05 - 11.00 mmol/l (4 - 973 mg/dl)
Extended measuring range after secondary dilution: 0.05 - 55.00 mmol/l (4 - 4867 mg/dl)

PERFORMANCE CHARACTERISTICS (7)
The results obtained in individual laboratories may differ from the given performance data.

Limit of Detection
Konelab 0.02 mmol/l (1.8 mg/dl)
The detection limit represents the lowest measurable concentration/activity that can be distinguished from zero. It is calculated as the concentration of zero sample + 3 SD (within run, n=24).
Indiko LoB 0.003 mmol/l (0.27 mg/dl)
Limit of Blank (LoB) represents the highest measurement result that is likely to be observed for an analyte-free sample (n = 60).
LoD: 0.02 mmol/l (1.8 mg/dl)
Limit of Detection (LoD) represents the lowest amount of analyte in sample that can be detected (n = 60).
LoQ: 0.02 mmol/l (1.8 mg/dl)
Limit of Quantitation (LoQ) represents the lowest actual concentration in a sample that can be quantitatively determined (n = 60).
Note: For Indiko, a study was performed using the CLSI Document EP17-A as a guideline.

Imprecision
The imprecision of the triglycerides assay is ≤ 4.0% Total CV.

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