# THE ROLE OF VITAMIN D AND CALCIUM SUPPLEMENTATION IN THE PATHOGENESIS OF TYPE 2 DIABETES MELLITUS

*Shahd Abdullah Alabdulkader*

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## Abstract

Mounting evidence suggests a crucial role for vitamin D in the pathogenesis of type 2 diabetes mellitus (T2DM). Our objectives were to examine the correlation between serum calcidiol and diabetes outcomes, and determine whether vitamin $D_3$ and calcium supplementation would attenuate the severity of T2DM. Eleven non-white, post-menopausal women with T2DM (age, 61 ± 11 y) were supplemented for 3 y with either placebo or 1800 IU $D_3$ + 720 mg calcium (CaD)/day. The relative change over 3 y in serum calcidiol significantly inversely correlated with the relative change in body weight, BMI, body fat (%), hip circumference, serum TC/HDL-C and serum PTH, whereas it positively correlated with serum calcium. Retrospective analysis showed differences between the CaD vs. placebo in hip circumference, serum calcidiol, serum PTH and systolic blood pressure. We conclude that modest improvements in vitamin D status may mitigate the decrement in T2DM-related sequelae in non-white, post-menopausal women.

## Dedication

I would like to dedicate this thesis to my parents: Abdullah Alabdulkader and Hussa Almulla.

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# Abbreviations

| | |
|---|---|
| 1-αOHase | 25-hydroxyvitamin $D_3$-1-αhydroxylase |
| $1,25(OH)_2D_3$ | Calcitriol |
| 25-OHase | Vitamin D-25-hydroxylase |
| $25(OH)D_3$ | Calcidiol |
| 7-DHC | 7-dehydrocholesterol |
| ALS | Amyotrophic lateral sclerosis |
| ALT | Alanine transaminase |
| AST | Aspartate transaminase |
| b-cell | Beta cells |
| BIA | Bioelectric impedance analysis |
| BMI | Body mass index |
| bw/d | Body weight per day |
| Ca | Calcium |
| CaD | Vitamin D and calcium |
| CaDDM | Calcium and vitamin D for diabetes mellitus |
| CHC | Community health centre |
| CHMS | Canadian health measure survey |
| CI | Confidence interval |
| CRP | C-reactive protein |
| CVD | Cardiovascular disease |
| DBP | Diastolic blood pressure |
| DCY | Vitamin D and calcium fortified yogurt |
| DY | Vitamin D fortified yogurt |
| FBG | Fasting blood glucose |
| FFAs | Free fatty acids |

| | |
|---|---|
| FPG | Fasting plasma glucose |
| FSG | Fasting serum glucose |
| GLUT-2 | Glucose transporters-2 |
| GLUT-4 | Glucose transporters-4 |
| GTA | Greater Toronto area |
| HbA1C | Glycated hemoglobin A1C |
| HC | Hip circumference |
| HDL | High-density lipoprotein |
| HDL-C | High-density lipoprotein cholesterol |
| HF | High fat |
| HOMA-2%S | Homeostatic model assessment of insulin sensitivity |
| HOMA-IR | Homeostatic model assessment of insulin resistance |
| HR | Hazard ratio |
| hsCRP | High sensitivity C-reactive protein |
| hsIL-6 | High sensitivity inerleukin-6 |
| hsTNF- α | High sensitivity tumor necrosis factor α |
| IGI | Insulinogenic index |
| IGT | Impaired glucose tolerance |
| IL-10 | Interleukin-10 |
| IL-1β | Interleukin-1α |
| IL-6 | Interleukin-6 |
| INF-γ | Interferon gamma |
| iNOS | Nitric oxide synthase |
| IOM | Institute of Medicine |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate |
| IU/d | International units per day |

| | |
|---|---|
| IVGTT | Intravenous glucose tolerance test |
| KATP | Potassium adenosine triphosphate |
| KIHD | Kuopio ischemic heart disease |
| LDL | Low-density lipoprotein |
| LDL-C | Low-density lipoprotein cholesterol |
| MS | Multiple sclerosis |
| MetS | Metabolic syndrome |
| NHANES | National Health and Nutrition Examination Survey |
| NO | Nitric oxide |
| OGIS | Oral glucose insulin sensitivity |
| OGTT | Oral glucose tolerance test |
| OR | Odds ratio |
| PG | Plasma glucose |
| PI3K | Phosphatidylinositol 3'-kinase |
| PTH | Parathyroid hormone |
| PY | Plain yogurt |
| QUICKI | Quantitative Insulin Sensitivity Check Index |
| RDA | Recommended dietary allowance |
| ROS | Reactive oxygen species |
| RR | Relative risk |
| RXR | Retinoid X receptor |
| SBP | Systolic blood pressure |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| SOD | Superoxide dismutase |
| T2DM | Type 2 diabetes mellitus |
| TBF | Total body fat |

| | |
|---|---|
| TC | Total cholesterol |
| TC/HDL-C | Total cholesterol HDL-C ratio |
| TG | Triglycerides |
| TNF-α | Tumor necrosis factor-alpha |
| UKPDS | United Kingdom prospective diabetes study |
| UPR | Unfolded protein response |
| UVB | Ultraviolet B rays |
| VDR | Vitamin D receptor |
| VDRE | Vitamin D response element |
| WC | Waist circumference |
| WHR | Waist-to-hip ratio |
| WHWH-CHC | Women's Health in Women's hands Community Health Centre |

# Introduction

## 1.0 Type 2 Diabetes Mellitus: Epidemiology and Background

Type 2 diabetes mellitus (T2DM) is one of the most prevalent metabolic disorders, with 171 million cases around the world (1). It is anticipated that this number will double by 2030 to 366 million (1). In Canada, it is expected that 3.7 million Canadians will suffer from diabetes by 2020, with a cost of more than 15 billion dollars (2). These numbers indicate an epidemic and call for immediate interventions. Diabetes patients also suffer from serious secondary illnesses such as coronary heart disease (CHD), blindness, kidney disease, amputation, and depression (3). The aetiology of T2DM is complex with a number of interacting genetic and environmental factors. These can be attenuated through lifestyle changes such as increasing physical activity and improving diet (4).

T2DM is characterized by altered macronutrient metabolism, specifically glucose, protein and lipids. Glucose homeostasis is a state in which plasma glucose concentrations are regulated to within normal ranges, even through periods of fasting (≤ 7.0 mmol/L) and feeding (≤ 11.1 mmol/L) (5). Normal glycaemia is mediated through two main physiological pathways: glucose production from the liver and glucose uptake by peripheral tissue, namely adipose tissue and muscle. Two key hormones regulate these physiological processes, respectively: glucagon and insulin. T2DM is diagnosed when fasting

plasma glucose (FPG) exceeds 7 mmol/L, or when plasma glucose (PG) of ≥11.1 mmol/L is detected 2h post-75 g oral glucose tolerance test (OGTT) (5). Insulin resistance (inability to respond properly to insulin) and β-cell dysfunction (inability to produce insulin) are the key defects in this endocrine disorder. Insulin resistance precedes β-cell dysfunction, whereby high circulating insulin levels are found in the plasma as a result of β-cell compensation for increased insulin demand. Nevertheless, as insulin resistance progresses β-cell function deteriorates. And by the time T2DM is diagnosed, individuals had lost approximately 80% of their β-cell function (6-7,179).

### 1.1 Risk Factors

Numerous risk factors contribute to the development of T2DM. Evidence suggests a crucial role of obesity in T2DM pathogenesis (8) as it contributes approximately 60% of total T2DM cost in the United States (9). Additionally, obesity is associated with a cluster of metabolic disorders that underlie T2DM pathogenesis such as: insulin resistance (10), hyperlipidemia (11), and inflammation (12). Other risk factors include: ethnicity (13), dietary habits, life style (14–17), and genetics (18).

The Nurses' Health Study is a prospective cohort study that examined the association between adiposity and T2DM risk in women, it included follow-up throughout 1976-1990. It demonstrated that the increase in BMI, an important

marker of adiposity, was associated with increased risk for T2DM (19). The increase in T2DM risk was more pronounced in those who had a BMI > 30 $kg/m^2$ (RR %95 CI = 27.6 [22.7 to 33.5]) and > 35 $kg/m^2$ (RR %95 CI = 93.2 [81.4 to 106.6]) as compared to those with a BMI < 29 $kg/m^2$ (19). Findings of the Nurses' Health Study were confirmed in another prospective study conducted in males (8). Waist circumference (WC), an adiposity index, was also positively correlated with insulin resistance (r = 0.88) in non-diabetic women (10). Hyperlipidemia is characterized by abnormally high lipid profile indices such as low-density lipoprotein (LDL) and total cholesterol (TC). High lipid indices correlate with obesity and contribute to T2DM pathogenesis (20).

Some ethnic backgrounds exhibit greater susceptibility to T2DM (21–24). A cross-sectional study by Goff et al. examined ethnic variation in insulin resistance and other biomarkers in three ethnic groups: South Asians, Black Africans, and white Europeans (21). Male South Asians had significantly higher HOMA-IR, homeostatic model of assessment insulin resistance; a marker of insulin resistance, than white European men by 49% (P < 0.001). Female South Asians and Black Africans showed higher HOMA-IR than white European women by 15.7% (P = 0.015) and 32.8% (P = 0.021), respectively (21). Black African women have 9.8% higher body fat (%) (P = 0.024) than South Asians, and significantly lower lipid profile indices: total cholesterol by 13%, LDL-C by 14%, triacylglycerol by 42%; than South Asian women.

Nevertheless, after adjustment for gender, age, and BMI, South Asians and Black Africans had significantly lower insulin sensitivity assessed by intra-venous glucose tolerance test (IVGTT) (21). High insulin resistance in South Asians was confirmed in another meta-analysis that examined the pathogenesis of T2DM in this group (22).

Lastly, genetic predisposition is a key risk factor in T2DM. The Finnish Twin Cohort Study examined the genetic aspects of chronic disease development, such as T2DM. Forty-one pairs of twins were included in the analysis to determine the heritability of insulin sensitivity and insulin secretion (25). IVGTT and euglycemic hyperinsulinaemic clamp were used to assess insulin secretion (first and late response) and insulin sensitivity. In monozygotic twins, insulin secretion positively correlated with heritability (first response $r = 0.55$, and late response $r = 0.66$) (25). Moreover, whole-body insulin sensitivity also positively correlated with heritability ($r = 0.46$) (25). Findings of this study confirmed the strong effect of heritability on two predominant contributors (decreased insulin secretion and insulin sensitivity) to T2DM pathogenesis.

## 1.2 Pathophysiology

T2DM is a multifaceted endocrine disorder in which alterations in different metabolic pathways and hormones contribute to the pathophysiology. Insulin resistance and β-cell dysfunction are the two main key defects in T2DM etiology.

Other underlying metabolic defects include: oxidative stress, lipotoxicity, glucotoxicity and systemic inflammation.

### 1.2.1 Insulin Resistance

Insulin, a potent anabolic hormone, is produced by the pancreatic β-cells of the islets of Langerhans. It regulates carbohydrates, protein and lipid metabolism. Insulin action is mediated through alterations in cell proliferation (26), gene expression (27), and apoptosis (28). For proper glucose uptake, multi-step insulin signaling must occur. Elevated plasma glucose concentration results in bi-phasic insulin release in an electrogenic fashion (29). It relies on two key ion channels: KATP and $Ca^{2+}$ voltage-sensitive channels. A rapid first response is initiated after the entry of glucose into the β-cells by glucose transporters-2 (GLUT-2), which is mediated by glucose-induced KATP channel inhibition causing cell depolarization. Cell membrane depolarization results in the opening of $Ca^{2+}$ voltage-sensitive channels, allowing $Ca^{2+}$ influx and insulin exocytosis (30,31).

Once insulin is released into circulation, it binds to the cell surface insulin receptor (IR) on peripheral tissues, resulting in the autophosphorylation of tyrosine β-subunits. Insulin receptor substrates (IRS1/2) are then phosphorylated, and hence activated, to act on phosphatidylinositol 3'-kinase (PI3K), an important signaling molecule. The activation of PI3K will ultimately

facilitate glucose entry into the cell through the translocation of GLUT-4 to the cell membrane (32). Insulin resistance develops when the above mechanism is altered and target peripheral tissues (muscle and adipose tissue) are unable to respond properly to insulin secretion (32). There are several proposed mechanisms underlie insulin resistance: alteration in lipid metabolism, activation of unfolded protein response (UPR), and systemic inflammation (33). Collectively, these mechanisms result in altered insulin receptor phosphorylation (an increase in serine and threonine phosphorylation and decrease in tyrosine phosphorylation) and signaling pathways (34).

### 1.2.2 β-cell Dysfunction

β-cells are an important component of the endocrine system, and play a key role in T2DM pathogenesis. Impaired β-cell function is pronounced in T2DM pathogenesis in both younger and older patients, and features high concentrations of plasma pro-insulin (an insulin precursor) (35). A recent study by Elder et al. compared β-cell preservation in T2DM adults (52.3 ± 2.8 y), healthy adolescents (14.5 ± 0.3 y) and newly T2DM-diagnosed adolescents (15.8 ± 0.5 y) (35). The disposition index, a measure of β-cell function, was significantly lower by ≈ 900% in both T2DM groups compared to healthy adolescents (35). In T2DM, β-cell function is compromised, and hence insulin secretion is reduced. The decline in β-cell function can be explained through the decrease in β-cell mass. A unique study by Butler and colleagues was

conducted on 124 human pancreata autopsies to understand the aetiology of β-cell deficit in T2DM (36). This study suggested that increased β-cell apoptosis is responsible for the observed decrease in β-cell mass. Frequency of β-cell apoptosis was significantly higher in lean diabetics by 571% compared to their BMI matched non-diabetic controls (36). In-vitro studies attribute β-cell apoptosis to the high expression of nitric oxide synthase (iNOS) and subsequent production of nitric oxide (NO) (37). NO plays an integral role in β-cell destruction, since it mediates the pro-apoptotic cytokines TNF-α, IL-1β, and INF-γ (37).

### 1.2.3 Other Underlying Metabolic Defects

Glucotoxicity, or persistent hyperglycemia, is believed to decrease insulin secretion (altered β-cell function) and insulin sensitivity (altered insulin signaling pathways) (38,39). Persistently high glucose aggravates β-cell dysfunction through: 1) the increased production of reactive oxygen species (ROS), which are deleterious at high concentrations, resulting in decreased antioxidant enzyme expression, such as that of superoxide dismutase (SOD 1 and 2) and glutathione peroxide (GPx-1) (11,38); 2) the disturbances in insulin signaling pathways that include decreased tyrosine, IRS-1 and PI3K phosphorylation, and increased serine and threonine phosphorylation (40); and 3) the activation of UPR, as an adaptive response of stressed endoplasmic reticulum (39).

Lipotoxicity: It is well-established that the accumulation of ectopic free fatty-acids, active fatty acid derivatives that are not stored in adipose tissues, contribute to insulin resistance and inflammation (41,42). The significance of insulin is not limited to its action on glucose metabolism but it is also an important hormone that regulates lipid metabolism. Lipotoxicity occurs when insulin's inhibitory effect on hormone sensitive lipase (HSL), which facilitates fat mobilization, is suppressed (43). Insulin action, signaling and receptor accessibility is highly affected by lipotoxicity (41,44).

Inflammation: Clinical evidence demonstrates increased production of inflammatory cytokines from adipose tissues, such as tumor necrosis factor (TNF- α), interleukin-6 (IL-6), and interleukin-10 (IL-10) (12).

### 1.3 Treatment and prognosis

Once an individual is diagnosed with T2DM by meeting the diagnostic criteria determined by the Canadian Diabetic Association (FPG ≥ 7.0 mmol/L, or 2h PG in a 75-g OGTT ≥ 11.1 mmol/L, or random PG ≥ 11.1 plus classic diabetes symptoms) (5), the main goal is to lower the elevated serum glucose levels (hyperglycemia) that are a hallmark in diabetic patients. This can be achieved through different mechanisms depending on the choice of pharmaceutical drugs. Insulin and oral hypoglycemic agents (i.e., metformin) are the main medical interventions for diabetic patients and are crucial for reducing

macro- and micro-vascular complications (45). Also of importance are non-medically based treatments such as exercise and improved nutrition. Nutritional- and lifestyle-based interventions demonstrate positive effect on diabetes outcome measures through attenuation of some of the underlying mechanisms, such as inflammation (inflammatory cytokines) (46,47) and dyslipidemia (lipid indices), or improving overall glycemic control (i.e. lowering glucose levels, decreasing HbA1c, and improving lipid profile) (14). Nutritional interventions aim to control carbohydrate and fat intake and distribution, increase physical activity, and promote weight loss. The aforementioned approaches resulted in a reduction of approximately 1-2% in HbA1c, an important indicator of glycemic control over the prior ~ 3 months (14), and a reduction in diabetes risk (15). In a clinical intervention, a very low-carbohydrate or a low-fat diet were assigned to obese men (mean ± SD for age 33.2 ±11.3 y, and BMI 34.3± 5.6 $kg/m^2$) for 8 weeks to study inflammation biomarkers (46). A significant decrease in absolute inflammatory biomarkers was observed in both the low-carbohydrate and low-fat groups: hsCRP (-55%, $P < 0.001$; and -48%, $P < 0.001$;respectively), hsTNF- α (-45%, $P < 0.001$; and -42%, $P < 0.001$; respectively), and hsIL-6 (-51%, $P < 0.001$; and -46%, $P < 0.001$; respectively) compared to baseline values (46). In another clinical trial, Dekker and colleagues concluded that increased physical activity results in decreased IL-6 concentration and waist circumference in obese and lean men with or without T2DM (16). Moreover, surgery- or dietary-

induced weight loss improved glycemic control (17). Diet and exercise have also shown to delay T2DM progression in subjects with impaired glucose tolerance (IGT) (48). In a meta-analysis that compared the effect of diet, exercise, and pharmacological interventions in reducing T2DM risk, diet (HR 95% CI = 0.67 [0.49 to 0.92]) and exercise (HR 95% CI = 0.49 [0.32 to 0.74]) interventions had comparable risk to that of pharmacological interventions (HR 95% CI = 0.70 [0.62 to 0.79]) (48).

## 2.0 Vitamin D

### 2.1 Vitamin D functions

Vitamin D is a fat-soluble vitamin, however its actions can be considered as those of a hormone. The traditional role of vitamin D is to maintain calcium and phosphorus homeostasis through facilitation of intestinal calcium absorption and renal reabsorption (49). The intestine is one of the main classical target organs for vitamin D action. Intestinal calcium absorption is mediated through genomic and non-genomic effects of circulating $1,25(OH)_2D_3$ (calcitriol, the most active form of vitamin D). Calbindin, a calcium binding-protein responsible for calcium transportation, is synthesized in response to increased gene expression in epithelial cells (50) as a result of vitamin D stimulation which exemplifies a genomic action of vitamin D. This is also the case in the kidneys which are another classical target organ for vitamin D (50). Nevertheless, due to the

presence of the vitamin D receptor (VDR) throughout body tissues, vitamin D function is not limited to mineral regulation and bone health, and is not limited to a few select organs (51,52). Empirical evidence suggests vitamin D plays a role in cell differentiation, cell proliferation, immunomodulation, muscle health, cardiovascular health, anti-inflammation, and fertility (52,53).

### 2.2 Vitamin D metabolism

Uniquely, vitamin D can be obtained through diet either in the form of ergocalciferol ($D_2$) from plants or from animal-based dietary sources in the form of cholecalciferol (Vitamin $D_3$). Vitamin D content in food is very limited, hence dietary sources are insufficient to fulfill vitamin D recommendations (54). Exposure to sunlight, specifically UVB rays (wavelengths ≥ 290-315 nm), is the main natural source of vitamin $D_3$ during the summer months (June- July) in the northern hemisphere (latitude ≥ 42°N) (55). Exposure to summer sunlight at noon for 15-90 minutes in northern latitudes will significantly increase serum calcidiol levels (55). Exposure to sunlight will result in photolytic conversion of 7-dehydrocholesterol (7-DHC), a precursor synthesized from cholesterol and found within the epidermal layer of the skin, to previtamin $D_3$. Subsequently, previtamin $D_3$ is isomerized by thermal induction to form vitamin $D_3$ (56,57). Vitamin $D_3$ receives two successive hydroxylations to achieve the highest degree of activation; one performed in the liver by vitamin D-25-hydroxylase (25-OHase) to form 25(OH)$D_3$ (also known as calcidiol) (58,59) and another performed in the

kidneys by 25-hydroxyvitamin $D_3$-1-αhydroxylase (1-αOHase) to form 1,25 dihydroxyvitamin $D_3$ (1,25 $(OH)_2D_3$) (59,60). The latter hydroxylation produces the most active metabolite of vitamin D, which is known as calcitriol (61); however, the former hydroxylation produces the most stable form. Furthermore, once 1,25$(OH)_2D_3$ is formed, it binds to the VDR to exert a wide-spectrum of biological effects in different body tissues that possess VDR. The production of calcitriol is not exclusive to renal production, as 1-αOHase is also found in other body organs such as colon (62), breast (61), parathyroid gland (62), and brain (63), allowing for the local production of this metabolite. Vitamin D action results in both genomic and rapid non-genomic actions (64). At the cell membrane, the binding of VDR ligands to the VDR results in rapid non-genomic actions; such as, an increase in intracellular Ca levels (65,66). In the cell, vitamin D and its metabolites bind to the VDR in the nucleus and form a heterodimer with retinoid X receptor (RXR). Consequently, the VDR-RXR complex binds to the vitamin D response element (VDRE) (67,68) and initiates a cascade of events ranging from calcium metabolism to increased gene expression (65).

### 2.3 Vitamin D deficiency

Worldwide, vitamin D deficiency is a major health risk. Initially, the recommended intake and optimal status for vitamin D were based on optimal bone health and fracture prevention (69). Generally, calcidiol levels below 50

nmol/L are considered deficient or insufficient, but not optimal (70–72). Vitamin D is involved in various metabolic pathways that underlie a number of chronic diseases (66). Higher levels of serum calcidiol levels in human and animal studies (> 75 nmol/L) improve health outcomes related to T2DM (73,74), multiple sclerosis (MS) (75), amyotrophic lateral sclerosis (ALS) (76–78), and some cancers (79,80). It is also suggested that an increase in serum calcidiol from 45 to 110 nmol/L may reduce global morality risk by approximately 20% from chronic diseases that have been linked to vitamin D deficiency (81). This is in agreement with another recent analysis of the NHANES III data which concluded that when combined with increased Mg intake serum calcidiol inversely associated with cardiovascular and colorectal mortality (82). A number of observational studies linked vitamin D deficiency to a wide range of chronic diseases, such as type 1 and 2 diabetes mellitus (83,84), and metabolic syndrome (85).

### 2.4 Vitamin D status controversy and recommended intake:

The Institute of Medicine (IOM) (71) published the most recent vitamin D Recommended Dietary Allowance (RDA) in 2010 (see Table 2). Nevertheless, to date there has been little agreement on what is considered deficient, sufficient, or optimal in terms of serum calcidiol concentrations. Suggested cut-off points for serum calcidiol status are summarized in Table 1. Difficulties arise; however, when an attempt is made to implement a general recommended intake of

vitamin D for a population with a wide range of health issues (see 'Vitamin D Deficiency'). There is, therefore, a definite need for specific recommendations for groups/subgroups with different ethnicities, ages, health/disease status and gender. Von Hurst et al concluded that optimal serum calcidiol concentrations that would reduce insulin resistance were between 80-119 nmol/L (86). In addition, serum calcidiol is affected by multiple factors: age, season, ethnicity, latitude, sun light exposure, and medical conditions (51,87).

In 2010, the Canadian Health Measure Survey (CHMS) reported vitamin D status by assessing serum calcidiol levels in 5,306 Canadians aged 6-79 y representing 28.2 million Canadians from all regions. Mean serum calcidiol was 67.7 nmol/L, with one-third with more than 75 nmol/L (87).

**Table 1:** Vitamin D status controversy (values refer to serum calcidiol concentrations) (71,72,88).

| Reference | Deficient | Sufficient | Optimal |
|---|---|---|---|
| Holick et al, 2011 | < 50 nmol/L | 52.5-72.5 nmol/L | > 72.5 nmol/L |
| Hanley et al, 2010 | < 25 nmol/L | 25-75 nmol/L | > 75 nmol/L |
| Ross et al, 2011 | < 27.5 nmol/L | 27.5-50 nmol/L | > 50 nmol/L |

**Table 2:** Vitamin D Recommended Dietary Allowance (RDA) by IOM (values are IU of vitamin D per day) (54).

| Age group | RDA |
|---|---|
| 0-1 years | 400 IU/d |
| 1-70 years | 600 IU/d |
| > 70 years | 800 IU/d |
| During pregnancy and Lactation | 600 IU/d |

### 2.4.1 Vitamin D and seasonal variation

According to CHMS, Canadians had lower concentrations of serum calcidiol in November-March than in April-October by 8.5% (87). A retrospective study conducted by Christensen et al intended to examine the variations in serum vitamin D and PTH resulting from age and seasonal changes in 1551 subjects in Western Norway. During the winter months and early spring, low serum calcidiol concentrations (25-49 nmol/L) were observed in 38% of the population while 43.7% of the population had serum calcidiol concentrations exceeding 75 nmol/L during summer months (89). Seasonal negative effect on serum calcidiol levels is confirmed in the literature (90–93) due to the insufficient UVB-radiation for dermal calcitriol production.

### 2.4.2 Vitamin D and age

7- dehydrocholestrol photolysis is the very first of multiple steps in generating calcitriol, however the production of this cholesterol derivative molecule decreases with aging (94). The cutaneous production of calcitriol is an age-dependent process that is decreased with increasing age, resulting in low levels of plasma calcitriol (94). Of interest, serum calcidiol concentration followed a U-shape pattern by age according to CHMS. Boys aged 6-11 y and male seniors 60-79 y had higher serum calcidiol (76.8 nmol/L and 70 nmol/L) than adult males aged 20-39 y (60.7 nmol/L) (87). A global meta-analysis revealed that the mean serum calcidiol levels are 17.5% lower in subjects aged > 75 y

than those who are 15-65 y (mean ± SEM: 47 ± 4.0 nmol/L vs. 57 ± 1.8 nmol/L; respectively) (95). These results coincide with those of the NHANES III whereby subjects who were 40-59 y and ≥ 60 y had lower serum calcidiol levels than those who were 20-39 y (71.7 ± 1.0 and 69.5 ± 0.9 nmol/L vs. 81.0 ± 1.1 nmol/L, respectively; $P < 0.0001$ for both) (84).

### 2.4.3 Vitamin D and Ethnicity

Ethnicity is a strong determinant of vitamin D status, and its effect on serum calcidiol concentrations has been examined in the literature in a wide range of ethnic backgrounds (96). Dark-skinned individuals have substantially lower levels of vitamin D than individuals with lighter skin, even after considering seasonal variations (93,97,98). CHMS reported that Canadians of white racial background had 27% higher calcidiol concentration than other ethnic backgrounds (87). In 126 healthy, normoglycemic individuals, whites had 47.9% higher calcidiol concentration than Asian Americans ($P = 0.0226$) (98). Harris and colleagues investigated plasma calcidiol seasonal fluctuations in two ethnic groups: white (n = 39 and age 31.7 ± 6.1 y) and Black (n = 51 and age 30.6 ± 5.9 y) women (99). During the winter (February-March) and summer (June-July) months, Black women had lower plasma calcidiol concentrations by 49.6% and 51.9% ($P < 0.005$), respectively (99). This difference is attributed to the decreased dermal production of vitamin $D_3$ in Black individuals due to increased skin pigmentation (96). Furthermore, non-hispanic Black and Mexican

Americans had significantly lower concentrations of serum calcidiol by 38.3% and 17%, respectively, than non-hispanic white ($P < 0.0001$) (84).

### 2.4.4 Vitamin D and Adiposity

Adiposity has a negative effect on vitamin D status. Studies have shown that body fat is inversely associated with serum calcidiol (90,98,100). A population-based study conducted by Snijder et al in 2005 using 453 participants aged 65 y or older found that total body fat (TBF) percentage was strongly associated with lower serum calcidiol and higher PTH levels for both men and women ($P < 0.001$) (100). In women, those in the highest serum calcidiol quartile (52.9 nmol/L) had lower total body fat (TBF 22.3%) compared to those in the lowest quartile (40.2 nmol/L; with TBF 48.2%) (100). These results coincide with another 16-week randomized clinical trial by Dong et al where 49 normotensive Black boys and girls aged 16.3 ± 1.4 y were randomly allocated to either a control (400 IU/d of vitamin $D_3$) or treatment group (2000 IU/d of vitamin $D_3$) (101). Results showed an exponential increase in vitamin D levels in the treatment group at 8 and 16 week (70.9 ± 22.0 and 85.7 ± 30.1 nmol/L, respectively) compared to baseline (33.1 ± 8.7 nmol/L) (101). TBF mass (kg) was inversely correlated with serum calcidiol in the treatment group at baseline and throughout the study (101). This strong association between adiposity and serum calcidiol might be explained by the fact that adipocytes are the main site

for vitamin D storage (102). Increased fat would decrease the amount of endogenously-produced vitamin D in the circulation.

### 2.4.5 Vitamin D and parathyroid hormone (PTH)

Parathyroid hormone (PTH) and vitamin D are extremely important regulators of calcium homeostasis. In response to low serum calcium levels, the parathyroid gland releases PTH into the circulation. Higher levels of PTH facilitates: 1) the second required hydroxylation of vitamin D in the kidneys to form calcitriol (103), 2) calcium mobilization from the bone (bone resorption) (104), and 3) calcium absorption in the intestine (105). Vitamin D and PTH have an inverse relationship (91,100).

## 3.0 Potential Mechanisms for the Effects of Vitamin D on T2DM

Vitamin D supplementation may have a significant effect on T2DM pathophysiology. This might be possible through enhancing insulin secretion via facilitation of β-cell biosynthetic capacity, improving insulin sensitivity, and reducing inflammatory response. These effects are not exclusive to the glycemic pathways but rather to multiple metabolic pathways that would indirectly attenuate T2DM severity.

Blood pressure: Blood pressure is regulated through the modulation of the renin-angiotensin system. In an animal study, mice were either fed vitamin D sufficient or deficient chow for 6 weeks, followed by a high fat (HF) diet for 8 weeks to

evaluate the effect of vitamin D deficiency on systolic and diastolic blood pressure (SBP and DBP) and atherosclerosis (106). At baseline and after 8 weeks of HF diet, vitamin D deficient mice had significantly higher SBP (by ≈ 15% and 10.7%, respectively) and DBP (by ≈ 25.9% and 7.7%, respectively) (106). Moreover, African women with low serum calcidiol levels (< 74.7 nmol/L) have higher SBP and DBP (by 9.5% and 6%, respectively) than those with sufficient serum calcidiol levels (> 74.7 nmol/L) (107).

Improving lipid profile: CVD complications in diabetic patients can be decreased through improving blood lipid profile, and lowering body weight through the suppression of PTH (108). Calcitriol suppresses macrophage cholesterol uptake and decreases foam cell formation (109). In vitamin D-deficient media, cultured macrophages of obese, hypertensive, diabetics had increased foam cell formation and increased cholesterol uptake compared to those cultured in vitamin D-supplemented media (110). Moreover, other lipid profile biomarkers (i.e. LDL-C and TGs) have negative association with serum calcidiol (85). Lastly, the consumption of vitamin D fortified yogurt (1000 IU/d) for 12 weeks resulted in significant decrease in WC and BMI ($P < 0.001$) (111).

### 3.1 Pancreatic β-cell function and insulin secretion

The vitamin D receptor has been identified in pancreatic β-cells (112). In-vitro studies suggest local production of calcitriol in pancreatic islets, through the action of 1-αOHase (113). Vitamin D facilitates the biosynthetic capacity of β-

cells through genomic modulation (114) and rapid non-genomic pathways (115). Chui et al investigated the association between vitamin D status and β-cell function in healthy, normoglycemic subjects (98). During OGTT, they found a negative correlation between calcidiol concentrations and glucose levels at fasting ($P = 0.0258$), 60 min ($P = 0.0011$), 90 min ($P = 0.0011$), and 120 min ($P = 0.0007$) (98). These findings indicate poor β-cell compensation under low calcidiol concentration, i.e. in low vitamin D status. Furthermore, vitamin D facilitates insulin secretion indirectly through intracellular calcium trafficking (115). In-vitro studies indicate that insulin secretion is a Ca-dependent process, and an acute increase in intracellular $Ca^{2+}$ induces insulin exocytosis (116) that represents the rapid non-genomic action of vitamin D. Animal studies have shown that insulin secretion is improved when vitamin D levels are normalized (114,117).

### 3.2 Insulin Resistance

Insulin resistance is a key player in the development of T2DM, and it is an important pathogenic factor underlying elevated fasting glucose levels. As insulin resistance progresses, there is an increase in the levels of free fatty acids (FFAs) due to the suppression of insulin's inhibitory effect on FFA release from the liver (118). In subjects with T2DM, plasma FFA concentrations are significantly higher by 10-70% ($P < 0.05$-$0.01$) during graded hyperinsulinemia tests than their matched controls after a 12 h overnight fast (119). Elevated

circulating FFAs decrease insulin-stimulated peripheral glucose uptake and increase hepatic glucose output into the blood (120). Furthermore, FFAs impair insulin release through their effects on pathways involved in cell signaling (121). Collectively, insulin resistance significantly contributes to the metabolic syndrome from a glycemic perspective. Moreover, it is associated with a number of metabolic abnormalities such as obesity (46) and dyslipidemia (5). Vitamin D facilitates gene transcription of the insulin receptor (IR) gene through its genomic actions (123) and up-regulates GLUT-4 translocation (124). In healthy, centrally obese men, vitamin $D_3$ supplementation (120,000 IU/d fortnightly for 6 weeks) improved insulin sensitivity calculated using the oral glucose insulin sensitivity (OGIS) in the treatment group by 138% (125).

### 3.3 Systemic Inflammation

Accumulating evidence suggests a strong association between T2DM and inflammation. Moreover, the immunomodulatory effect of vitamin D has been established. Calcitriol suppresses the expression of inflammatory cytokines through its genomic action on specific tissues (126). In a 12-week randomized clinical trial by Shab-bidar et al., subjects who were assigned to vitamin D-fortified yogurt (500 IU vitamin $D_3$ and 170 mg calcium/250 mL, twice per day) exhibited a significant decrease in TNF-α, IL-6 and CRP (45%, 89% and 51%, respectively) compared to those who were assigned plain yogurt (127). Furthermore, they had higher concentration of IL-10 (25%), an anti-inflammatory

cytokine, compared to the control group (127). A recent cross-sectional study has revealed a negative correlation between serum calcidiol concentrations and oxidative stress/inflammation markers such as oxidized LDL ($r = -0.413$, $P = 0.001$) and advanced oxidation protein products ($r = -0.475$, $P < 0.001$) in subjects with diabetes or IFG (128).

## 4.0 Literature Review

A considerable amount of literature has been published on the association between vitamin D and T2DM. The Nurse Health study conducted by Pittas and colleagues examined the relative risk for T2DM incidence (73). The lowest relative risk (RR = 0.67) for T2DM incidence was achieved when vitamin $D_3$ and calcium intake exceeded 800 IU/d and 1200 mg/d, respectively (73). This section of the thesis reviews the literature concerning the effectiveness of vitamin D repletion on glycemia, starting with epidemiological studies, through prospective and human clinical trials, and lastly, animal models.

### 4.1 Epidemiological Studies

In the Third National Health and Nutrition Examination Survey (NHANES III), Scragg et al. investigated the association between serum calcidiol and the risk of T2DM, and whether this association, if any, varies by ethnicity (84). This survey showed an inverse association between diabetes (risk factors or prevalence) and serum calcidiol in non-Hispanic whites (OR 95% CI = 0.25 [0.11

to 0.60]) and Mexican Americans (OR 95% CI = 0.17, [0.08 to 0.37]) after adjusting for age, sex, BMI, leisure activity and seasonality (84). This association was confirmed by other epidemiological studies that examined the association between calcidiol levels and T2DM incidence or glycemic biomarkers (i.e. fasting insulin and fasting glucose, etc.) (129–132). The Kuopio Ischaemeic Heart Disease Risk Factor study (KIHD) examined the association between diabetes biomarkers, T2DM risk and vitamin D deficiency (129). This cross-sectional study analyzed serum calcidiol levels of a total of 850 men and 906 women, aged 53-73 y. Subjects were classified according to their vitamin D levels. Those who had higher serum calcidiol levels (51 - 112.8 nmol/L), had significantly lower levels of fasting insulin, fasting blood glucose, and OGTT 2h glucose by 16.7%, 1.4%, and 5.1%, respectively, than those with lower calcidiol levels (8.5 - 34.4 nmol/L) after adjusting for age, gender, and examination year (129). These associations with fasting insulin and fasting blood glucose were weakened by further adjustment for BMI, WHR, smoking, leisure-time physical activity, intake of fruits and vegetables, family history of diabetes, and examination month (129). Findings of the KIHD study indicate an important possible role for vitamin D in glucose metabolism; it is an essential nutrient for health that modulates glucose homeostasis. Nevertheless, vitamin D levels did not affect diabetes incidence or prevalence in the KIHD study which coincides with another cross-sectional study in the general population in Denmark (130). Using an ethnically diverse sample

in Toronto and London, Ontario, Canada, serum calcidiol levels were inversely associated with traditional components of the metabolic syndrome; namely, fasting insulin ($P < 0.001$) and TGs ($P < 0.001$) after adjusting for age, sex, ethnicity, season, PTH, physical activity, nutrient supplementation, and waist circumference (85).

### 4.2 Prospective Studies

There is a large volume of published prospective studies assessing the role of vitamin D in T2DM pathogenesis. A longitudinal observational follow-up study by Joerginson and colleagues evaluated the effectiveness of vitamin D status as a predictor of albuminurea progression and all-cause and cardiovascular mortality in T2DM patients (133). Over 15 y of follow-up, severe vitamin D deficiency was associated with 2 fold greater all-cause and cardiovascular mortality (HR = 1.96 [1.29–2.98] and 1.95 [1.11–3.44], respectively) (133). However, severe vitamin D deficiency failed to predict diabetic kidney disease (133). Another 5-year prospective study was conducted on non-diabetic Asians at high risk for T2DM to assess vitamin D deficiency's effect on T2DM incidence (134). Participants were divided according to their vitamin D status as follows: deficient (< 24 nmol/L), insufficient (25-50 nmol/L), and sufficient (≥ 50 nmol/L). After adjusting for BMI, WC, IGI and HOMA-IR, a negative correlation was found between poor vitamin D status and T2DM incidence (134). Those who were vitamin D deficient were 3 fold more likely to

develop T2DM than their sufficient counterparts (HR 95% CI = 3.23 [1.66 to 3.60]) (134). These results corroborate another 6-y prospective study in which those who developed T2DM had lower serum calcidiol concentration by 11% compared to those who did not develop T2DM (135). Serum calcidiol has also been examined as a predictor for future glycemic status in 524 non-diabetic men and women (mean ± SD, age 52.9 ± 7.7 y) (136). Age- and sex-adjusted baseline mean serum calcidiol levels were 60.2 ± 25.3 nmol/L (136). After 10 y follow-up, serum calcidiol was inversely associated with the 2h glucose tolerance test (β 95% CI = -0.0094 [-0.004 to 0.0002]), fasting insulin (β 95% CI = -0.1447 [-0.261 to -0.028]), and HOMA-IR (β 95% CI = -0.005 [-0.01 to -0.001]), after adjusting for age, sex, smoking, season, BMI, PTH, calcium, and IGF-1 (136). This association was confirmed in another study that examined the association of baseline serum calcidiol with insulin resistance, β-cell function (evaluated using IGI/IR which is calculated by dividing insulinogenic index IGI by HOMA-IR and by using insulin secretion sensitivity index-2 ISSI-2), and glucose regulation in pre-diabetic 489 participants (M/F, age 50 ± 10 y; BMI 30.33 ± 4 $kg/m^2$) (137). At the 3 year follow-up and with respect to baseline serum calcidiol, IGI/IR and ISS-2, measurements used to asses β-cell function, had a positive association per unit increase in calcidiol (β 95% CI = 0.005 [0.0009 to 0.008] and 0.002 [0.003 to 0.003], respectively) and a negative association with $AUC_{glucose}$ during OGTT (β 95% CI = -0.001 [-0.002 to -0.0003]), after adjusting for age, sex,

ethnicity, and season of blood draw to measure 25(OH)$D_3$. The study also revealed that participants who remained at normal glucose tolerance had higher serum calcidiol concentration by 12% in comparison to participants who became dysglycemic at the follow up (59.84 ± 23.07 nmol/L vs. 53.03 ± 23.1 nmol/L, respectively; P = 0.0041) (137). Prospectively, the association between serum calcidiol and T2DM or MetS risk has been demonstrated (138–141), whereas in others it has disappeared after further adjustment for adiposity (137)(142), or has been rejected (143).

### 4.3 Human Clinical Trials

Clinical trials are needed to establish the role of vitamin D supplementation with respect to glycemia. In the last decade, clinical interventions that examined the effect of correcting vitamin D deficiency on diabetic markers were conducted in a wide range of ethnic groups: Blacks (144), whites (145), South Asians (86) and in Middle Eastern countries (111,146). Vitamin $D_3$ dosage in these trials ranged from a fairly low dose (400-800 IU/d) (145,147) to doses reaching the upper tolerable intake (2000-4000 IU/d) (101,148). In these studies, baseline serum calcidiol concentrations were either deficient or insufficient (15-50 nmol/L), and most values increased following supplementation to optimal levels (75-120 nmol/L). Von hurst et al. conducted a randomized, placebo controlled, double-blind intervention study, with subjects assigned to either a 4000 IU/d vitamin $D_3$ or placebo for 6 months (86). Vitamin

$D_3$ supplementation significantly increased the serum calcidiol median (from 21 to 75 nmol/L) as compared to the placebo group (from 19 to 29 nmol/L) (86). As well, supplementation improved insulin sensitivity (measured by homeostatic model assessment for insulin sensitivity HOMA-2%S) by 41% at 6 months in subjects (n = 16) whose calcidiol levels reached > 80 nmol/L (P = 0.003). Insulin resistance (measured by HOMA-IR) was also significantly reduced by 11.7% (P = 0.02), however no differences were observed in insulin secretion between the two groups (86). In a randomized clinical trial by Nikooyeh et al., subjects were randomly divided into three groups: vitamin D + calcium fortified yogurt (DCY; 500 IU vitamin $D_3$ and 250 mg Ca/250 mL), vitamin D fortified yogurt (DY; 500 IU vitamin $D_3$ and 150 mg Ca/250 mL), or plain yogurt (PY; no vitamin $D_3$ and 150 mg Ca/250 mL) twice per day for 12 weeks (111). The DY and DCY groups experienced a significant decrease in fasting serum glucose (FSG= -12.9 ± 33.7 mg/dL, P = 0.015; and -9.6 ± 46.9 mg/dL P = 0.035, respectively), HbA1c (-0.4 ± 1.2%, P < 0.001; and -0.4 ± 1.9%, P < 0.001, respectively) and HOMA-IR (-0.6 ± 1.4, P = 0.001; and -0.6 ± 3.2, P < 0.001, respectively) compared to PY. Furthermore, a significant decrease in BMI (-0.9 ± 0.6 $kg.m^2$, P < 0.001; and -0.4 ± 0.7 $kg/m^2$, P = 0.005, respectively) and waist circumference (-3.6 ± 2.7 cm; and -2.9 ± 3.3 cm; P < 0.001 for both, respectively) was observed in DY and DCY compared to PY (111). The 16-week randomized, placebo controlled, double-blind Calcium and Vitamin D for Diabetes Mellitus (CaDDM) trial

assigned 92 white adults at high risk of T2DM to either calcium (400 mg twice daily), vitamin $D_3$ (2000 IU once daily), or their matching placebo (101). Vitamin $D_3$ supplementation significantly improved the disposition index (a marker of β-cell function; derived as the product of insulin secretion and insulin sensitivity) by 138% (adjusted mean change ± SE: 300 ± 130 compared with -126 ± 127 for placebo P = 0.011), and increased insulin secretion (62 ± 39 mU• L-1 •min compared with -63 ± 37 mU• L-1 •min for placebo; P = 0.046), with a non-significant decrease in HbA1C (%) (149). It is worth noting that calcium supplementation did not change any of the measured outcomes.

To examine the effect of vitamin D and calcium supplementation on T2DM incidence in postmenopausal women, The Women's Health Initiative was conducted in 2008 and considered one of the largest intervention human studies with 33,951 healthy post-menopausal women aged 50-79 y (73). Women were assigned to either the treatment (1000 mg elemental calcium and 400 IU vitamin $D_3$ daily) or placebo group (73). The authors concluded that vitamin D and calcium supplementation had no effect on T2DM incidence. However, the vitamin D dose (400 IU/d) was relatively low (lower than the current RDA for vitamin D) and may not have been enough to detect an effect on T2DM incidence. Recently, researchers investigated the safety and efficacy of the upper tolerable dosage of vitamin $D_3$ supplementation (4000 IU/d) (86,144,148). Harris and colleagues investigated the effect of 12 weeks of vitamin $D_3$

supplementation on glycemic markers, namely insulin sensitivity, insulin secretion and the disposition index, in 89 overweight and obese African Americans with diabetes or prediabetes (148). Following a randomized, placebo controlled design; subjects were allocated to either the treatment (4000 IU/d of vitamin $D_3$) or placebo group. A significant increase ($P < 0.001$) in vitamin D status in the treatment group (40 to 81 nmol/L) was observed (148). Unexpectedly, insulin sensitivity significantly decreased ($P < 0.034$) in the treatment group by 4% and increased by 12% in the placebo group (148). A dosage of vitamin $D_3$ supplementation of 4000 IU/d for 6 months reduced HOMA-IR by 27% ($P = 0.033$) and improved QUICKI, a measure of insulin sensitivity, by 18% ($P = 0.016$) in obese adolescents (144). However, there was no effect on FPG, HbA1c, CRP, and IL-6 (144).

### 4.4 Animal Studies

What we know about vitamin D and its relation to diabetes is largely based upon animal studies that investigated the underlying mechanisms of vitamin D action. In 1980, Norman and colleagues conducted the very first study that examined vitamin D's potential in controlling glycemia (150). In response to a glucose-arginine perfusion, uremic vitamin D deficient rats experienced 49% decrease in 1$^{st}$ phase insulin response and 47% decrease in 2$^{nd}$ phase insulin response compared to vitamin D replete rats ($P < 0.05$) (150). These findings were promising and opened a new window in this field. Another animal model

study compared FBG between 3 groups of mice: control (n = 8), diabetic (n = 8), diabetic and supplemented with vitamin $D_3$ (0.28 IU/mg/d; n = 8) (151). After 15 days, animals were sacrificed and those that were supplemented with vitamin $D_3$ had a 24% significant decrease in FBG compared to their diabetic counterparts (190 mg/dL vs. 250 mg/dL, respectively) (151). These findings are suggestive of vitamin D's role in attenuating diabetes severity through improving glycemic biomarkers. Importantly, vitamin D was examined from a therapeutic perspective as well as from a preventative perspective. In another animal model study, mice were divided in 4 groups, 10 mice in each: control (Cont), alloxan-induced diabetic (Diab), diabetic that received 5000 IU/kg bw/d calcitriol after diabetes induction (VDther), and diabetic that received 5000 IU/kg bw/d calcitriol prior to alloxan injection (VDprev) (152). Plasma insulin was significantly lower by 59% and 44% in the Diab group and VDther compared to the Cont group ($P < 0.001$ for both) (152). However, VDprev experienced a significant increase in plasma insulin by 98% ($P < 0.01$) accompanied by a significant 45.7% decrease in plasma glucose ($P < 0.01$) compared to the Diab group. The decrease in plasma glucose was also significant in VDther compared to Diab group ($P < 0.01$) (152). When SOD activity was examined in renal and hepatic tissues, VDther and VDprev groups had significantly higher activity ($P < 0.05$ and $P < 0.001$, respectively) compared to the Diab Group. In addition, significantly higher GPx activity was also observed (152).

## 6.0 Rationale

Current research suggests an association between vitamin D deficiency and type 2 diabetes (153). There is a gap in the literature regarding the effect of vitamin D and calcium supplementation in T2DM patients of diverse ethnicities. In our study, we aim to investigate the effect of vitamin D and calcium supplementation in T2DM patients of ethnic minorities in the GTA, Ontario.

This study is designed to determine whether vitamin D and calcium supplementation would attenuate the severity of T2DM through improving fasting insulin, fasting glucose, glycemic control, and HOMA-IR, as well as changes in medications, anthropometric measures and other outcome measures.

## 7.0 Objectives

The objectives of this study are to determine whether:

1- Vitamin D status correlates with T2DM outcomes,

2- Vitamin $D_3$ and calcium supplementation attenuates the severity of T2DM by improving glycemic biomarker (fasting insulin, fasting glucose, HbA1c, and HOMA-IR), lipid profile (total cholesterol, LDL-C, HDL-C, TGs, and total cholesterol/HDL-C ratio), and anthropometric measures (body weight, waist and hip circumference, systolic and diastolic blood pressure) in ethnically diverse, post-menopausal women.

## 8.0 Hypothesis

We hypothesized that subjects assigned to the treatment group would experience an improvement in serum calcidiol, fasting serum glucose and insulin, insulin resistance and β-cell function measured by homeostatic model assessment, lipid profile, and anthropometric measures.

## 9.0 Methods

### 9.1 Study Design and Subject Recruitment

T2DM patients were recruited at the Women's Health in Women's Hands Community Health Centre (WHWH-CHC), and allocated to two groups following a double-blind, placebo-controlled fashion: vitamin D and calcium (CaD; AgaeCal Inc, Vancouver, BC, Canada) or placebo. For ethical reasons,T2DM patients with deficient levels of vitamin D were assigned to the CaD group; however, subjects remained blinded. The rest of the subjects were assigned randomly to either placebo or CaD group. Recruitment was done by coordinating with WHWH-CHC personnel. The study details were clearly explained to the study subjects and written consent was provided before study commenced (see Appendix B). This study was approved by both the York University Research Ethics Board (certificate # 2009-055) and Health Canada (Protocol #220).

Baseline blood work and anthropometric measures were recorded prior to supplementation at baseline and at 3 years. Demographic data were pulled from

WHWH-CHC intake forms (Table 3). Compliance was measured verbally by asking subjects if they were taking the placebo and CaD pills. Subjects completed a 3-day dietary record at baseline and at the 3-year timepoint. All participants were not on insulin therapy throughout the study duration.

**Table 3:** Demographic characteristics of trial participants (n = 11).

| | | n (%) |
|---|---|---|
| Ethnicity | | |
| | South Asian | 1 (9.1) |
| | Caribbean | 7 (63.6) |
| | Black | 3 (27.3) |
| Education | | |
| | Elementary (grades 1-8) | 5 (45.5) |
| | High school (grades 9-12 or 13) | 5 (45.5) |
| | University, community college, trade school apprenticeship | 1 (9.1) |
| Household | | |
| | Couple without children | 1 (9.1) |
| | Live alone | 4 (36.4) |
| | Siblings | 1 (9.1) |
| | Extended family | 1 (9.1) |
| | Grandparents with grandchildren | 1 (9.1) |
| | Single parent family (mother) | 3 (27.3) |
| Income | | |
| | 1000-14999 | 3 (27.3) |
| | 15000-19999 | 5 (45.5) |
| | 20000-24999 | 1 (9.1) |
| | 30000-34999 | 2 (18.2) |

## 9.2 Subject Selection and Withdrawal

### 9.2.1 Subject Inclusion Criteria

Non-white, post-menopausal women diagnosed with T2DM, who have an attending physician, and use the services of WHWH-CHC.

### 9.2.2 Subject Exclusion Criteria

Subjects diagnosed with the following were excluded from the study: 1) Chronic (such as liver, kidney, cancers, etc) or neurodegenerative diseases (such as ALS, Alzheimer's disease, multiple sclerosis), 2) any disease/condition that can affect T2DM, insulin function or glucose metabolism, 3) osteomalacia, osteopenia and/or osteoporosis, or 4) celiac disease or allergies toward gluten. Furthermore, participants who already supplemented with vitamin $D_3$ and calcium (except for a multi vitamin/mineral supplement) were excluded from the study.

### 9.2.3 Subject Withdrawal Criteria

Subjects were withdrawn from the study if they 1) reported adverse effects, or 2) were diagnosed with chronic disease that may affect T2DM, insulin function or glucose metabolism.

### 9.3 Main Outcome Measures

At baseline and the 3-year follow-up visit, a wide range of blood tests and anthropometric measurements were performed for each participant, as follows.

#### 9.3.1 Anthropometric Measurements

The registered nurse at the WHWH-CHC performed anthropometric measurements for participants at the clinic. Weight and height were assessed using weight scale and height rod, and body mass index was calculated. Total body fat (kg) and body fat percentage was measured by using bioelectrical impedance analysis (BIA) (OMRON® HBF-306CCAN) (154). Waist and hip circumference were measured by using measuring tape: waist circumference was measured an inch above the belly button and hip circumference was measured at the widest area of the hips, and waist-to-hip ratio was calculated. Blood pressure (systolic and diastolic) readings were measured while seated.

#### 9.3.2 Blood Tests

Fasting blood was analyzed for: serum calcidiol to assess vitamin D status, glycemic biomarkers (fasting serum glucose and insulin, and glycated hemoglobin) HOMA-IR (a measure of insulin resistance) and HOMA-B (a measure of beta-cell function) were calculated from fasting glucose and insulin values (155); lipid profile (triglycerides-TG, total cholesterol-TC, LDL-C, HDL-C, and TC/HDL-C ratio); parathyroid hormone (PTH); liver function tests (aspartate

transaminase-AST, alanine transaminase-ALT,); and serum calcium and albumin, and kidney function tests (serum creatinine, and estimated glomerular filtration rate-eGFR). Participants received lab requisitions either through mail or when they presented for their regular follow up visits at the center.

### 9.3.3 Nutritional Assessment

All participants completed a 3-day (2 weekdays and 1 weekend) diet record at baseline and 3-year follow-up visit.

## 9.4 Supplementation Dosage

Participants received a 3-month supply of a daily dose of four pills, containing either placebo or CaD. Treatment pills each contained each, 450 IU of vitamin $D_3$ and 180 g of calcium, for a total of 1800 IU of vitamin $D_3$ and 720 mg of calcium per day. The intervention dosage was based on previous clinical interventions that detected an improvement in glycemic biomarkers (86,111,149). Vitamin $D_3$ and calcium intake among Canadians was also considered. The mean intake of vitamin $D_3$ and calcium from dietary sources and supplements is 412 IU/d and 1063 mg/d (156,157). The supplementation in this study does not exceed the tolerable upper level established by the IOM.

## 9.5 Statistical Analysis

A total of 11 subjects were included in the final analysis; these included those who had completed all blood tests and anthropometric measurements at

baseline and at the 3-year follow-up visit. Due to the small sample size, non-parametric tests were used to analyze pre and post treatment data. One-tailed Spearman's rank correlation coefficient was used to examine the association between calcidiol levels and all dependent outcome measures, because we a *priori* hypothesized that the treatment group would achieve an improvement in glycemic control based on previous studies (86,111,125,144,146,147,149). All subjects were included in the following one-tailed bivariate correlations: serum calcidiol levels vs body weight, body mass index (BMI), total body fat (kg), % body fat, waist circumference (WC), hip circumference (HC), waist-to-hip ratio (WHR), systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting serum glucose (FSG), fasting serum insulin, glycated hemoglobin A1c (HbA1c), HOMA-IR, HOMA-B, triglycerides (TG), total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), total cholesterol/HDL-C ratio (TC/HDL-C), parathyroid hormone (PTH), aspartate transaminase (AST), alanine transaminase (ALT), serum calcium, serum albumin, serum creatinine, and estimated glomerular filtration rate (eGFR). Bivariate correlations were performed for values at baseline, 3 years, and both absolute and relative change over 3 years vs baseline.

The Wilcoxon-signed rank test was used to detect within-group differences, and the Mann-Whitney test was used to detect the between-group differences. Analysis was conducted twice: following the *per-protocol* method

based on the original treatment group assignment, and following the *retrospective* method based on the increase in serum calcidiol concentration. The percentage change in serum calcidiol levels was calculated for all subjects, and those who had at least a 20% increase in serum calcidiol levels at the 3-y mark as compared to their baseline values were included in the treatment group. After implementing per-protocol method, 2 subjects who had 2% and 9% increase in serum calcidiol were reassigned to the placebo group. One subject who had a 42.9% increase in serum calcidiol reassigned to the treatment group; the subject was contacted and confirmed the consumption of 2000 IU $D_3$/day for the last 3 years. Compliance was also appraised by considering the decrease in PTH levels in all subjects who were included in the treatment group after the per-protocol method was implemented. Statistical analyses were performed using SPSS 21 (version 21, IBM). Significance was considered at $P \leq 0.10$, and trends were considered at $0.10 < P \leq 0.15$. Data are presented as means ± standard deviation (SD) and medians (percentiles 25%-75%). (Check appendix A for parametric tests).

Diet intake was analyzed using Diet Analysis™+ (version 8.0) for the 3 d diet record at baseline and at the 3-year follow-up. Mean intake of macronutrients (kilocalories, total carbohydrates, total protein, total fat, mono- and poly- unsaturated fat, cholesterol) and micronutrients (vitamin D and

calcium) was calculated. Descriptive statistics were used to describe demographic data of trial participants.

### 10.0 Challenges and limitations

The main weakness of the current pilot study was the small sample size, which limited the applicability of its results to the general population. The most important challenge lies in the fact that CHC policy did not allow the researcher to contact CHC clients for follow-up. This resulted in low-response rate, poor compliance, and missing outcome measures. A number of additional issues were not addressed in the study design and also contributed to poor compliance. Firstly, the low socio-economic status of the participants presented several challenges regarding adhering to follow-up visits to the CHC, as the transportation expenses caused an economic burden. Secondly, the study subjects verbally self-reported compliance to the registered nurse without providing empty bottles to confirm compliance, violating our instructions as per the study design; a matter made difficult to remedy because the CHC would not allow our researchers contact with the study subjects. Thirdly, supplementation dosage was divided into four pills per day which was likely difficult for the subjects to adhere to; this was a matter related to pill manufacturing by AgaeCal Inc (Vancouver, BC, Canada). Lastly, although the length of the current study would establish the role of chronic CaD supplementation on aspects of T2DM pathology (a disease characterized by systemic anomalies in metabolism), and

hence strengthen the results in theory, the long duration of this human clinical trial resulted in a high rate of drop out.

## 11.0 Results

Simple descriptive statistical analysis was used to investigate the effect of vitamin $D_3$ and calcium supplementation on a wide range of glycemic control biomarkers. Baseline characteristics are presented as medians and percentiles following per-protocol (Table 4) and retrospective (Table 5) methods. Three-year, subject characteristics are presented as medians percentiles following per-protocol (Table 6) and retrospective (Table 7). Median serum calcidiol concentrations were 54 and 106 nmol/L at baseline and 66 and 79 nmol/L after 3 years; for CaD and placebo, respectively for per-protocol method.

**Table 4:** Baseline characteristics presented as medians (percentiles 25%-75%) following **per-protocol** method, for T2DM subjects (n = 11).

| Measurement | Placebo n = 4<br>Median<br>(Percentiles 25%-75%) | CaD n = 7<br>Median<br>(Percentiles 25%-75%) | P |
|---|---|---|---|
| Age (years) | 68 (50.5-72.7) | 57 (49-69) | |
| Body weight (kg) | 91.9 (82.9-95.0) | 73.5 (58.0-84.7) | 0.109 |
| BMI (kg/m$^2$) | 34.7 (33.8-36.6) | 27.7 (24.8-34.9) | 0.164 |
| Total body fat (kg) | 30.9 (27.9-31.9) | 24.7 (19.5-28.5) | 0.109 |
| Body fat (%) | 35.4 (29.3-42.1) | 32.5 (28.5-42.5) | 1.000 |
| WC (cm) | 101.0 (90.3-108.0) | 89.5 (83.0-109.0) | 0.527 |
| HC (cm) | 126.0 (101.5-131.4) | 116.0 (101.0-121.0) | 0.164 |
| WHR | 0.84 (0.77-0.92) | 0.79 (0.76-0.92) | 0.788 |
| SBP (mmHg) | 126.0 (119.5-144.5) | 135.0 (115.0-140.0) | 0.927 |
| DBP (mmHg) | 80.0 (78.5-84.5) | 85.0 (80.0-90.0) | 0.527 |
| Serum Calcidiol (nmol/L) | 106.5 (92.3-125.3) | 54.0 (43.0-67.0) | **0.006** |
| FSG (mmol/L) | 7.3 (6.8-8.9) | 7.0 (6.0-9.1) | 0.788 |
| Fasting serum insulin (pmol/L) | 67.0 (47.3-561.5) | 65.0 (21.0-143.0) | 0.788 |
| HbA1C (%) | 7.1 (6.7-7.9) | 7.5 (6.8-9.4) | 0.527 |
| HOMA-IR | 21.7 (14.3-232.5) | 20.2 (5.6-43.2) | 0.527 |
| HOMA-B | 384.0 (260.4-1946.7) | 178.6 (23.5-866.7) | 0.315 |
| TG (mmol/L) | 0.83 (0.71-1.07) | 1.01 (0.87-1.45) | 0.315 |
| TC (mmol/L) | 3.91 (2.83-5.08) | 5.17 (4.60-5.86) | 0.109 |
| LDL-C (mmol/L) | 2.23 (1.11-3.36) | 3.33 (2.56-3.79) | 0.230 |
| HDL-C (mmol/L) | 1.44 (1.36-1.56) | 1.40 (1.13-1.77) | 1.000 |
| TC/HDL-C (mmol/L) | 2.63 (2.01-3.51) | 3.56 (2.60-4.20) | 0.164 |
| PTH (pmol/L) | 7. 5 (6.6-9.1) | 5.0 (4.1-8.3) | 0.230 |
| AST (U/L) | 20.5 (16.8-22.8) | 22.0 (21.0-30.0) | 0.412 |
| ALT (U/L) | 15.0 (13.5-20.3) | 21.0 (19.0-38.0) | 0.230 |
| Serum calcium (mmol/L) | 2.4 (2.3-2.5) | 2.4 (2.3-2.5) | 0.927 |
| Serum albumin (g/L) | 43.0 (39.0-45.5) | 41.0 (37.8-45.0) | 0.648 |
| Serum creatinine (µmol/L) | 70.5 (68.3-95.3) | 79.0 (70.0-84.0) | 0.412 |

| | | | |
|---|---|---|---|
| eGFR ($ml/min/1.73m^2$) | 73.5 (53.5-86.0) | 69.0 (64.0-78.0) | 0.648 |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol, TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. *P is significant ≤ 0.10.

**Table 5:** Baseline characteristics presented as medians (percentiles 25%-75%), following **retrospective** method, for T2DM subjects (n = 11).

| Measurement | Placebo n = 5<br>Median<br>(Percentiles 25%-75%) | CaD n = 6<br>Median<br>(Percentiles 25%-75%) | P |
|---|---|---|---|
| Body weight (kg) | 90.8 (82.5-97.1) | 73.0 (55.9-80.4) | **0.030** |
| BMI ($kg/m^2$) | 35.5 (34.4-36.8) | 27.1(23.9-33.8) | **0.004** |
| Total body fat (kg) | 30.5 (27.7-32.6) | 24.5 (18.8-27.0) | **0.030** |
| Body fat (%) | 39.3 (30.0-44.1) | 32.3 (26.6-36.0) | 0.429 |
| WC (cm) | 109.0 (92.5-111.5) | 89.3 (79.8-95.3) | 0.082 |
| HC (cm) | 123.5 (106.3-126.0) | 112.5 (98.0-123.9) | 0.429 |
| WHR | 0.92 (0.82-0.93) | 0.78 (0.76-0.82) | 0.126 |
| SBP (mmHg) | 124.0 (116.5-145.0) | 132.5 (122.3-140.0) | 0.931 |
| DBP (mmHg) | 80.0 (79.0-88.0) | 82.5 (77.5-86.3) | 1.000 |
| Serum calcidiol (nmol/L) | 94.0 (58.0-114.0) | 56.5 (39.3-80.3) | 0.247 |
| FSG (mmol/l) | 7.4 (7.0-8.5) | 6.8 (5.4-9.8) | 0.429 |
| Fasting serum insulin (pmol/L) | 86.0 (56.5-431.5) | 48.5 (18.3-108.3) | 0.177 |
| HbA1C (%) | 6.9 (6.5-7.3) | 7.9 (7.3-9.7) | **0.030** |
| HOMA-IR | 27.5 (18.7-172.0) | 13.3 (5.5-29.7) | **0.082** |
| HOMA-B | 464.9 (285.6-1653.7) | 173.3 (17.6-494.6) | **0.082** |
| TG (mmol/l) | 0.87 (0.75-1.40) | 1.00 (0.78-1.19) | 1.000 |
| TC (mmol/l) | 3.93 (2.96-4.89) | 5.44 (4.63-6.09) | **0.017** |
| LDL-C (mmol/l) | 2.05 (1.16-3.28) | 3.48 (2.81-3.83) | **0.052** |
| HDL-C (mmol/l) | 1.43 (1.23-1.52) | 1.42 (1.32-2.02) | 0.792 |
| TC/HDL-C | 3.22 (2.02-3.52) | 3.82 (2.55-4.23) | 0.126 |
| PTH (pmol/L) | 6.5 (4.6-8.7) | 6.1 (4.2-8.7) | 1.000 |
| AST (U/L) | 23.0 (20.5-29.5) | 21.5 (15.5-34.8) | 0.329 |

| | | | |
|---|---|---|---|
| ALT (U/L) | 22.0 (14.0-30.0) | 19.0 (14.5-40.3) | 0.792 |
| Serum calcium (mmol/l) | 2.4 (2.3-2.5) | 2.3 (2.3-2.4) | 0.247 |
| Serum albumin (g/L) | 40.0 (37.9-45.0) | 43.0 (39.7-45.3) | 0.662 |
| Serum creatinine (μmol/L) | 76.0 (68.5-91.5) | 75.5 (69.8-84.0) | 0.792 |
| eGFR (ml/min/1.73m$^2$) | 69.0 (56.5-79.5) | 73.0 (63.8-79.3) | 0.792 |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol, TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. *P is significant ≤ 0.10.

Table 6: Subjects characteristics at 3 y, presented as medians percentiles, following **per-protcol** assignment, for T2DM subjects (n= 11)

| Measurement | Placebo n = 4<br>Median<br>(25 -75% percentiles) | CaD n = 7<br>Median<br>(25 -75% percentiles) |
|---|---|---|
| Body weight (kg) | 92.6 (88.6-98.5) | 76.0 (57.5-84.5) |
| BMI | 37.1 (33.8-38.2) | 32.0 (24.6-35.1) |
| Total body fat (kg) | 20.1 (19.2-21.4) | 16.5 (12.5-18.3) |
| Body fat (%) | 44.6 (42.6-45.8) | 38.1 (21.7-44.1) |
| WC (cm) | 104.1 (101.2-108.4) | 96.5 (71.0-104.0) |
| HC (cm) | 121.9 (117.4-128.2) | 108.0 (106.0-116.8) |
| WHR | 0.86 (0.80-0.91) | 0.89 (0.84-0.96) |
| SBP (mmHg) | 135.5 (125.3-160.0) | 137.0 (110.0-147.0) |
| DBP (mmHg) | 77.5 (68.5-87.25) | 91.0 (71.0-95.0) |
| Serum calcidiol (nmol/L) | 79.0 (50.3-145.3) | 66.0 (58.0-88.0) |
| FSG (mmol/L) | 6.9 (4.3-9.7) | 6.4 (5.8-6.8) |
| Fasting serum insulin (pmol/L) | 53.5 (29-281.25) | 83.0 (40.0-108.0) |
| HBA1C (%) | 7.9 (7.4-8.8) | 7.5 (6.7-9.6) |
| HOMA-IR | 21.3 (8.8-49.1) | 27.8 (6.3-31.6) |
| HOMA-B | 213.0 (151.1-53618.0) | 242.4 (71.4-765.5) |
| TG (mmol/L) | 0.82 (0.75-1.08) | 1.14 (0.90-1.27) |
| TC (mmol/L) | 3.25 (2.57-4.97) | 4.58 (2.82-5.12) |
| LDL-C (mmol/L) | 1.48 (1.01-2.84) | 2.25 (1.04-3.17) |
| HDL-C (mmol/L) | 1.41 (1.29-1.73) | 1.40 (1.17-1.51) |
| TC/HDL-C | 2.45 (1.88-2.87) | 2.59 (2.20-3.76) |
| PTH (pmol/L) | 7.5 (5.3-10.7) | 3.7 (3.5-5.2) |
| AST (U/L) | 18.0 (14.3-18.8) | 17.0 (13.0-25.0) |

| | | |
|---|---|---|
| ALT (U/L) | 14.0 (13.0-21.0) | 17.0 (15.0-23.0) |
| Serum calcium (mmol/L) | 2.5 (2.3-2.6) | 2.4 (2.3-2.4) |
| Serum albumin (g/L) | 44.5 (44.0-45.8) | 41.0 (41.0-47.0) |
| Serum creatinine | 65.5 (58.0-94.0) | 72.0 (67.0-75.0) |
| eGFR | 78.5 (52.3-95.8) | 73.0 (61.0-75.0) |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol, TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate.

**Table 7:** Subject characteristics at 3 y, presented as medians (percentiles), following **per-protocol**, for T2DM subjects (n = 11)

| Measurement | Placebo n = 5 Median (Percentiles 25%-75%) | CaD n = 6 Median (Percentiles 25%-75%) |
|---|---|---|
| Body weight (kg) | 95.0 (86.3-99.4) | 74.5 (55.5-85.5) |
| BMI (kg/m$^2$) | 37.1 (35.8-37.8) | 29.7 (23.8-32.5) |
| Total body fat (kg) | 20.6 (18.7-21.6) | 16.2 (12.0-18.5) |
| Body fat (%) | 44.1 (42.4-45.6) | 34.6 (21.5-42.7) |
| WC (cm) | 104.0 (102.8-107.8) | 92.7 (63.3-104.1) |
| HC (cm) | 121.9 (116.8-127.0) | 107.0 (101.1-117.4) |
| WHR | 0.86 (0.82-0.91) | 0.87 (0.83-0.96) |
| SBP (mmHg) | 136.0 (122.0-163.0) | 129.5 (114.5-143.3) |
| DBP (mmHg) | 74.0 (70.5-86.5) | 91.0 (67.5-95.5) |
| Serum calcidiol (nmol/L) | 69.0 (47.0-81.0) | 77.0 (59.5-113.0) |
| FSG (mmol/L) | 6.8 (4.7-9.0) | 6.4 (5.5-7.8) |
| Fasting serum insulin (pmol/L) | 100.0 (53.5-232.5) | 44.5 (18.3-90.0) |
| HbA1C (%) | 7.5 (7.1-8.6) | 8.2 (7.0-9.8) |
| HOMA-IR | 27.8 (21.3-43.7) | 9.2 (5.1-33.7) |
| HOMA-B | 606.1 (213.0-36169.6) | 187.2 (-354.8-373.2) |
| Triglycerides (mmol/L) | 0.90 (0.78-1.21) | 1.08 (0.74-1.33) |
| TC (mmol/L) | 2.67 (2.54-4.43) | 4.85 (3.58-5.41) |
| LDL-C (mmol/L) | 1.05 (0.98-2.54) | 2.43 (1.81-3.28) |
| HDL-C (mmol/L) | 1.29 (1.21-1.41) | 1.50 (1.32-1.96) |
| TC/HDL-C | 2.20 (1.97-3.33) | 2.75 (2.43-3.42) |
| PTH (pmol/L) | 6.3 (4.8-10.0) | 3.8 (3.5-5.1) |
| AST (U/L) | 18.0 (13.0-22.0) | 17.5 (12.5-23.3) |
| ALT (U/L) | 17.0 (13.0-23.0) | 16.0 (13.0-23.0) |
| Serum calcium (mmol/L) | 2.5 (2.3-2.5) | 2.4 (2.3-2.4) |
| Serum albumin (g/L) | 44.0 (42.5- 46.0) | 43.5 (40.5-46.5) |
| Serum creatinine (µmol/L) | 70.0 (62.0-88.5) | 70.5 (61.0-76.0) |
| eGFR (mL/min/1.73 m$^2$) | 71.0 (58.0-86.5) | 74.0 (67.8-86.5) |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total

cholesterol, TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate

One-tailed bivariate correlations were performed including all subjects at baseline, all subjects at 3 years, as well as with both absolute change and percentage change from baseline. At baseline, serum calcidiol negatively correlated with TG, TC, LDL-C, TC/HDL-C ($P = 0.095$, $P = 0.051$, $P = 0.063$, and $P = 0.071$) (Figure 2). A negative trend was observed between serum calcidiol and HbA1c ($P = 0.148$) (Figure 2). Non-significant negative correlations were observed between serum calcidiol and SBP, DBP, FSG, HbA1c, HDL-C, AST, ALT, and serum creatinine. Also, non-significant positive correlations were observed between serum calcidiol and body weight, BMI, TBF (%), WC, HC, WHR, fasting serum insulin, HOMA-IR, HOMA-B, serum calcium, serum albumin, and eGFR (See Table 8 and figures 1, 2, and 3).

**Table 8:** Spearman's rank coefficient bivariate correlations at baseline, for serum calcidiol, with the different outcome measures (n = 11).

| Measurement | Correlation coefficient | P (1-tailed) | P (2-tailed) |
|---|---|---|---|
| Body weight (kg) | 0.355 | 0.142 | 0.284 |
| BMI (kg/m$^2$) | 0.355 | 0.142 | 0.284 |
| Total body fat (kg) | 0.355 | 0.142 | 0.285 |
| Body fat (%) | 0.036 | 0.458 | 0.916 |
| WC (cm) | 0.105 | 0.380 | 0.760 |
| HC (cm) | 0.336 | 0.156 | 0.312 |
| WHR | 0.127 | 0.355 | 0.710 |
| SBP (mmHg) | -0.119 | 0.363 | 0.726 |
| DBP (mmHg) | -0.248 | 0.231 | 0.463 |
| FSG (mmol/L) | -0.064 | 0.426 | 0.852 |
| Fasting serum insulin (pmol/L) | 0.255 | 0.225 | 0.450 |
| HbA1c (%) | -0.346 | 0.148 | 0.296 |
| HOMA-IR | 0.236 | 0.242 | 0.484 |
| HOMA-B | 0.300 | 0.185 | 0.370 |
| TG (mmol/L) | -0.427 | **0.095** | 0.190 |
| TC (mmol/L) | -0.519 | **0.051** | 0.102 |
| LDL-C (mmol/L) | -0.491 | **0.063** | 0.126 |
| HDL-C (mmol/L) | -0.232 | 0.246 | 0.492 |
| TC/HDL-C | -0.473 | **0.071** | 0.142 |
| PTH (pmol/L) | 0.264 | 0.217 | 0.434 |
| AST (U/L) | -0.156 | 0.323 | 0.646 |
| ALT (U/L) | -0.275 | 0.206 | 0.412 |
| Serum calcium (mmol/L) | 0.255 | 0.224 | 0.448 |
| Serum albumin (g/L) | 0.329 | 0.162 | 0.324 |
| Serum creatinine (µmol/L) | -0.210 | 0.268 | 0.536 |
| eGFR (mL/min/1.73 m$^2$) | 0.114 | 0.369 | 0.738 |

BMI, body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; PTH, parathyroid hormone; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. *P is significant ≤ 0.10.

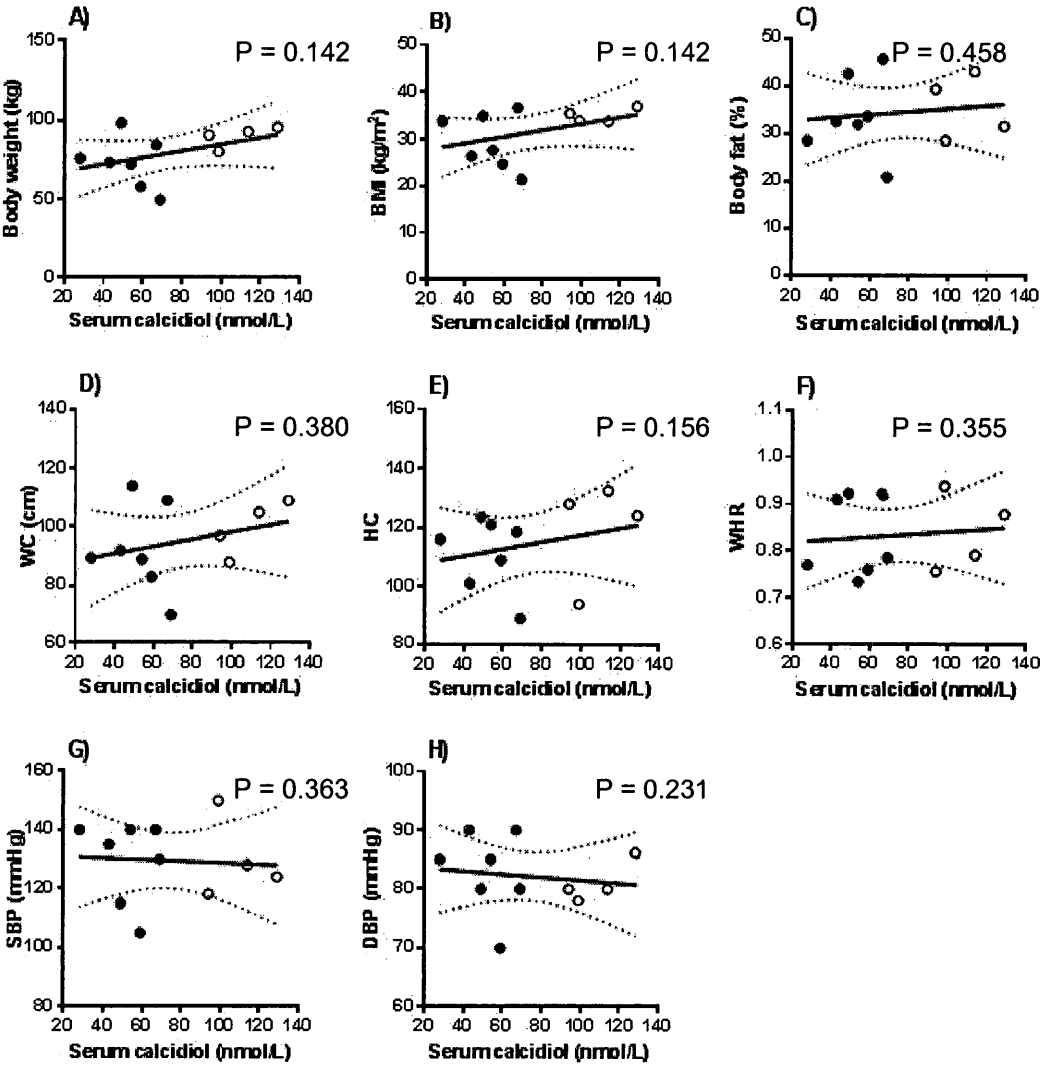


**Figure 1:** Correlations between baseline serum calcidiol (nmol/L) and baseline A) body weight (kg), B) body mass index (BMI, kg/m$^2$), C) body fat (%), D) waist circumference (WC, cm), E) hip circumference (HC, cm), F) waist-to-hip ratio (WHR), G) systolic blood pressure (SBP, mmHg), and H) diastolic blood pressure (DBP, mmHg), for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

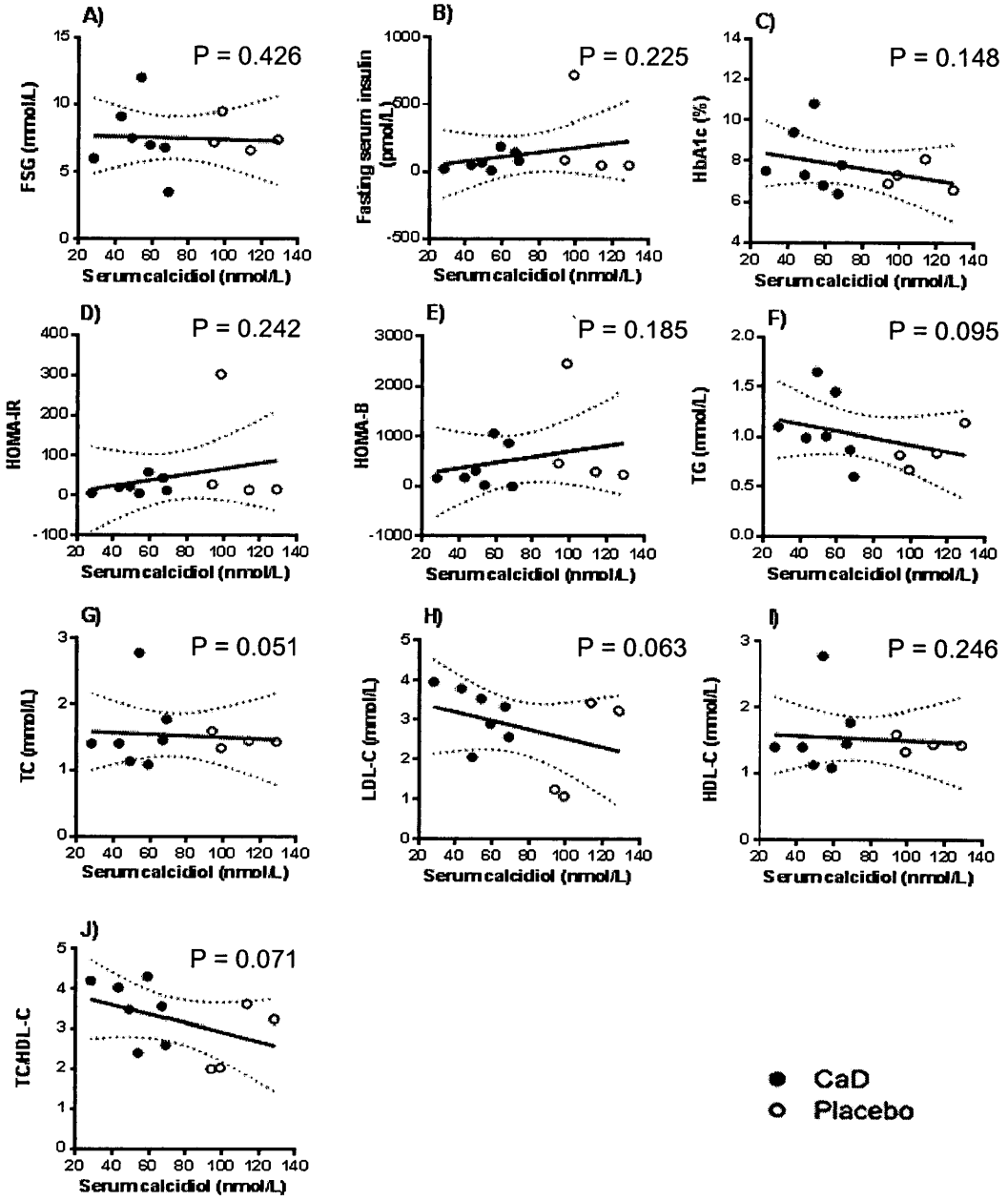


**Figure 2:** Correlations between baseline serum calcidiol (nmol/L) and baseline A) fasting serum glucose (mmol/L), B) fasting serum insulin (pmol/L), C) glycated hemoglobinA1c (HbA1c, %), D) homeostatic model assessment of insulin resistance (HOMA-IR), E) homeostatic model assessment of β-cell function (HOMA-B), F) triglycerides (TG, mmol/L), G) total cholesterol (TC, mmol/L), H) low-density lipoprotein cholesterol (LDL-C, mmol/L), I) high-density lipoprotein cholesterol (HDL-C, mmol/L), and J) total cholesterol/HDL-C ratio (TC/HDL-C, mmol/L), for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

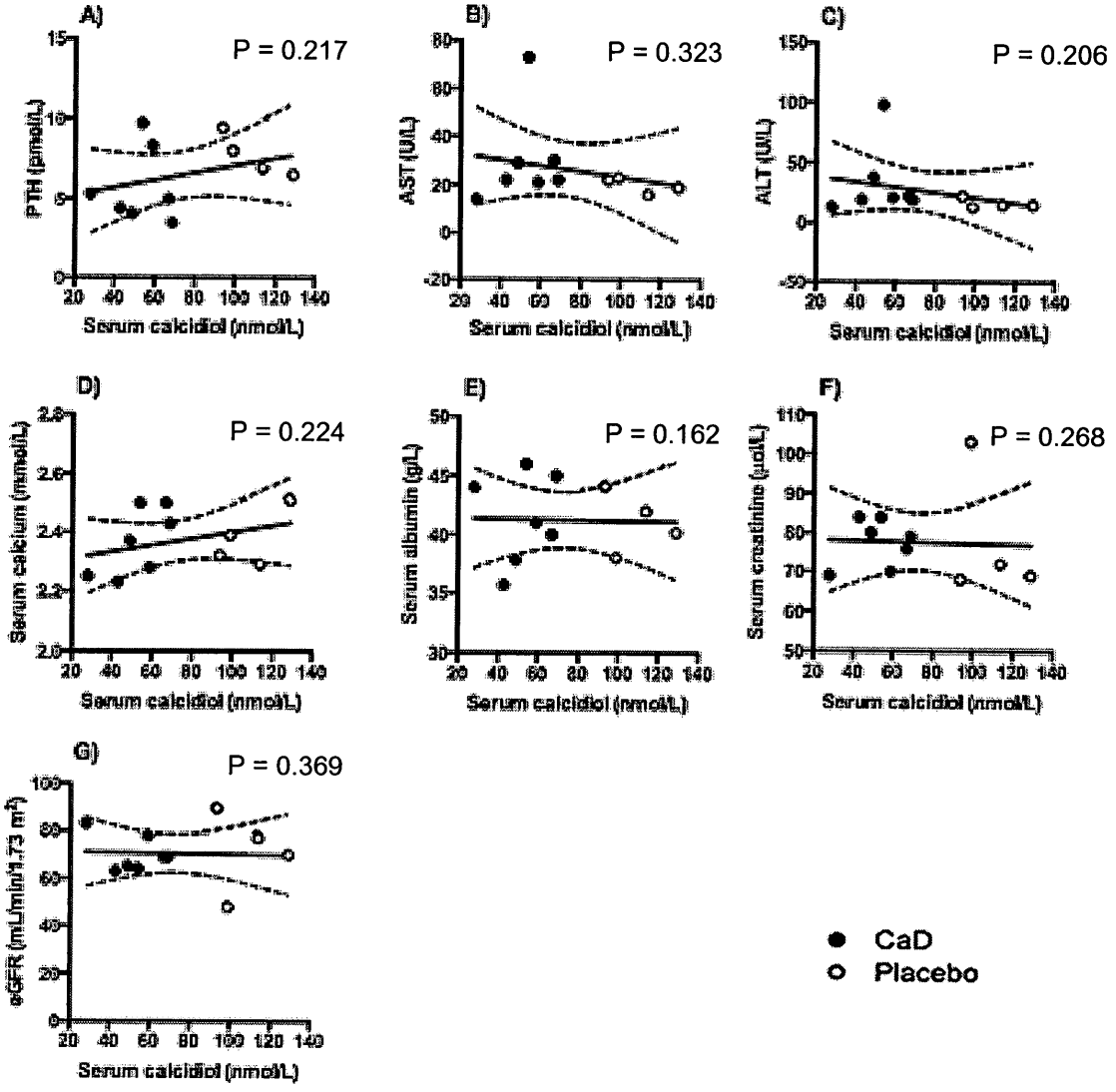


**Figure 3:** Correlations between baseline serum calcidiol (nmol/L) and baseline A) parathyroid hormone (PTH, pmol/L), B) aspartate transaminase (AST, U/L), C) alanine transaminase (ALT, U/L), D) serum calcium (mmol/L), E) serum albumin (g/L), F) serum creatinine (µmol/L), and G) estimated glomerular filtration rate (eGFR, mL/min/1.73 $m^2$) for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

At the 3-year time point, serum calcidiol was negatively correlated with DBP and HbA1c (P = 0.073 and P = 0.046) (Figures 4 and 5), and positively correlated with serum calcium (P = 0.078) (Figure 6). A negative trend was observed between serum calcidiol and FSG (P = 0.119) (Figure 5). Negative non-significant correlations were observed between serum calcidiol and body weight, BMI, TBF, WC, HC, WHR, SBP, FSG, HOMA-IR, HOMA-B, ALT, and serum creatinine. Moreover, positive non-significant correlations were observed between serum calcidiol and fasting serum insulin, TG, TC, LDL-C, HDL-C, TC/HDL-C, PTH, AST, serum albumin, and eGFR (See Table 9 and Figures 4, 5, and 6).

**Table 9:** Spearman's rank coefficient bivariate correlations at 3 y, for serum calcidiol with the different outcome measures (n = 11).

| Measurement | Correlation coefficient | P (1-tailed) | P (2-tailed) |
|---|---|---|---|
| Body weight (kg) | -0.164 | 0.315 | 0.630 |
| BMI (kg/m$^2$) | -0.155 | 0.325 | 0.650 |
| Total body fat (kg) | -0.164 | 0.315 | 0.631 |
| Body fat (%) | -0.009 | 0.489 | 0.978 |
| WC (cm) | -0.087 | 0.400 | 0.800 |
| HC (cm) | -0.239 | 0.239 | 0.478 |
| WHR | -0.182 | 0.296 | 0.592 |
| SBP (mmHg) | -0.127 | 0.355 | 0.710 |
| DBP (mmHg) | -0.469 | **0.073** | 0.146 |
| FSG (mmol/L) | -0.388 | 0.119 | 0.238 |
| Fasting serum insulin (pmol/L) | 0.036 | 0.458 | 0.916 |
| HbA1c (%) | -0.533 | **0.046** | 0.092 |
| HOMA-IR | -0.118 | 0.365 | 0.730 |
| HOMA-B | -0.200 | 0.278 | 0.556 |
| TG (mmol/L) | 0.045 | 0.447 | 0.894 |
| TC (mmol/L) | 0.278 | 0.204 | 0.408 |
| LDL-C (mmol/L) | 0.155 | 0.325 | 0.650 |
| HDL-C (mmol/L) | 0.209 | 0.269 | 0.538 |
| TC/HDL-C | 0.287 | 0.196 | 0.392 |
| PTH (pmol/L) | 0.041 | 0.452 | 0.904 |
| AST (U/L) | 0.212 | 0.266 | 0.532 |
| ALT (U/L) | -0.170 | 0.309 | 0.618 |
| Serum calcium (mmol/L) | 0.458 | **0.078** | 0.156 |
| Serum albumin (g/L) | 0.083 | 0.404 | 0.808 |
| Serum creatinine (µmol/L) | -0.073 | 0.416 | 0.832 |
| eGFR (mL/min/1.73 m$^2$) | 0.005 | 0.495 | 0.990 |

BMI, body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; PTH, parathyroid hormone; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. P is significant ≤ 0.10.

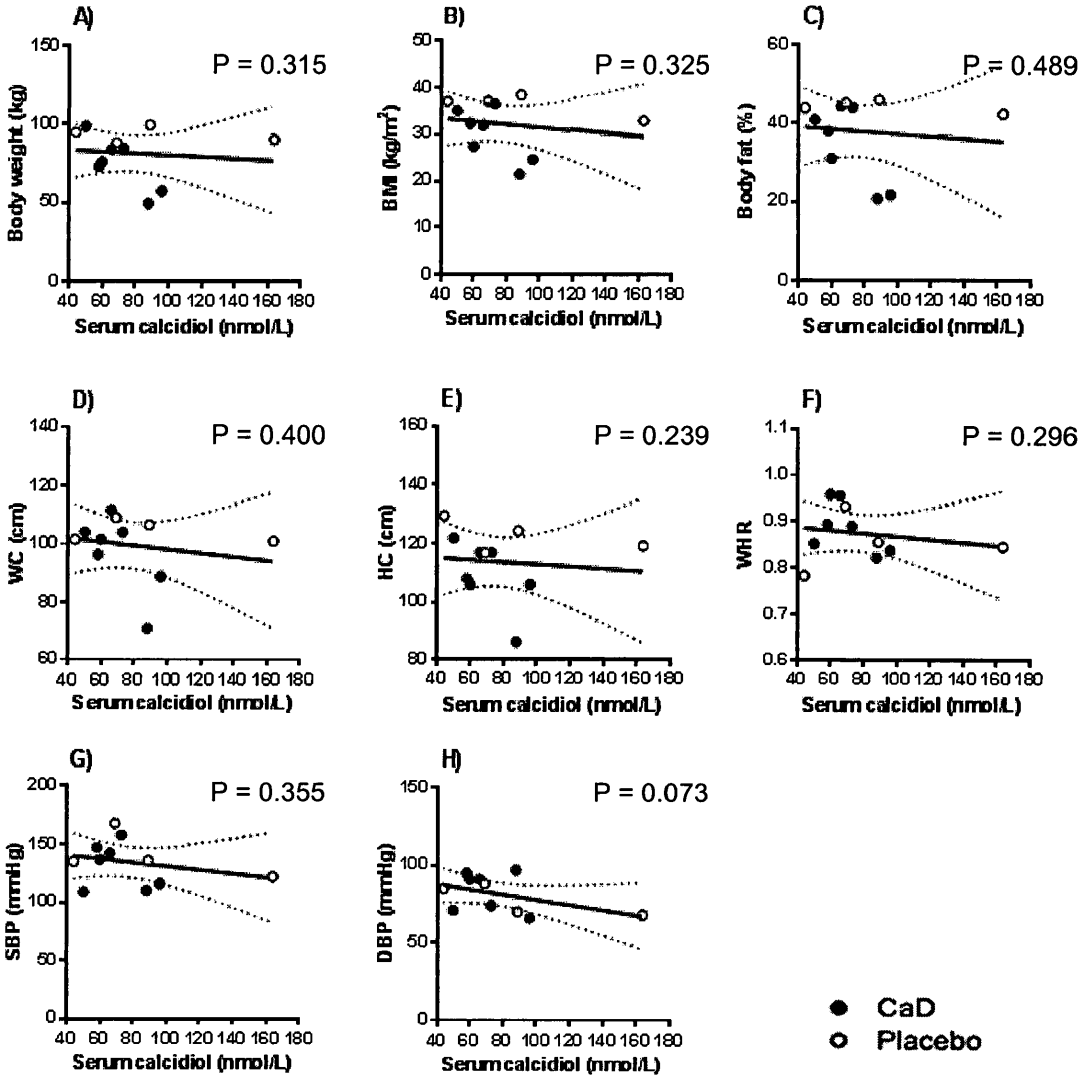


**Figure 4:** Correlations at 3 years between serum calcidiol (nmol/L) and A) body weight (kg), B) body mass index (BMI, kg/m$^2$), C) body fat (BF, %), D) waist circumference (cm), E) hip circumference (cm), F) waist-to-hip ratio (WHR), G) systolic blood pressure (SBP, mmHg), and H) diastolic blood pressure (DBP, mmHg), for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

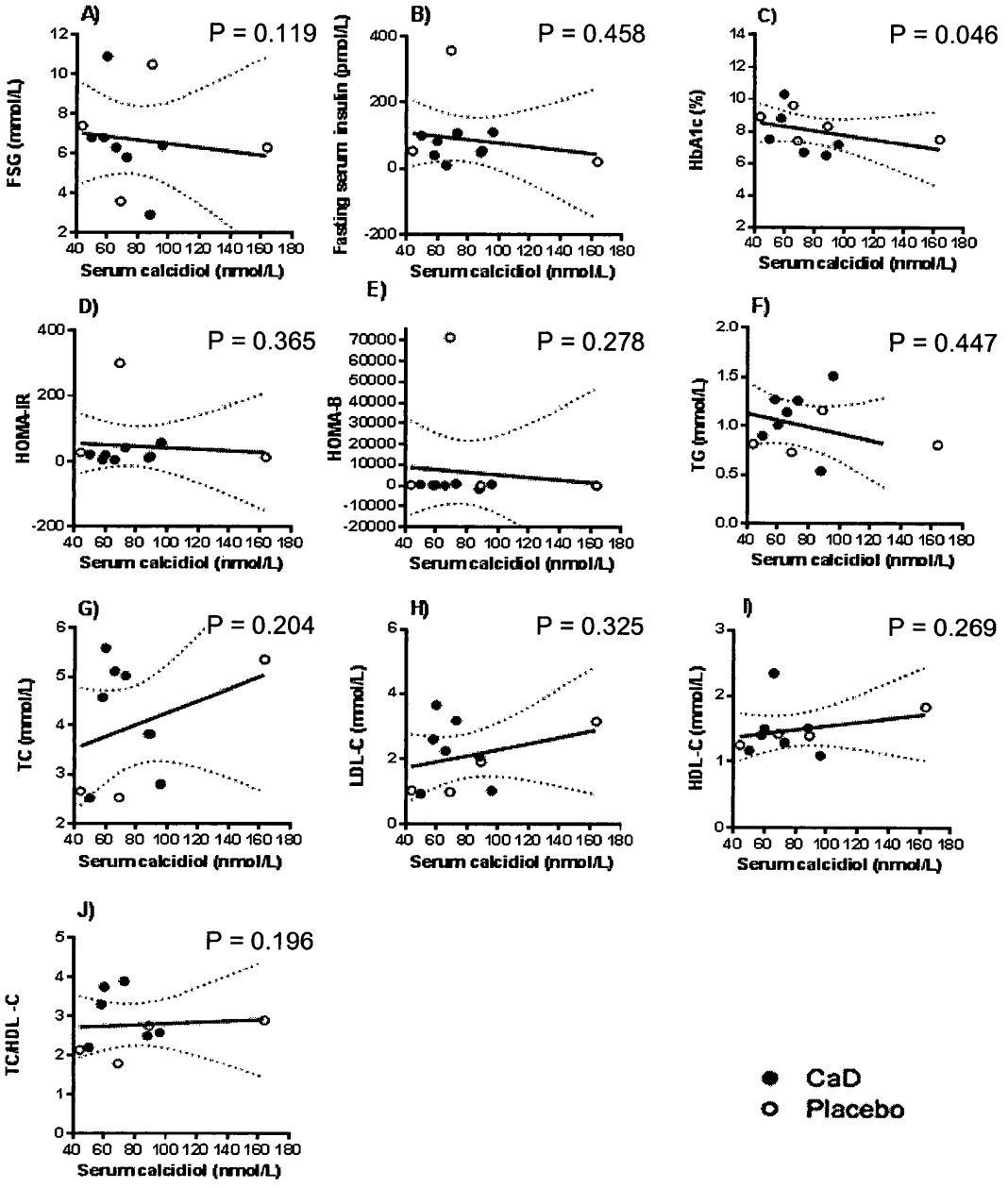


**Figure 5:** Correlations at 3 years between serum calcidiol (nmol/L) and A) fasting serum glucose (FSG, mmol/L), B) fasting serum insulin (pmol/L), C) glycated hemoglobinA1c (HbA1c, %), D) homeostatic model assessment of insulin resistance (HOMA-IR), E) homeostatic model assessment of β-cell function (HOMA-B), F) triglycerides (TG, mmol/L), G) total cholesterol (TC, mmol/L), H) low-density lipoprotein cholesterol (LDL-C, mmol/L), I) high-density lipoprotein cholesterol (HDL-C, mmol/L), and J) total cholesterol/HDL-C ratio (TC/HDL-C, mmol/L), for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

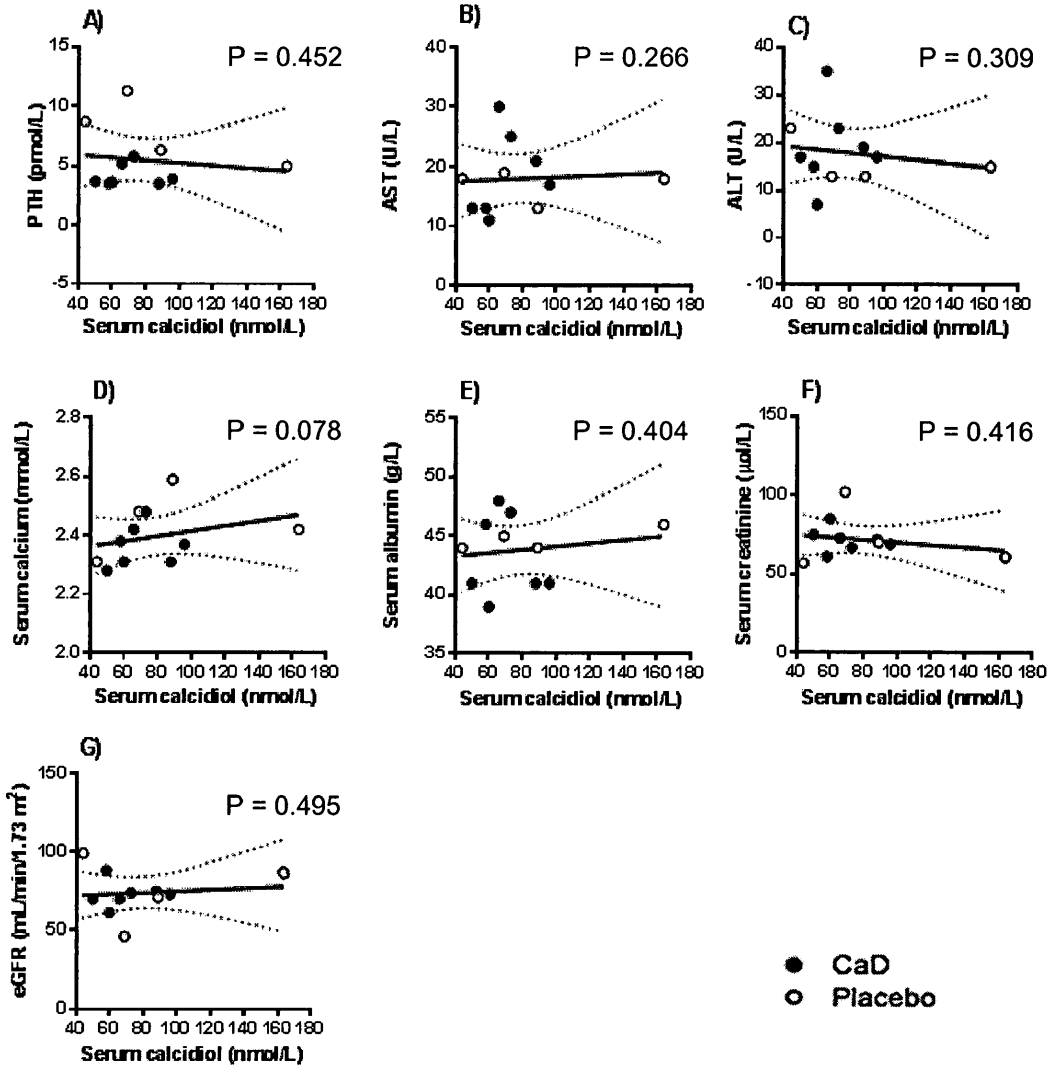


**Figure 6:** Correlations at 3 years between serum calcidiol (nmol/L) and A) parathyroid hormone (PTH, pmol/L), B) aspartate transaminase (AST, U/L), C) alanine transaminase (ALT, U/L), D) serum calcium (mmol/L), E) serum albumin (g/L), F) serum creatinine (µmol/L), and G) estimated glomerular filtration rate (eGFR, mL/min/1.73 $m^2$) for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

Absolute change in serum calcidiol over 3 years was negatively correlated with the absolute change in body weight, BMI, TBF, HC, SBP, and TC/HDL-C, PTH, AST ($P = 0.005$, $P = 0.005$, $P = 0.094$, $P = 0.004$, $P = 0.043$, $P = 0.085$, $P = 0.038$, $P = 0.066$, respectively) and positively correlated with the absolute change in WHR ($P = 0.014$) (Figures 7,8, and 9). A negative trend was observed between serum calcidiol and HbA1c ($P = 0.117$), LDL-C ($P = 0.156$), and a positive trend was observed between serum calcidiol and HDL-C ($P = 0.148$), and serum calcium ($P = 0.122$) (See Table 10 and Figures 7, 8, and 9).

**Table 10:** Spearman's rank coefficient bivariate correlation for the absolute change over 3 y in serum calcidiol with the different outcome measures (n = 11).

| Measurement | Correlation coefficient | P (1-tailed) | P (2-tailed) |
|---|---|---|---|
| Body weight (kg) | -0.736 | **0.005** | 0.010 |
| BMI ($kg/m^2$) | -0.736 | **0.005** | 0.010 |
| Total body fat (kg) | 0.173 | 0.306 | 0.612 |
| Body fat (%) | -0.428 | **0.094** | 0.188 |
| WC (cm) | 0.055 | 0.437 | 0.874 |
| HC (cm) | -0.755 | **0.004** | 0.007 |
| WHR | 0.655 | **0.014** | 0.029 |
| SBP (mmHg) | -0.540 | **0.043** | 0.086 |
| DBP (mmHg) | 0.114 | 0.369 | 0.736 |
| FSG (mmol/L) | 0.064 | 0.426 | 0.852 |
| Fasting serum insulin (pmol/L) | 0.014 | 0.484 | 0.968 |
| HbA1c (%) | -0.391 | 0.117 | 0.235 |
| HOMA-IR | -0.036 | 0.458 | 0.916 |
| HOMA-B | -0.227 | 0.251 | 0.502 |
| TG (mmol/L) | 0.087 | 0.400 | 0.800 |
| TC (mmol/L) | -0.036 | 0.458 | 0.916 |
| LDL-C (mmol/L) | -0.336 | 0.156 | 0.312 |
| HDL-C (mmol/L) | 0.346 | 0.148 | 0.296 |
| TC/HDL-C | -0.445 | **0.085** | 0.170 |
| PTH (pmol/L) | -0.555 | **0.038** | 0.077 |
| AST (U/L) | 0.483 | **0.066** | 0.133 |
| ALT (U/L) | -0.009 | 0.489 | 0.979 |
| Serum calcium (mmol/L) | 0.384 | 0.122 | 0.244 |
| Serum albumin (g/L) | -0.192 | 0.286 | 0.572 |
| Serum creatinine (µmol/L) | -0.129 | 0.353 | 0.706 |
| eGFR ($mL/min/1.73\ m^2$) | -0.046 | 0.446 | 0.892 |

BMI, body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; PTH, parathyroid hormone; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. P is significant ≤ 0.10.

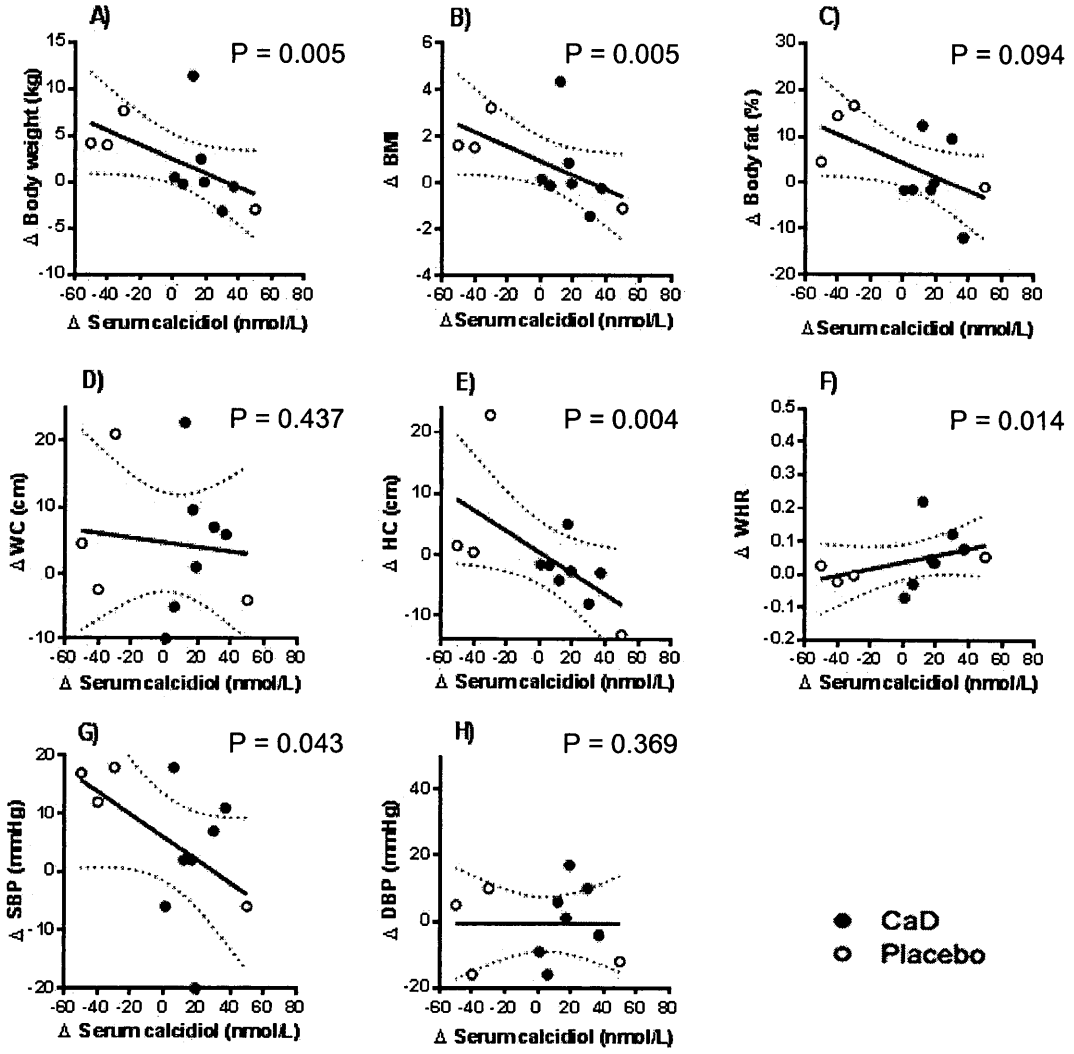


**Figure 7**: Correlations between the absolute change Δ in serum calcidiol (nmol/L) and the absolute change Δ in A) body weight (kg), B) body mass index (BMI, kg/m$^2$), C) body fat (BF, %), D) waist circumference (WC, cm), E) hip circumference (HC, cm), F) waist-to-hip ratio (WHR), G) systolic blood pressure (SBP, mmHg), and H) diastolic blood pressure (DBP, mmHg), for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

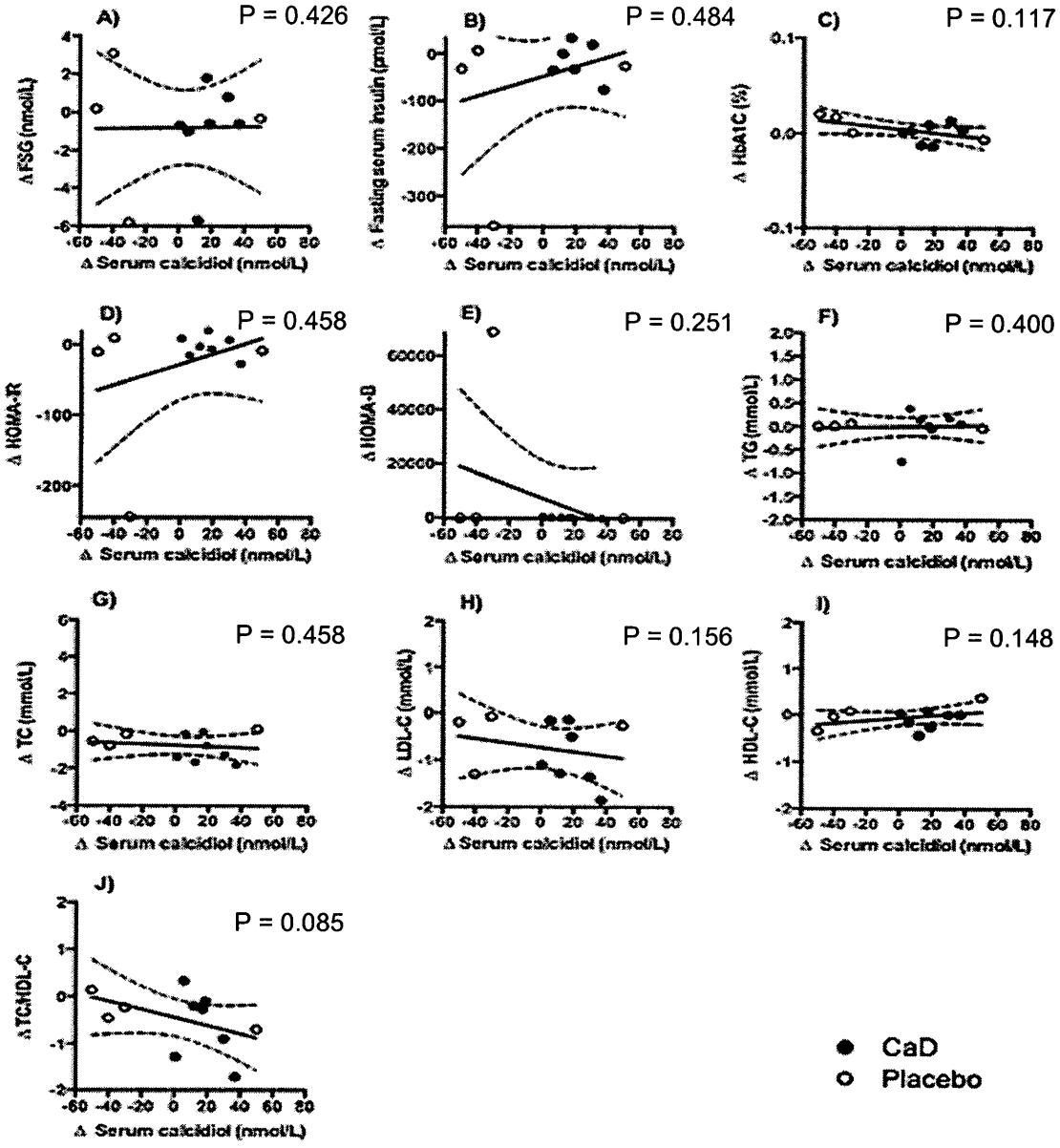


**Figure 8:** Correlations between the absolute change Δ in serum calcidiol (nmol/L) and the absolute change Δ in A) fasting serum glucose (FSG, mmol/L), B) fasting serum insulin (pmol/L), C) glycated hemoglobinA1c (HbA1c, %), D) homeostatic model assessment of insulin resistance (HOMA-IR), E) homeostatic model assessment of β-cell function (HOMA-B), F) triglycerides (TG, mmol/L), G) total cholesterol (TC, mmol/L), H) low-density lipoprotein cholesterol (LDL-C, mmol/L), I) high-density lipoprotein cholesterol (HDL-C, mmol/L), and J) total cholesterol/HDL-C ratio (TC/HDL-C, mmol/L),

at 3 years for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

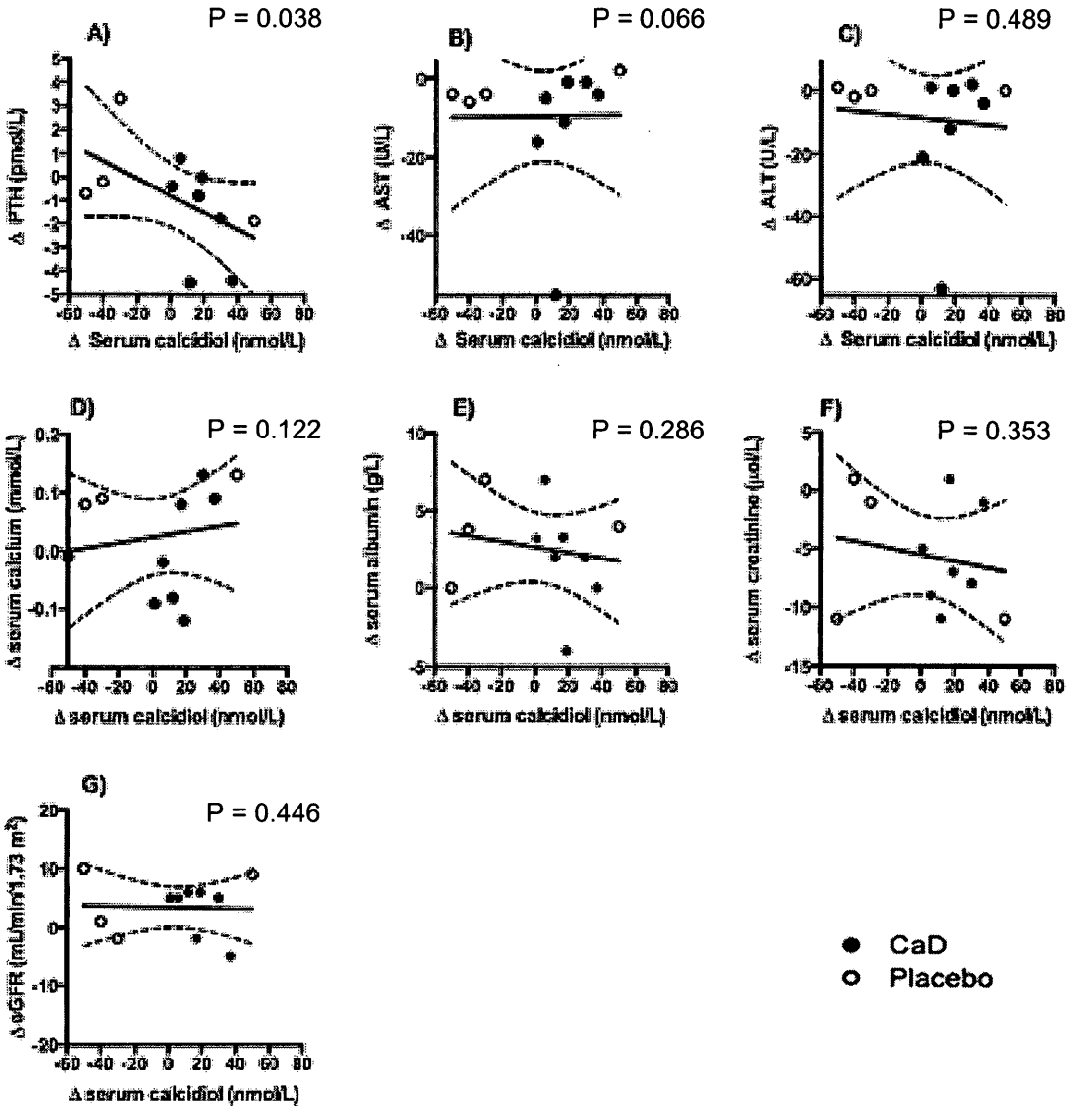


**Figure 9:** Correlations between the absolute change Δ in serum calcidiol (nmol/L) and the absolute change Δ in A) parathyroid hormone (PTH, pmol/L), B) aspartate transaminase (AST, U/L), C) alanine transaminase (ALT, U/L), D) serum calcium (mmol/L), E) serum albumin (g/L), F) serum creatinine (μmol/L), and G) estimated glomerular filtration rate (eGFR, mL/min/1.73 $m^2$) for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

Relative change in serum calcidiol over 3 years was negatively correlated with the relative change in body weight, BMI, TBF, HC, TC/HDL-C, and PTH ($P = 0.005$, $P = 0.005$, $P = 0.085$, $P = 0.013$, $P = 0.095$, and $P = 0.014$, respectively), and positively with WHR and serum calcium ($P = 0.008$ and $P = 0.059$) (See figures 10, 11, and 12). A negative trend was observed between serum calcidiol and SBP ($P = 0.123$). In addition, a positive trend was observed between serum calcidiol and DBP ($P = 0.123$) and HDL-C ($P = 0.156$) (Table 11).

**Table 11:** Spearman's rank coefficient bivariate correlation for the relative change over 3 y in serum calcidiol, with the different outcome measures (n = 11).

| Measurement | Correlation coefficient | P (1-tailed) | P (2-tailed) |
|---|---|---|---|
| Body weight (kg) | -0.736 | **0.005** | 0.010 |
| BMI (kg/m$^2$) | -0.736 | **0.005** | 0.010 |
| Total body fat (kg) | -0.736 | **0.005** | 0.010 |
| Body fat (%) | -0.445 | **0.085** | 0.170 |
| WC (cm) | 0.182 | 0.296 | 0.592 |
| HC (cm) | -0.664 | **0.013** | 0.026 |
| WHR | 0.700 | **0.008** | 0.016 |
| SBP (mmHg) | -0.382 | 0.123 | 0.246 |
| DBP (mmHg) | 0.164 | 0.315 | 0.631 |
| FSG (mmol/L) | 0.109 | 0.375 | 0.750 |
| Fasting serum insulin (pmol/L) | 0.064 | 0.426 | 0.852 |
| HbA1c (%) | -0.218 | 0.260 | 0.520 |
| HOMA-IR | 0.109 | 0.375 | 0.750 |
| HOMA-B | -0.009 | 0.489 | 0.978 |
| TG (mmol/L) | 0.218 | 0.260 | 0.520 |
| TC (mmol/L) | -0.041 | 0.452 | 0.904 |
| LDL-C (mmol/L) | -0.064 | 0.426 | 0.852 |
| HDL-C (mmol/L) | 0.336 | 0.156 | 0.312 |
| TC/HDL-C | -0.427 | **0.095** | 0.190 |
| PTH (pmol/L) | -0.655 | **0.014** | 0.028 |
| AST (U/L) | 0.318 | 0.170 | 0.340 |
| ALT (U/L) | -0.009 | 0.489 | 0.978 |
| Serum calcium (mmol/L) | 0.500 | **0.059** | 0.118 |
| Serum albumin (g/L) | -0.087 | 0.400 | 0.546 |
| Serum creatinine (µmol/L) | -0.045 | 0.447 | 0.894 |
| e-GFR (mL/min/1.73 m$^2$) | -0.145 | 0.335 | 0.670 |

BMI, body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; PTH, parathyroid hormone; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. P is significant ≤ 0.10.

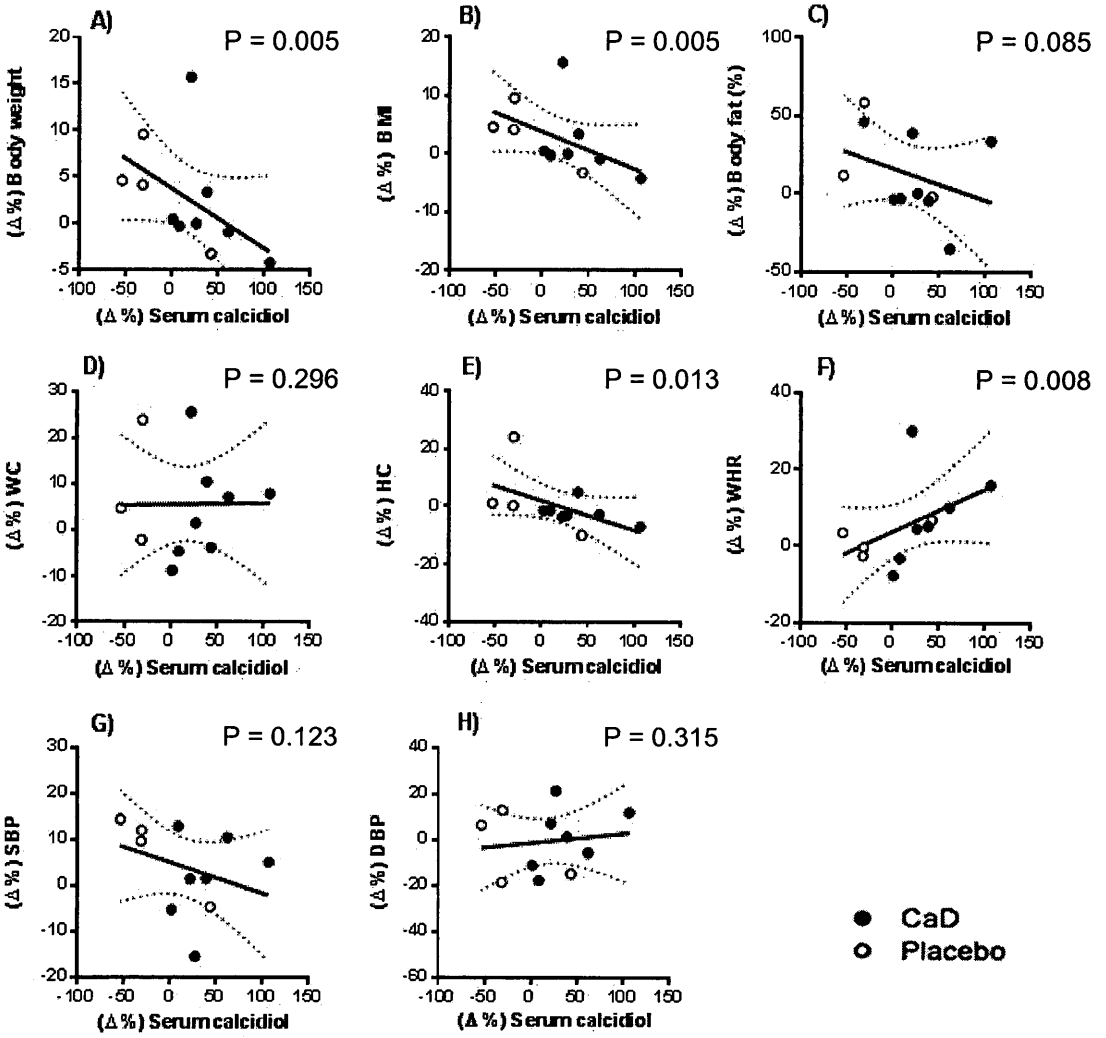


**Figure 10:** Correlations between the relative change (Δ%) in serum calcidiol (nmol/L) and the relative change (Δ%) in A) body weight (kg), B) body mass index (BMI, kg/m$^2$), C) body fat (BF, %), D) waist circumference (WC, cm), E) hip circumference (HC, cm), F) waist-to-hip ratio (WHR), G) systolic blood pressure (SBP, mmHg), and H) diastolic blood pressure (DBP, mmHg), for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

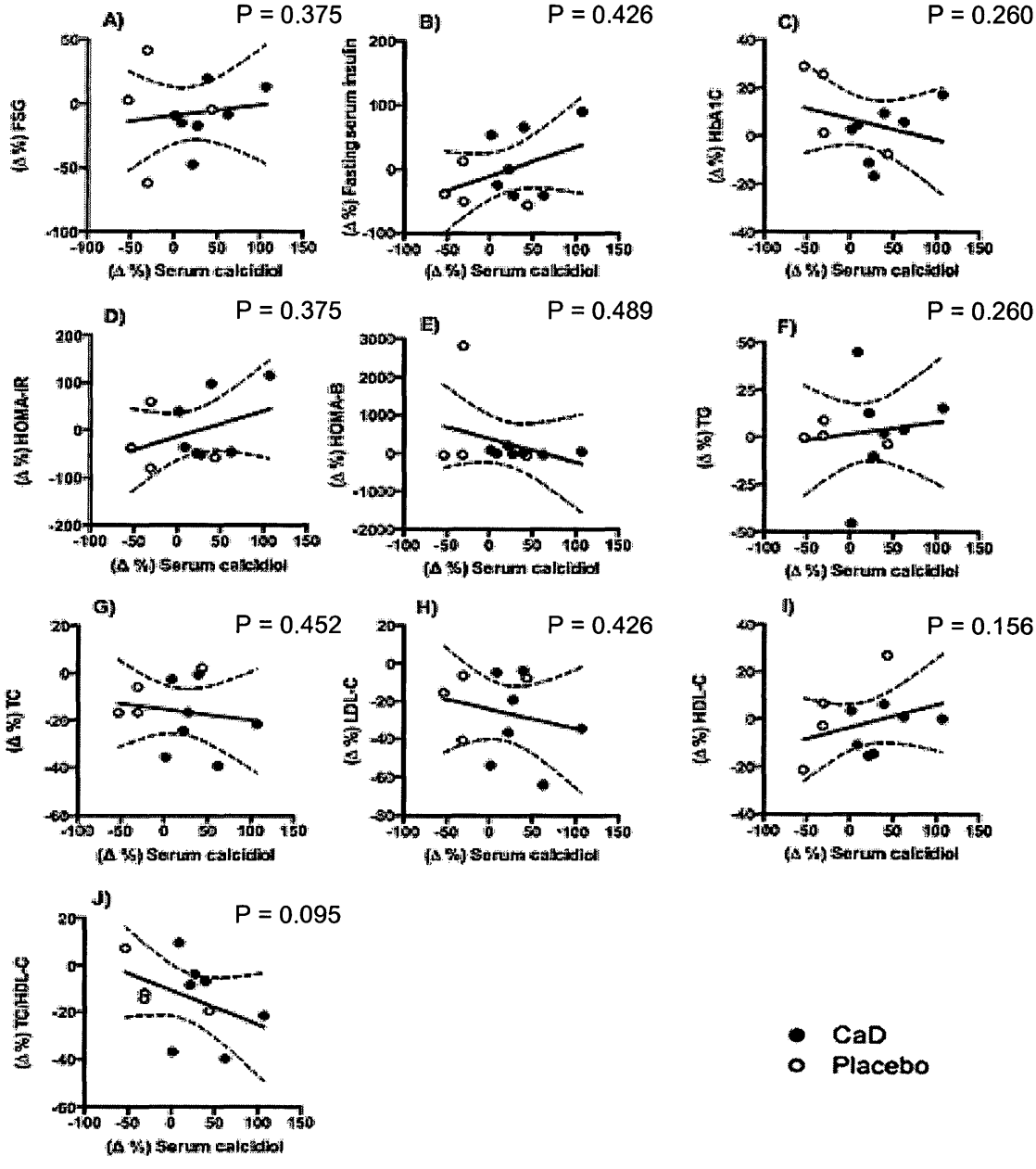


**Figure 11:** Correlations between the relative change (Δ%) in serum calcidiol (nmol/L) and the relative change (Δ%) in A) fasting serum glucose (FSG, mmol/L), B) fasting serum insulin (pmol/L), C) glycated hemoglobinA1c (HbA1c, %), D) homeostatic model assessment of insulin resistance (HOMA-IR), E) homeostatic model assessment of β-cell function (HOMA-B), F) triglycerides (TG, mmol/L), G) total cholesterol (TC, mmol/L), H) low-density lipoprotein cholesterol (LDL-C, mmol/L), I) high-density lipoprotein cholesterol (HDL-C, mmol/L), and J) total cholesterol/HDL-C ratio (TC/HDL-C, mmol/L),

for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

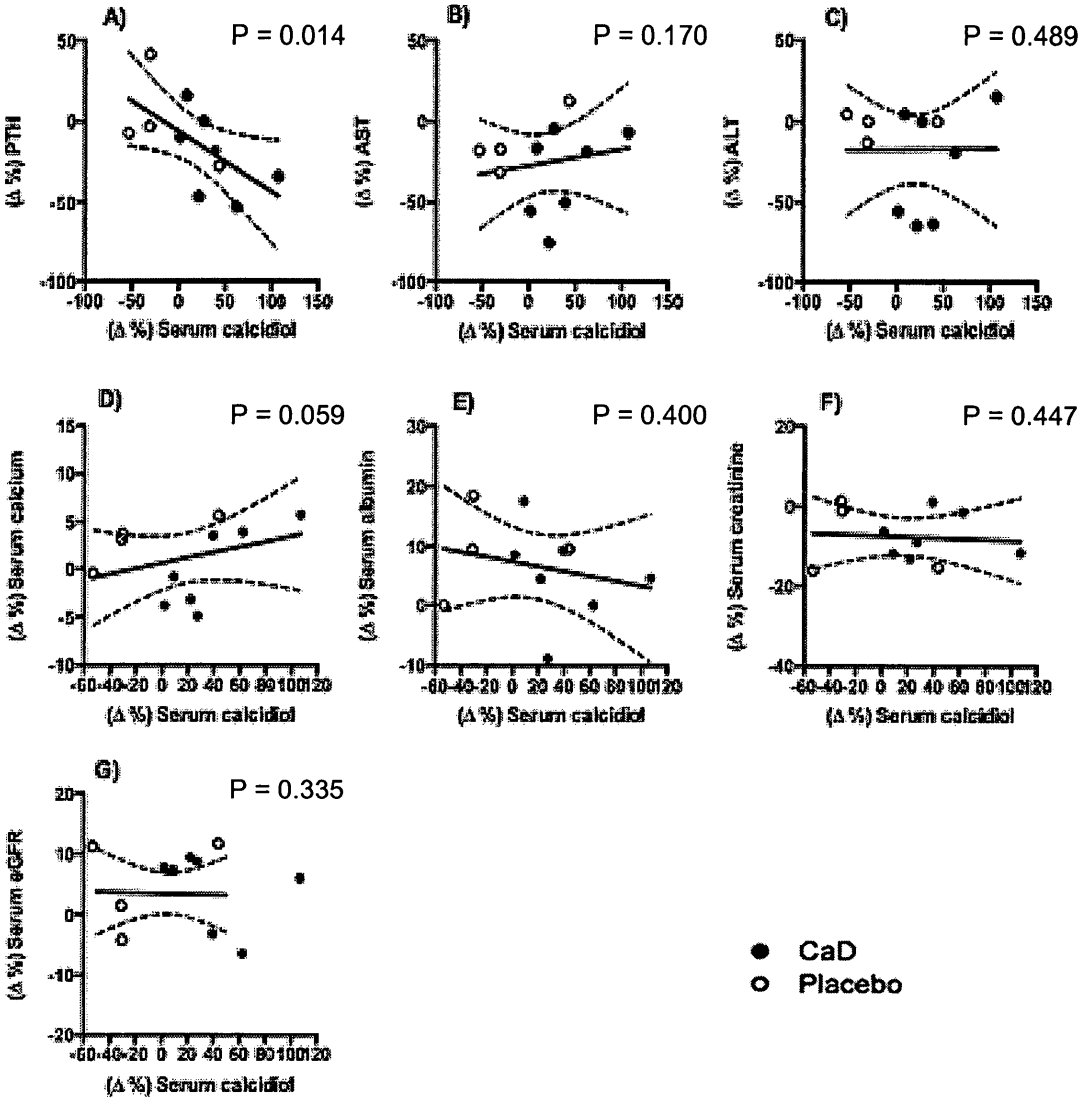


**Figure 12:** Correlations between the relative change (Δ%) in serum calcidiol (nmol/L) and the relative change (Δ%) in A) parathyroid hormone (PTH, pmol/L), B) aspartate transaminase (AST, U/L), C) alanine transaminase (ALT, U/L), D) serum calcium (mmol/L), E) serum albumin (g\L), F) serum creatinine (µmol/L), and G) estimated glomerular filtration rate (eGFR, mL/min/1.73 $m^2$) for 11 T2DM patients (open circles, placebo; solid circles, CaD; according to per-protocol method).

Table 12 depicts the results based on the per-protcol, in the CaD group, serum calcidiol increased significantly (P = 0.018), while the following measures decreased significantly over 3 years total body fat (kg) (P = 0.018), TC (P = 0.018), LDL-C (P = 0.034), TC/HDL (P = 0.091), PTH (P = 0.093), AST (P = 0.018) and serum creatinine (P = 0.041), compared to their baseline levels. In the placebo group, total body fat decreased significantly (P = 0.068), and HbA1c increased significantly (P = 0.068), compared to their baseline levels. Moreover, after 3 years, CaD group had significantly lower body weight (P = 0.042), BMI (P = 0.024), total body fat (P = 0.042), body fat (%) (P = 0.073), HC (P = 0.042), and PTH (P = 0.024), compared to the placebo group

**Table 12:** Comparison of the medians in outcome measures in participants, both within group and between groups, following **per-protocol** analysis, for T2DM

| Measurement | Placebo n = 4 | | | CaD n = 7 | | | P*† |
|---|---|---|---|---|---|---|---|
| | Median | | P | Median | | P | |
| | Baseline | 3 years | | Baseline | 3 years | | |
| Body weight (kg) | 91.9 | 92.6 | 0.144 | 73.5 | 76.0 | 0.674 | **0.042** |
| BMI (kg/m$^2$) | 34.7 | 37.1 | 0.144 | 27.7 | 32.0 | 0.753 | **0.024** |
| Total body fat (kg) | 30.9 | 20.1 | **0.068** | 24.7 | 16.5 | **0.018** | **0.042** |
| Body fat (%) | 35.4 | 44.6 | 0.144 | 32.5 | 38.1 | 0.866 | **0.073** |
| WC (cm) | 101.0 | 104.1 | 0.465 | 89.5 | 96.5 | 1.000 | 0.315 |
| HC (cm) | 126.0 | 121.9 | 0.465 | 116.0 | 108.0 | 0.176 | **0.042** |
| WHR | 0.84 | 0.86 | 0.581 | 0.79 | 0.89 | 0.108 | 0.527 |
| SBP (mmHg) | 126.0 | 135.5 | 0.144 | 135.0 | 137.0 | 0.498 | 0.788 |
| DBP (mmHg) | 80.0 | 77.5 | 0.465 | 85.0 | 91.0 | 0.310 | 0.315 |
| Serum calcidiol (nmol/L) | 106.5 | 79.0 | 0.581 | 54.0 | 66.0 | **0.018** | 0.648 |
| FSG (mmol/L) | 7.3 | 6.9 | 0.715 | 7.0 | 6.4 | 0.498 | 0.788 |
| Fasting serum insulin (pmol/L) | 67.0 | 53.5 | 0.144 | 65.0 | 83.0 | 0.598 | 1.000 |
| HbA1C (%) | 7.1 | 7.9 | **0.068** | 7.5 | 7.5 | 0.672 | 0.788 |
| HOMA-IR | 21.7 | 21.3 | 0.273 | 20.2 | 27.8 | 0.866 | 1.000 |
| HOMA-B | 384.04 | 213.0 | 0.715 | 178.6 | 242.4 | 0.866 | 1.000 |
| TG (mmol/L) | 0.83 | 0.82 | 0.593 | 1.01 | 1.14 | 0.446 | 0.230 |
| TC (mmol/L) | 3.91 | 3.25 | 0.144 | 5.17 | 4.58 | **0.018** | 0.527 |
| LDL-C (mmolL/L) | 2.23 | 1.48 | 0.680 | 3.33 | 2.25 | **0.018** | 0.527 |
| HDL-C (mmol/L) | 1.44 | 1.41 | 0.715 | 1.40 | 1.40 | 0.345 | 1.000 |
| TC/HDL-C | 2.63 | 2.45 | 0.144 | 3.56 | 2.59 | **0.091** | 0.315 |
| PTH (pmol/L) | 7.5 | 7.5 | 0.715 | 5.0 | 3.7 | **0.093** | **0.024** |
| AST (U/L) | 20.5 | 18.0 | 0.144 | 22.0 | 17.0 | **0.018** | 1.000 |
| ALT (U/L) | 15.0 | 14.0 | 0.285 | 21.0 | 17.0 | 0.116 | 0.412 |
| Serum calcium (mmol/L) | 2.4 | 2.5 | 0.144 | 2.4 | 2.4 | 0.751 | 0.164 |
| Serum albumin (g/L) | 43.0 | 44.5 | 0.285 | 41.0 | 41.0 | 0.18 | 0.648 |
| Serum creatinine (µmol/L) | 70.5 | 65.5 | 0.194 | 79.0 | 72.0 | **0.041** | 0.527 |
| eGFR (ml/min/1.73m$^2$) | 73.5 | 78.5 | 0.273 | 69.0 | 73.0 | 0.667 | 0.788 |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG;

triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. P is significant ≤ 0.10. P* denotes significance between groups.

**Table 13:** Comparison of the medians of the percent change differences in outcome measures in participants, following **per-protocol** analysis.

| Measurement | Placebo n = 4 | CaD n = 7 | P |
|---|---|---|---|
| Body weight | 4.4 | 0.0 | 0.412 |
| BMI | 4.4 | 0.0 | 0.412 |
| Total body fat | -32.6 | -35.4 | 0.412 |
| Body fat (%) | 28.9 | -3.3 | 0.109 |
| WC | 1.3 | 7.1 | 0.927 |
| HC | 0.7 | -2.8 | 0.412 |
| WHR | 1.6 | 5.2 | 0.527 |
| SBP | 10.8 | 1.5 | 0.315 |
| DBP | -4.4 | 7.1 | 0.412 |
| Serum calcidiol | -30.7 | 27.5 | 0.109 |
| FSG | -0.9 | -9.3 | 0.648 |
| Fasting serum insulin | -44.4 | 0.0 | 0.164 |
| HbA1C | 13.6 | 4.7 | 0.527 |
| HOMA-IR | -47.1 | -35.6 | 0.315 |
| HOMA-B | -39.4 | 25.6 | 0.230 |
| TG | 0.4 | 4.1 | 0.527 |
| TC | -11.3 | -21.8 | 0.230 |
| LDL-C | -11.5 | -34.3 | 0.648 |
| HDL-C | 2.0 | 0.0 | 0.648 |
| TC/HDL-C | -13.1 | -8.3 | 1.000 |
| PTH | -5.3 | -18.2 | 0.315 |
| AST | -17.8 | -19.0 | 0.527 |
| ALT | 0.0 | -19.0 | 0.412 |
| Serum calcium | 3.5 | -0.8 | 0.412 |
| Serum albumin | 9.5 | 4.6 | 0.527 |
| Serum creatinine | -8.1 | -8.9 | 0.927 |
| eGFR | 6.3 | 7.3 | 0.648 |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. *P is significant ≤ 0.10.

Table 14 depicts the results based on the retrospective method, compared to baseline, the CaD group had a significant decrease in total body fat (P = 0.028), TC (P = 0.075), LDL-C (P = 0.028), TC/HDL-C (P = 0.028), PTH (P = 0.043), and serum creatinine (P = 0.058), and increase in WHR (P = 0.028), and serum calcidiol (P = 0.028), compared to baseline. While the placebo group had a significant increase in body weight (P = 0.080), SBP (P = 0.078), and HbA1c (P = 0.068), and a significant decrease in total body fat (P = 0.043), TC (P = 0.068), AST (P = 0.066), and serum creatinine (P = 0.068). After 3 years, subjects who were assigned to the CaD group, had significantly lower body weight (P = 0.017), BMI (P = 0.004), total body fat (P = 0.017), body fat (%) (P = 0.052), WC (P = 0.082), HC (P = 0.030), and PTH (P = 0.030), compared to the placebo group. Retrospective analysis also showed differences between the CaD vs. placebo in hip circumference (-3.3% vs. +0.3%, respectively, P = 0.052), serum calcidiol (+41.7% vs. -30.3%, respectively, P = 0.004), serum PTH (-30.8% vs. -3.1 %, respectively, P = 0.003), systolic blood pressure (-1.5% vs. +12.0%, respectively, P = 0.126) (Table 15).

Table 14: Comparison of the medians in outcome measures in both, within group and between groups, following **retrospective** analysis, for T2DM (n = 11).

| Measurement | Placebo n = 5 | | | CaD n = 6 | | | P* |
|---|---|---|---|---|---|---|---|
| | Median | | P | Median | | P | |
| | Baseline | 3 years | | Baseline | 3 years | | |
| Body weight (kg) | 90.8 | 95.0 | **0.080** | 73.0 | 74.5 | 0.893 | **0.017** |
| BMI (kg/m$^2$) | 35.5 | 37.1 | 0.655 | 27.1 | 29.7 | 0.893 | **0.004** |
| Total body fat (kg) | 30.5 | 20.6 | **0.043** | 24.5 | 16.2 | **0.028** | **0.017** |
| Body fat (%) | 39.3 | 44.1 | 0.180 | 32.3 | 34.6 | 0.893 | **0.052** |
| WC (cm) | 109.0 | 104.0 | 0.180 | 89.3 | 92.7 | 0.600 | **0.082** |
| HC (cm) | 123.5 | 121.9 | 0.893 | 112.5 | 107.0 | 0.173 | **0.030** |
| WHR | 0.92 | 0.86 | 0.225 | 0.78 | 0.87 | **0.028** | 0.792 |
| SBP (mmHg) | 124.0 | 136.0 | **0.078** | 132.5 | 129.5 | 0.752 | 0.662 |
| DBP (mmHg) | 80.0 | 74.0 | 0.343 | 82.5 | 91.0 | 0.173 | 0.429 |
| Serum calcidiol (nmol/L) | 94.0 | 69.0 | 0.465 | 56.5 | 77.0 | **0.028** | 0.429 |
| FSG (mmol/L) | 7.4 | 6.8 | 0.144 | 6.8 | 6.4 | 0.752 | 0.792 |
| Fasting insulin (pmol/L) | 86.0 | 100.0 | 0.357 | 48.5 | 44.5 | 0.416 | 0.126 |
| HbA1C (%) | 6.9 | 7.5 | **0.068** | 7.9 | 8.2 | 0.833 | 0.662 |
| HOMA-IR | 27.5 | 27.8 | 0.500 | 13.3 | 9.2 | 0.463 | 0.247 |
| HOMA-B | 464.9 | 606.1 | 0.500 | 173.3 | 187.2 | 0.345 | **0.082** |
| TG (mmol/L) | 0.87 | 0.90 | 1.000 | 1.00 | 1.08 | 0.293 | 0.792 |
| TC (mmol/L) | 3.93 | 2.67 | **0.068** | 5.44 | 4.85 | **0.075** | **0.052** |
| LDL-C (mmolL/L) | 2.05 | 1.05 | 0.109 | 3.48 | 2.43 | **0.028** | 0.177 |
| HDL-C (mmol/L) | 1.43 | 1.29 | 0.465 | 1.42 | 1.50 | 0.893 | 0.126 |
| TC/HDL-C | 3.22 | 2.20 | 0.715 | 3.82 | 2.75 | **0.028** | 0.329 |
| PTH (pmol/L) | 6.5 | 6.3 | 0.465 | 6.1 | 3.8 | **0.043** | **0.030** |
| AST (U/L) | 23.0 | 18.0 | **0.066** | 21.5 | 17.5 | 0.115 | 1.000 |
| ALT (U/L) | 22.0 | 17.0 | 1.000 | 19.0 | 16.0 | 0.144 | 1.000 |
| Serum calcium (mmol/L) | 2.4 | 2.5 | 0.581 | 2.3 | 2.4 | 0.141 | 0.429 |
| Serum albumin (g/L) | 40.0 | 44.0 | 0.180 | 43.0 | 43.5 | 0.581 | 1.000 |
| Serum creatinine (µmol/L) | 76.0 | 70.0 | **0.068** | 75.5 | 70.5 | **0.058** | 1.000 |
| eGFR (ml/min/1.73m$^2$) | 69.0 | 71.0 | 0.102 | 73.0 | 74.0 | 0.140 | 0.662 |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function;

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. P is significant ≤ 0.10. P* denotes significance between groups.

**Table 15:** Comparison of the medians of the percent change differences in outcome measures in participants, following **retrospective** analysis.

| Measurement | Placebo n = 5 | CaD n = 6 | P |
|---|---|---|---|
| Body weight | 4.2 | -0.4 | 0.247 |
| BMI | 4.2 | -0.4 | 0.247 |
| Total body fat | -32.7 | -35.7 | 0.247 |
| Body fat (%) | 11.7 | -1.1 | 0.429 |
| WC | -2.0 | 7.5 | 0.247 |
| HC | 0.3 | -3.3 | **0.052** |
| WHR | -2.52 | 8.49 | **0.004** |
| SBP | 12.0 | 1.5 | 0.126 |
| DBP | -11.3 | 4.1 | 0.329 |
| Serum calcidiol | -30.3 | 41.7 | **0.004** |
| FSG | -9.3 | -6.6 | 0.931 |
| Fasting serum insulin | -24.5 | -20.1 | 1.000 |
| HbA1C | 4.7 | -0.8 | 0.329 |
| HOMA-IR | -35.6 | -46.6 | 1.000 |
| HOMA-B | 8.4 | 12.8 | 1.000 |
| TG | 0.9 | 3.1 | 0.931 |
| TC | -16.7 | -19.3 | 1.000 |
| LDL-C | -15.3 | -26.7 | 1.000 |
| HDL-C | -2.8 | 0.5 | 0.792 |
| TC/HDL-C | -11.8 | -13.9 | 0.537 |
| PTH | -3.1 | -30.8 | **0.030** |
| AST (U/L) | -18.2 | -13.1 | 0.662 |
| ALT (U/L) | 0.0 | -9.5 | 0.537 |
| Serum calcium | -0.4 | 3.8 | 0.429 |
| Serum albumin | 9.5 | 4.5 | 0.429 |
| Serum creatinine | -6.3 | -10.2 | 0.792 |
| eGFR | 7.3 | 7.4 | 0.931 |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein

cholesterol; TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. *P is significant ≤ 0.10.

Diet intake was assessed by 3-day (2 weekdays and 1 weekend) diet record at baseline and at the 3-year follow-up. Mean intake of the macro- and micro- nutrients is summarized in Table 16. Vitamin D and calcium intake through the diet were lower than the recommended daily allowance (600 IU/d and 1200 mg, respectively).

**Table 16:** Macronutrients intake at baseline and at 3 years for all T2DM patients (n =11), presented as means ± SD.

| Macronutrients | Baseline Mean ± SD | 3 years Mean ± SD |
|---|---|---|
| Energy (Kcal) | 1458 ± 578 | 1394 ± 522 |
| Carbohydrates (g) | 228 ± 123 | 210 ± 110 |
| Total protein (g) | 63 ± 14 | 37 ± 13 |
| Total fat (g) | 36 ± 14 | 62 ± 15 |
| Saturated fat (g) | 9 ± 4 | 10 ± 4 |
| Monounsaturated | 12 ± 5 | 12 ± 4 |
| Polyunsaturated | 8 ± 4 | 8 ± 4 |
| Cholesterol (mg) | 279 ± 301 | 296 ± 330 |
| Vitamin D (IU) | 91.0 ± 48.8 | 101.0 ± 47.9 |
| Calcium (mg) | 492 ± 264 | 537 ± 264 |

## 12. Discussion

This clinical trial investigated the effect of vitamin $D_3$ and calcium supplementation on glycemic control and other sequelae in type 2 diabetic patients from multi-ethnic background. In the present study, absolute and relative change in serum calcidiol (vitamin D status) negatively correlated with the absolute and relative change in body weight and BMI, total body fat (only in the relative change), body fat (%), HC, SBP, TC/HDL-C, PTH, and positively correlated with absolute and relative change in WHR and serum calcium (only in the relative change). At 3 years, there was a negative correlation between serum calcidiol and DBP and HbA1C. When the data were analyzed using the per-protocol method was used, the CaD group had a significant increase in serum calcidiol, and decrease in TC, LDL-C, TC/HDL, PTH, AST, and serum creatinine, compared to baseline values. However, when the data were analyzed using the retrospective method, the CaD group had a significant increase in WHR and serum calcidiol, and decrease in total body fat, TC, LDL-C, TC/HDL-C, PTH, and serum creatinine, compared to baseline values.

Serum calcidiol was measured in all participants during the summer months (May-June) at baseline and after 3 years. Although, median serum calcidiol levels increased significantly in the CaD group by 27%, it did not reach optimal levels (> 75 nmol/L) (72,88) when analyzed using per-protocol, likely due to poor compliance and poor adherence to the study protocol. However, when

using the retrospective method, all subjects had serum calcidiol levels > 50 nmol/L, that accounted for a modest 41% increase, reaching an average of 77 nmol/L, a value considered optimal according to the IOM (71). Additionally, the retrospective method showed differences between the CaD vs. placebo in serum calcidiol (+41.7% vs. -30.3%, respectively, P = 0.004). The poor compliance could be attributed to participants requiring consuming 4 pills per day combined with quite lengthy study duration. Dietary vitamin D intake was assessed by 3-day food record; mean vitamin D intake at baseline (90 IU/d) and at 3 years (101 IU/d) does not meet vitamin D RDA (600 IU/d) (54). In another human clinical trial, the consumption of 2000 IU/d $D_3$ for 12 weeks, allowed for optimal (> 75 nmol/L) serum calcidiol levels in Black youth who had baseline levels of 33.1 nmol/L (101). Moreover, 1000 IU/d of vitamin $D_3$ and calcium fortified yogurt increased serum calcidiol levels to > 70 nmol/L from baseline values of 44 nmol/L, after 12 weeks in Middle-Eastern diabetic patients (age 50.7 ± 6.1 y) (111). However, diabetic patients who had serum calcidiol baseline levels of 29 nmol/L and were supplemented with the same 1000 IU/d dose for 12 months did not achieve optimal levels (43.8 nmol/L); authors reported a large number of drop-outs (158). In Black youth, daily consumption of 2000 IU $D_3$ increased serum calcidiol levels from 33.1 to 86 nmol/L after 16 weeks (90). Finally, in clinical trials that used the current upper tolerable intake (4000 IU/$D_3$), serum calcidiol levels consistently reached optimal levels after 12 weeks (144,148,159)

or 16 weeks (160). Findings of the present study confirm the need to revise the vitamin D recommended daily allowance while considering ethnicity and health conditions.

Serum calcidiol and body composition

The change in serum calcidiol (absolute and relative vs. baseline) negatively correlated with body weight, BMI, body fat (%), and HC. These findings are consistent with those of Nikooyeh et al, in which changes in serum calcidiol negatively correlated with weight ($r = -0.331$, $P = 0.001$), BMI ($r = -0.358$, $P = 0.001$), and fat mass ($r = -0.219$, $P = 0.038$) (93,111). Moreover, absolute and relative change in serum calcidiol levels negatively correlated with PTH, as corroborated by previous studies (85,89,161,162). The beneficial effect of vitamin D on adiposity could be explained by its effect on PTH and, therefore, serum calcium (108). High intracellular $Ca^{2+}$ concentrations promote lipogenesis and, consequently, weight gain (108,163,164). In a population-based study, serum calcidiol was inversely associated with BMI, TBF%, and WC (standardized β-values -0.096, -0.194, and -0.109, $P < 0.05$; respectively); with these variables being positively associated with PTH (standardized β-values 0.126, 0.214, and 0.071 $P < 0.005$, respectively) (100). In the Framingham Heart study, vitamin D deficiency (< 49.8 nmol/L) was higher among those who had a BMI of ≥ 30 $kg/m^2$, and serum calcidiol concentration was inversely associated with WC and BMI (regression coefficient [SE] -3.11, $P < 0.0001$ and -2.81, $P <$

0.0001, respectively) after adjustment for age, sex, and season (165). Vitamin D is a fat soluble vitamin which is stored in the body fat compartments, which explains the association between low circulating calcidiol concentrations and increased fat mass (180). The positive correlation observed between serum calcidiol and WHR in the current analysis is an artifact due to a significant negative correlation with HC, but not WC.

In both analyses (per-protocol and retrospective), those who were in the CaD group had significantly lower body weight, BMI, total body fat, and body fat (%), WC (only in retrospective), and HC after 3 years as compared to the placebo group. The observed non-significant increase in BMI in the CaD group, compared to baseline, is due to the increase in body weight not body fat; hence, the significant decrease in total body fat indicates that subjects in the CaD group became leaner by 36% (retrospective). Vitamin $D_3$ and calcium supplementation attenuated the increase in HC (retrospective: -3.25% vs +0.32%, P = 0.052), body weight and other body composition measures (body weight, BMI, body fat (%)).

Absolute change in serum calcidiol negatively correlated with the absolute change in SBP. At 3 years, serum calcidiol negatively correlated with DBP. Hypertension is highly prevalent in diabetic patients, and contributes to increased risk of cardiovascular disease (166). These findings further support the hypothesis that vitamin D is a negative regulator of the renin-angiotensin

system, generated from animal model studies (167). In vivo, VDR knock-out mice exhibit significant increases in SBP and DBP compared to wild type (≈ 29% and 27%, respectively) (167). This is also in agreement with a cross-sectional study which found serum calcitriol to be inversely correlated with DBP ($r = -0.41$) in healthy, middle-aged men (mean age 63 y) (168). However, results from human clinical trials are inconclusive and equivocal. In one of the largest human clinical trials, vitamin $D_3$ and calcium supplementation (400 IU $D_3$/d and 1000 mg of elemental calcium) did not affect SBP or DBP in multi-ethnic postmenopausal women (169), perhaps due to the low amount of supplemental vitamin $D_3$. Another randomized clinical trial investigated the effect of 5000 IU $D_3$/d for 12 weeks on endothelial function in 50 diabetic patients (170). After 12 weeks, 5000 IU of vitamin $D_3$ did not affect SBP or DBP (170). In the current study, subjects who were in the placebo group had significantly higher SBP by 12% after 3 years compared to baseline values, according to retrospective analysis. Additionally, vitamin $D_3$ supplementation attenuated the increase in SBP in CaD vs placebo (retrospective: +1% vs +12, respectively, $P = 0.126$)

Moreover, PTH decreased significantly in the CaD by 31% after 3 years compared to baseline values, according to retrospective analysis, (vs. -3% in the placebo group).

Serum calcidiol and glycemic biomarkers

The correlation between serum calcidiol levels and glycemic control biomarkers (FSG, fasting serum insulin, HbA1c, and HOMA-IR) was investigated in the current study. At 3 years, a negative trend in the correlation between serum calcidiol and fasting serum glucose was detected. Serum calcidiol negatively correlated with fasting plasma glucose ($r = -0.28$, $P = 0.02$) after adjusting for BMI and race in cross-sectional study in obese female adolescents (132) and in another clinical trial that supplemented 1000 IU/d $D_3$ to diabetic patients (Middle-eastern, age 50.7 ± 6.1 y) for 12 weeks ($r = -0.208$, $P = 0.049$) (111). Vitamin D's role in controlling glycemia is based on its ability to enhance insulin sensitivity through the facilitation of glucose uptake in adipose tissue (171) and muscle (172), both tissues possess the VDR. Therefore, vitamin D stimulates insulin receptor transcription and insulin signaling pathways in these tissues, ultimately lowering blood glucose levels (173). Moreover, VDR presence in pancreatic β-cells facilitates insulin production and secretion (150). Indeed, supplementing South Asian, vitamin D deficient women (mean age 41.5 y) with 4000 IU $D_3$/d for 6 months increased serum calcidiol from baseline values of 21 nmol/L to > 80 nmol/L, improving insulin sensitivity by 41%(86). Vitamin D repletion (from 26.3 nmol/L to 63 nmol/L) improved fasting serum glucose in a subgroup of vitamin D deficient, obese, female adolescents by 5.2% (132).

Fasting serum insulin positively correlated (non-significantly) with serum calcidiol levels in the current analysis (at baseline, r = 0.255; at 3 years, r = 0.036; absolute change, r = 0.014; and relative change, r = 0.064). However, in a cross-sectional study, fasting insulin negatively correlated with serum calcidiol (r = -0.42, P = 0.03) after adjusting for BMI in Caucasian Americans, but not in African Americans (132). Another multi-ethnic, cross-sectional study also indicated inverse adjusted association of serum calcidiol and fasting insulin (P = 0.019) (85). There are several possible explanations for these results. For example, in the former study (132), mean baseline fasting insulin levels were relatively higher than in our participants at baseline (232 pmol/L vs 132 pmol/L in our study), and in the latter (85), the association was derived from multi-ethnic population, including whites, Hispanics and others. Most importantly, FSG positively correlated with serum calcidiol in our analysis for both the absolute and relative change from baseline, which would explain the positive correlation with fasting insulin.

Our study also showed that serum calcidiol levels are negatively correlated with HbA1c at 3 years. In addition, a negative trend between serum calcidiol and HbA1c at baseline and the absolute change from baseline was observed. This negative correlation was observed in another cross-sectional study, in which serum calcidiol levels were inversely associated with HbA1c ($r^2$ = 0.058, P = 0.008) in diabetic patients and non-diabetic controls ($r^2$ = 0.086, P =

0.001) (174). In a human clinical trial, HbA1c significantly decreased by 6.4% in diabetic patients after 12 weeks of vitamin $D_3$ and calcium fortified yogurt (1000 IU/d $D_3$ and 500 mg Ca) (111). In the current study, HbA1c increased significantly in the placebo group by 5% compared to baseline values, according to retrospective analysis. Vitamin $D_3$ supplementation attenuated the increase in HbA1c in CaD vs placebo (per protocol: -0.8% vs 5%).

Finally, in the current study, correlations between serum calcidiol and HOMA indices (HOMA-IR and HOMA-B) were negative (but not significant) at 3 years and when the absolute change from baseline was calculated. One unanticipated finding in the current study was the significant increase in HOMA-B in the CaD group when using retrospective analysis and the decrease in HOMA-IR in the placebo group when using per-protocol analysis. Vitamin $D_3$ and calcium supplementation did not improve insulin resistance and β-cell function in this sample of diabetic patients. Another clinical trial in pre-diabetic, obese African Americans whereby the treatment group (4000 $D_3$ IU/d) exhibited decreased insulin sensitivity (measured by Mastuda Insulin Sensitivity Index MISI) in contrast to a significant increase in insulin sensitivity for the placebo group (148). However, improvements in HOMA-IR and HOMA-%S indices by 11.7% and 13.3% were observed when serum calcidiol levels reached 80-119 nmol/L (86,175). These findings further support the potential role of vitamin D in attenuating glycemic biomarkers in diabetic patients.

Serum calcidiol and lipid profile

The change in serum calcidiol (absolute and relative vs. baseline) negatively correlated with TC/HDL-C. Moreover, at baseline, serum calcidiol levels negatively correlated with TG, TC, LDL-C, and TC/HDL-C. A positive trend was observed in the correlation between serum calcidiol and HDL-C with regard to both the absolute and relative change from baseline. Subjects who were in the CaD group (for both intent-to treat and retrospective analysis) experienced a significant decrease in TC, LDL-C, and TC/HDL-C, compared to baseline. In a weight reduction intervention, vitamin $D_3$ supplementation (400 IU/d) for 15 weeks, was associated with a 13% decrease in LDL-C (145), but not in diabetic patients who received 1000 IU/d for 12 months (158). The positive correlation between serum calcidiol and HDL-C in the current study is in agreement with previous studies (85,131,145,176), as is the negative correlation with TG (85). Moreover, in a 12-week clinical trial intervention that determined the effect of high dose of vitamin $D_3$ supplementation (5000 IU/d) on endothelial function in diabetic patients, serum TG significantly decreased by 8.1% (170). Vitamin D's beneficial effect on lipid profile in diabetic patients might be explained through its role in suppressing foam cell formation (109,110).

Serum calcidiol and other biomarkers

Serum calcidiol positively correlated with serum calcium at 3 years and with its relative change from baseline. It is worth noting that participants in our

study were not diagnosed with liver or kidney disease, hence AST, ALT, serum creatinine, serum albumin, serum calcium, and eGFR, were within normal ranges throughout the study. This was despite the significant decrease in AST in the CaD group, and the positive correlation between AST and serum calcidiol. Lower serum calcidiol (175) and calcitriol (177) concentrations were observed concurrently with very low eGFR (15-29 mL/min/1.73 $m^2$) as part of stage 4 chronic kidney disease. Similarly, the negative correlation between serum calcidiol and eGFR in the current study, is in agreement with another Canadian cross-sectional study that investigated the association between serum calcidiol and MetS components (85).

## 13.0 Summary and conclusion

Insulin resistance and elevated fasting serum glucose are fundamental aspects in T2DM; evidence suggests a potential role for vitamin D in controlling glycemia and attenuating T2DM. Vitamin D deficiency and T2DM share similar risk factors such as: non-white ethnicity (21,84,87), obesity (8,10,19,20,90,165), and increased age (84,94,95). In addition, seasonal variations influence vitamin D status (89–92). Vitamin D deficiency is associated with poor glucose tolerance and reduced insulin secretion. This association is based on the fact that VDR, which is necessary for vitamin D action, is located in pancreatic β-cells (responsible for insulin secretion) and peripheral tissues (responsible for insulin sensitivity). Cross-sectional and prospective studies have shown that low serum

calcidiol levels were associated with increased risk of diabetes (83,84,134,135,141), metabolic syndrome (85,131,132,138), insulin resistance and glucose intolerance (129,130,136,137,139), increased mortality (81,82,133), and increased adiposity (100,165). In clinical trials that provided a higher dose than the RDA (600 IU/d) in diabetics, pre-diabetics, or subjects at high risk of T2DM, including a wide range of ethnicities, and combined or not with Ca supplementation, favourable effects were observed on glycemic biomarkers (86,111,144,148,149), lipid profile (146), and body composition (111). However, clinical trials that supplemented relatively lower vitamin $D_3$ (< 800 IU/d) showed no effect of vitamin $D_3$ supplementation on T2DM outcomes (178).

This pilot study demonstrates that vitamin $D_3$ supplementation may attenuate T2DM severity in postmenopausal women of multi-ethnic backgrounds even in with modest increases in serum calcidiol level. We suggest a revision of the current vitamin D RDA with particular consideration for diverse ethnicities and different health conditions. We have also confirmed the negative association between serum calcidiol and adiposity, PTH, and LDL-C. Nevertheless, findings of this study are weakened due to poor compliance. Further research is indeed warranted to produce more robust results as well as investigate the underlying mechanisms of vitamin D action in diabetes control.

## 14. Significance of research

Despite the limitations, the current clinical intervention indeed suggests that a modest increase in serum calcidiol mitigates diabetes outcomes and provides us with some insight into the potential role of vitamin $D_3$ and calcium supplementation in attenuating diabetes severity. Our participant sample was relatively small, however it included individuals from three different ethnic groups (Caribbean, South Asian, and BlackBlack) that reside in Toronto, Ontario. The results of this study serve as the basis for other larger clinical trials to further elucidate the role of vitamin D supplementation in mitigating T2DM

Although the Institute of Medicine (IOM) concluded its revision of the vitamin D RDA in 2010, the current study suggests that the committee should reconsider the benefit that could be derived from vitamin D supplementation in those suffering vitamin D-related diseases such as T2DM.

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# Appendix A

## Presentation of data as means ± SD with parametric tests.

**Table 17:** Baseline characteristics presented as means ± SD following **per-protocol** assignment, for T2DM subjects (n = 11).

| Measurement | All n = 11 | Placebo n = 4 | CaD n = 7 |
|---|---|---|---|
| Age (y) | 61 ± 10 | 63 ± 12 | 59 ± 10 |
| Body weight (kg) | 79.3 ± 15.5 | 90.0 ± 6.7 | 73.3 ± 16.2 |
| BMI (kg/m$^2$) | 31.4 ± 5.3 | 35.0 ± 1.5 | 29.4 ± 5.8 |
| Total Body fat (kg) | 26.7 ± 5.2 | 30.2 ± 2.3 | 24.6 ± 5.4 |
| Body fat (%) | 34.3 ± 7.5 | 35.6 ± 6.7 | 33.6 ± 8.3 |
| WC (cm) | 95.0 ± 13.2 | 99.8 ± 9.3 | 92.4 ± 15.0 |
| HC (cm) | 114.2 ± 14.2 | 119.6 ± 17.4 | 111.1 ± 12.4 |
| WHR | 0.83 ± 0.08 | 0.84 ± 0.08 | 0.83 ± 0.08 |
| SBP (mmHg) | 129.5 ± 13.2 | 130.0 ± 14.0 | 129.3 ± 14.0 |
| DBP (mmHg) | 82.2 ± 5.8 | 81.0 ± 3.5 | 82.9 ± 7.0 |
| Serum Calcidiol (nmol/L) | 73.2 ± 31.7 | 109.0 ± 15.8 | 52.7 ± 14.3 |
| FSG (mmol/L) | 7.5 ± 2.1 | 7.7 ± 1.2 | 7.4 ± 2.6 |
| Fasting insulin (pmol/L) | 132.6 ± 201.5 | 225.3 ± 330.3 | 79.7 ± 64.5 |
| HbA1C (%) | 7.7 ± 1.3 | 7.2 ± 0.7 | 8.0 ± 1.6 |
| HOMA-IR | 47.7 ± 85.4 | 89.5 ± 141.0 | 23.8 ± 19.9 |
| HOMA-B | 553 ± 708 | 864 ± 1055 | 376 ± 422 |
| TG (mmol/L) | 1.01 ± 0.32 | 0.87 ± 0.20 | 1.10 ± 0.35 |
| TC (mmol/L) | 4.76 ± 1.18 | 3.94 ± 1.18 | 5.23 ± 0.95 |
| LDL-C (mmol/L) | 2.82 ± 0.99 | 2.24 ± 1.25 | 3.16 ± 0.69 |
| HDL-C (mmol/L) | 1.53 ± 0.46 | 1.45 ± 0.11 | 1.58 ± 0.58 |
| TC/HDL-C (mmol/L) | 3.22 ± 0.84 | 2.71 ± 0.82 | 3.51 ± 0.76 |
| PTH (pmol/L) | 6.5 ± 2.2 | 7.7 ± 1.3 | 5.8 ± 2.3 |
| AST (U/L) | 26.4 ± 16.1 | 20.0 ± 3.16 | 30.1 ± 19.6 |
| ALT (U/L) | 26.8 ± 24.6 | 16.3 ± 3.9 | 32.9 ± 29.7 |
| Serum Calcium (mmol/L) | 2.4 ± 0.1 | 2.4 ± 0.1 | 2.4 ± 0.1 |
| Serum albumin (g/L) | 41.8 ± 3.6 | 42.5 ± 3.4 | 41.4 ± 3.8 |
| Serum Creatinine (µmol/L) | 77.6 ± 10.3 | 78.0 ± 16.8 | 77.4 ± 6.1 |
| eGFR (ml/min/1.73m$^2$) | 70.5 ± 11.1 | 71.0 ± 17.2 | 70.1 ± 7.6 |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol, TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate.

**Table 18:** Baseline characteristics presented as means ± SD following **retrospective** assignment, for T2DM subjects (n = 11).

| Measurement | Placebo n = 5 Mean ± SD | CaD n = 6 Mean ± SD |
|---|---|---|
| Body weight (kg) | 90.0 ± 7.5 | 70.5 ± 15.2 |
| BMI (kg/m$^2$) | 35.6 ± 1.3 | 28.0 ± 5.0 |
| Total body fat (kg) | 30.2 ± 2.5 | 23.7 ± 5.1 |
| Body fat (%) | 37.5 ± 7.3 | 31.7 ± 7.2 |
| WC (cm) | 103.4 ± 10.6 | 88.1 ± 11.5 |
| HC (cm) | 117.6 ± 13.6 | 111.4 ± 15.3 |
| WHR | 0.88 ± 0.07 | 0.79 ± 0.06 |
| SBP (mmHg) | 129.4 ± 15.0 | 129.7 ± 13.1 |
| DBP (mmHg) | 82.8 ± 5.0 | 81.7 ± 6.8 |
| Serum calcidiol (nmol/L) | 87.6 ± 30.8 | 61.2 ± 29.5 |
| FSG (mmol/L) | 7.7 ± 1.0 | 7.4 ± 2.9 |
| Fasting serum insulin (pmol/L) | 212.4 ± 286.0 | 66.2 ± 64.3 |
| HbA1C (%) | 6.9 ± 0.4 | 8.4 ± 1.5 |
| HOMA-IR | 81.8 ± 122.9 | 19.3 ± 19.8 |
| HOMA-B | 869 ± 911 | 290 ± 397 |
| TG (mmol/L) | 1.03 ± 0.39 | 1.00 ± 0.28 |
| TC (mmol/L) | 3.92 ± 1.00 | 5.46 ± 0.82 |
| LDL-C (mmol/L) | 2.18 ± 1.06 | 3.36 ± 0.54 |
| HDL-C (mmol/L) | 1.39 ± 0.17 | 1.65 ± 0.60 |
| TC/HDL-C | 2.86 ± 0.78 | 3.52 ± 0.83 |
| PTH (pmol/L) | 6.6 ± 2.2 | 6.4 ± 2.4 |
| AST (U/L) | 24.6 ± 4.7 | 28.0 ± 22.3 |
| ALT (U/L) | 22.0 ± 9.8 | 30.8 ± 33.0 |
| Serum calcium (mmol/L) | 2.4 ± 0.1 | 2.3 ± 0.1 |
| Serum albumin (g/L) | 41.2 ± 3.7 | 42.3 ± 3.7 |
| Serum creatinine (µmol/L) | 79.2 ± 14.2 | 76.3 ± 6.9 |
| eGFR (ml/min/1.73m$^2$) | 68.2 ± 14.6 | 72.3 ± 8.2 |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol, TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate.

**Table 19:** subjects characteristics at 3 y, presented as means ± SD, following **per-protocol** assignment, for T2DM subjects (n = 11).

| Measurement | Placebo n = 4 | | CaD n = 7 | |
|---|---|---|---|---|
| | Mean | ± SD | Mean | ± SD |
| Body weight (kg) | 93.2 | ± 5.2 | 74.8 | ± 16.9 |
| BMI (kg/m$^2$) | 36.4 | ± 2.5 | 29.9 | ± 5.6 |
| Total body fat (kg) | 20.2 | ± 1.1 | 16.2 | ± 3.7 |
| Body fat (%) | 44.3 | ± 1.7 | 43.4 | ± 10.1 |
| WC (cm) | 104.7 | ± 3.7 | 88.0 | ± 25.0 |
| HC (cm) | 122.5 | ± 5.6 | 108.8 | ± 11.7 |
| WHR | 0.86 | ± 0.06 | 0.89 | ± 0.06 |
| SBP (mmHg) | 140.3 | ± 19.6 | 131.3 | ± 19.5 |
| DBP (mmHg) | 77.8 | ± 10.2 | 83.6 | ± 12.8 |
| Serum calcidiol (nmol/L) | 91.5 | ± 51.7 | 70.1 | ± 16.7 |
| FSG (mmol/L) | 7.0 | ± 2.9 | 6.6 | ± 2.3 |
| Fasting serum insulin (pmol/L) | 121.3 | ± 157.9 | 71.6 | ± 39.0 |
| HbA1C (%) | 8.0 | ± 0.7 | 8.1 | ± 1.5 |
| HOMA-IR | 26.4 | ± 22.0 | 21.6 | ± 14.4 |
| HOMA-B | 17994 | ± 35604 | 173 | ± 857 |
| TG (mmol/L) | 0.88 | ± 0.19 | 1.09 | ± 0.31 |
| TC (mmol/L) | 3.60 | ± 1.30 | 4.22 | ± 1.2 |
| LDL-C (mmol/L) | 1.78 | ± 1.01 | 2.25 | ± 1.01 |
| HDL-C (mmol/L) | 1.47 | ± 0.25 | 1.47 | ± 0.42 |
| TC/HDL-C | 2.40 | ± 0.52 | 2.92 | ± 0.72 |
| PTH (pmol/L) | 7.8 | ± 2.8 | 4.2 | ± 0.9 |
| AST (U/L) | 17.0 | ± 2.7 | 18.6 | ± 7.1 |
| ALT (U/L) | 16.0 | ± 4.8 | 19.0 | ± 8.6 |
| Serum calcium (mmol/L) | 2.5 | ± 0.1 | 2.4 | ± 0.1 |
| Serum albumin (g/L) | 44.8 | ± 1.0 | 43.3 | ± 3.6 |
| Serum creatinine (µmol/L) | 72.5 | ± 20.4 | 71.7 | ± 7.5 |
| eGFR (ml/min/1.73m$^2$) | 75.5 | ± 22.8 | 73.0 | ± 18.1 |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol, TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate.

**Table 20:** Subject characteristics at 3 y, presented as means ± SD, following **retrospective** assignment, for T2DM subjects (n = 11).

| Measurement | Placebo n = 5<br>Mean ± SD | CaD n = 6<br>Mean SD |
|---|---|---|
| Body weight | 93.2 ± 6.7 | 71.7 ± 15.5 |
| BMI ($kg/m^2$) | 36.9 ± 1.2 | 28.4 ± 4.7 |
| Total body fat (kg) | 20.2 ± 1.5 | 15.5 ± 3.6 |
| Body fat (%) | 44.0 ± 1.9 | 33.0 ± 10.2 |
| WC (cm) | 105.0 ± 2.8 | 84.9 ± 25.9 |
| HC (cm) | 121.9 ± 5.4 | 107.1 ± 11.7 |
| WHR | 0.86 ± 0.05 | 0.89 ± 0.06 |
| SBP (mmHg) | 141.2 ± 22.9 | 129.0 ± 15.1 |
| DBP(mmHg) | 77.6 ± 8.3 | 84.7 ± 13.9 |
| Serum calcidiol (nmol/L) | 65.0 ± 18.2 | 88.7 ± 40.0 |
| FSG (mmol/L) | 6.8 ± 2.5 | 6.6 ± 2.5 |
| Fasting serum insulin (pmol/L) | 134.4 ± 127.0 | 52.3 ± 38.3 |
| HbA1C (%) | 7.8 ± 0.9 | 8.3 ± 1.5 |
| HOMA-IR | 31.6 ± 15.1 | 16.5 ± 15.6 |
| HOMA-B | 14674 ± 31712 | -30 ± 823 |
| TG (mmol/L) | 0.97 ± 0.23 | 1.05 ± 0.34 |
| TC (mmol/L) | 3.32 ± 1.10 | 4.55 ± 1.06 |
| LDL-C (mmol/L) | 1.62 ± 0.95 | 2.46 ± 0.91 |
| HDL-C (mmol/L) | 1.30 ± 0.10 | 1.61 ± 0.43 |
| TC/HDL-C | 2.56 ± 0.8 | 2.88 ± 0.57 |
| PTH (pmol/L) | 7.2 ± 2.9 | 4.1 ± 0.8 |
| AST (U/L) | 17.6 ± 5.0 | 18.3 ± 6.7 |
| ALT (U/L) | 17.8 ± 5.0 | 18.0 ± 9.3 |
| Serum calcium (mmol/L) | 2.4 ± 0.1 | 2.4 ± 0.1 |

| | | |
|---|---|---|
| Serum albumin (g/L) | 44.2 ± 2.2 | 43.5 ± 3.6 |
| Serum creatinine (μmol/L) | 74.2 ± 16.9 | 70.2 ± 9.0 |
| eGFR (ml/min/1.73m$^2$) | 72.0 ± 18.8 | 75.5 ± 10.1 |

BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol, TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate.

**Table 21:** Comparison of outcome measures, presented as means ± SD, in participants within-group, following **retrospective** assignment, for T2DM subjects (n = 11).

| Measurement | Placebo n = 5 | | P | CaD n = 6 | | P |
|---|---|---|---|---|---|---|
| | Baseline Mean ± SD | 3 years Mean ± SD | | Baseline Mean SD | 3 years Mean SD | |
| Body weight | 90.0 ± 7.5 | 93.2 ± 6.7 | **0.086** | 70.5 ± 15.2 | 71.7 ± 15.5 | 0.600 |
| BMI (kg/m$^2$) | 35.6 ± 1.3 | 36.9 ± 1.2 | **0.095** | 28.0 ± 5.0 | 28.4 ± 4.7 | 0.644 |
| Total body fat (kg) | 30.2 ± 2.5 | 20.2 ± 1.5 | **< 0.005** | 23.7 ± 5.1 | 15.5 ± 3.4 | **< 0.005** |
| Body fat (%) | 37.5 ± 7.3 | 44.0 ± 1.9 | 0.167 | 31.7 ± 7.2 | 33.0 ± 10.2 | 0.733 |
| WC (cm) | 103.4 ± 10.6 | 105 ± 2.8 | 0.776 | 88.1 ± 11.5 | 84.9 ± 25.9 | 0.775 |
| HC (cm) | 117.6 ± 13.6 | 121.9 ± 5.4 | 0.411 | 111.4 ± 15.3 | 107.1 ± 11.7 | 0.139 |
| WHR | 0.88 ± 0.07 | 0.86 ± 0.05 | 0.287 | 0.79 ± 0.06 | 0.89 ± 0.06 | **0.017** |
| SBP (mmHg) | 129.4 ± 15.0 | 141.2 ± 22.9 | **0.062** | 129.7 ± 13.1 | 129.0 ± 15.1 | 0.888 |
| DBP (mmHg) | 82.8 ± 5.0 | 77.6 ± 8.3 | 0.390 | 81.7 ± 6.8 | 84.7 ± 13.9 | 0.215 |
| Serum calcidiol (nmol/L) | 87.6 ± 30.8 | 65.0 ± 18.2 | 0.112 | 61.2 ± 29.5 | 88.7 ± 40.0 | **0.005** |
| FSG (mmol/L) | 7.7 ± 1.0 | 6.8 ± 2.5 | 0.258 | 7.4 ± 2.9 | 6.6 ± 2.5 | 0.502 |
| Fasting serum insulin (pmol/L) | 212.4 ± 286.0 | 134.4 ± 127.0 | 0.342 | 66.2 ± 64.3 | 52.3 ± 38.3 | 0.431 |
| HbA1C (%) | 6.9 ± 0.4 | 7.8 ± 0.9 | 0.103 | 8.4 ± 1.5 | 8.3 ± 1.49 | 0.861 |
| HOMA-IR | 81.8 ± 122.9 | 31.6 ± 15.1 | 0.360 | 19.3 ± 19.8 | 16.4 ± 15.6 | 0.672 |
| HOMA-B | 869 ± 911 | 14674 ± 31712 | 0.373 | 290 ± 397 | -30 ± 823 | 0.288 |
| TG (mmol/L) | 1.03 ± 0.39 | 0.97 ± 0.23 | 0.772 | 1.00 ± 0.28 | 1.05 ± 0.34 | 0.244 |
| TC (mmol/L) | 3.92 ± 1.00 | 3.32 ± 1.10 | **0.060** | 5.46 ± 0.82 | 4.55 ± 1.06 | **0.042** |
| LDL-C (mmol/L) | 2.18 ± 1.06 | 1.62 ± 0.95 | **0.098** | 3.36 ± 0.54 | 2.46 ± 0.91 | **0.025** |
| HDL-C (mmol/L) | 1.39 ± 0.17 | 1.3 ± 0.10 | 0.347 | 1.65 ± 0.60 | 1.61 ± 0.43 | 0.786 |

| | | | | | | |
|---|---|---|---|---|---|---|
| TC/HDL-C | 2.86 ± 0.78 | 2.54 ± 0.8 | 0.348 | 3.52 ± 0.83 | 2.88 ± 0.57 | **0.048** |
| PTH (pmol/L) | 6.6 ± 2.2 | 7.2 ± 2.9 | 0.486 | 6.4 ± 2.4 | 4.1 ± 0.8 | **0.032** |
| AST (U/L) | 24.6 ± 4.7 | 17.6 ± 5.0 | **0.037** | 28.0 ± 22.3 | 18.3 ± 6.7 | 0.221 |
| ALT (U/L) | 22.0 ± 9.8 | 17.8 ± 5.0 | 0.378 | 30.8 ± 33.0 | 18.0 ± 9.3 | 0.265 |
| Serum calcium (mmol/L) | 2.4 ± 0.1 | 2.4 ± 0.1 | 0.688 | 2.3 ± 0.1 | 2.4 ± 0.0 | 0.102 |
| Serum albumin (g/L) | 41.2 ± 3.7 | 44.2 ± 2.2 | 0.170 | 42.3 ± 3.7 | 42.6 ± 3.2 | 0.502 |
| Serum creatinine (µmol/L) | 79.2 ± 14.2 | 74.2 ± 16.9 | **0.093** | 76.3 ± 6.9 | 70.2 ± 9.0 | **0.031** |
| eGFR (ml/min/1.73m$^2$) | 68.2 ± 14.6 | 72.0 ± 18.8 | 0.135 | 72.3 ± 8.2 | 75.5 ± 10.1 | 0.212 |

Data presented as means ± SD. BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. Within group, a paired t-test was performed to determine if there were significant differences between values at 3 years vs. baseline. P is significant ≤ 0.10.

**Table 22:** Comparison of outcome measures, presented as means ± SD, in participants between groups at 3 years, following **retrospective** assignment, for T2DM subjects (n = 11).

| Measurement | Placebo n = 5 | CaD n = 6 | P* |
|---|---|---|---|
| | 3 years | 3 years | |
| | Mean ± SD | Mean SD | |
| Body weight | 93.2 ± 6.7 | 71.7 ± 15.5 | **0.019** |
| BMI (kg/m$^2$) | 36.9 ± 1.2 | 28.4 ± 4.7 | **0.006** |
| Total body fat (kg) | 20.2 ± 1.5 | 15.5 ± 3.4 | **0.018** |
| Body fat (%) | 44.0 ± 1.9 | 33.0 ± 10.2 | **0.045** |
| WC (cm) | 105.0 ± 2.8 | 84.9 ± 25.9 | 0.116 |
| HC (cm) | 121.9 ± 5.4 | 107.1 ± 11.7 | **0.028** |
| WHR | 0.86 ± 0.05 | 0.89 ± 0.06 | 0.505 |
| SBP (mmHg) | 141.2 ± 22.9 | 129.0 ± 15.1 | 0.316 |
| DBP(mmHg) | 77.6 ± 8.3 | 84.7 ± 13.9 | 0.347 |
| Serum calcidiol (nmol/L) | 65.0 ± 18.2 | 88.7 ± 40.0 | 0.256 |
| FSG (mmol/L) | 6.8 ± 2.5 | 6.6 ± 2.5 | 0.889 |
| Fasting serum insulin (pmol/L) | 134.4 ± 127.0 | 52.3 ± 38.3 | 0.164 |
| HbA1C (%) | 7.8 ± 0.9 | 8.3 ± 1.49 | 0.479 |
| HOMA-IR | 31.6 ± 15.1 | 16.4 ± 15.6 | 0.139 |
| HOMA-B | 14674 ± 31712 | -30 ± 823 | 0.281 |
| TG (mmol/L) | 0.97 ± 0.23 | 1.05 ± 0.34 | 0.695 |
| TC (mmol/L) | 3.32 ± 1.10 | 4.55 ± 1.06 | **0.092** |
| LDL-C (mmol/L) | 1.62 ± 0.95 | 2.46 ± 0.91 | 0.168 |
| HDL-C (mmol/L) | 1.3 ± 0.10 | 1.61 ± 0.43 | 0.157 |
| TC/HDL-C | 2.54 ± 0.8 | 2.88 ± 0.57 | 0.472 |
| PTH (pmol/L) | 7.2 ± 2.9 | 4.1 ± 0.8 | **0.079** |
| AST (U/L) | 17.6 ± 5.0 | 18.3 ± 6.7 | 0.845 |
| ALT (U/L) | 17.8 ± 5.0 | 18.0 ± 9.3 | 0.967 |
| Serum calcium (mmol/L) | 2.4 ± 0.1 | 2.4 ± 0.0 | 0.366 |
| Serum albumin (g/L) | 44.2 ± 2.2 | 42.6 ± 3.2 | 0.702 |
| Serum creatinine (µmol/L) | 74.2 ± 16.9 | 70.2 ± 9.0 | 0.623 |

| | | | |
|---|---|---|---|
| eGFR (ml/min/1.73m$^2$) | 72.0 ± 18.8 | 75.5 ± 10.1 | 0.702 |

Data presented as means ± SD. BMI; body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PTH, parathyroid hormone; FSG, fasting serum glucose; HbA1C, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-B, homeostatic model assessment of β-cell function; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC/HDL-C, total cholesterol and high-density lipoprotein cholesterol ratio; TG; triglycerides; AST, aspartate transaminase; ALT, alanine transaminase; and eGFR, estimated glomerular filtration rate. An unpaired t-test was performed to determine if there were significant differences between values at 3 years in CaD vs. placebo. P is significant ≤ 0.1.

# Appendix B

## Consent forms

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**Research participant Consent Form**
**Women's Health in Women's Hands Community Health Centre**

**Date**: May 25, 2010

**Study Name**: The role of vitamin D and calcium supplementation in modulating the pathogenesis of type 2 diabetes mellitus (T2DM).

**Researchers**: Mazen J Hamadeh, Jesse Solomon, and Shahd Alabdulkader

**Collaborators:**

**Qualified Investigator**: Dr. Sonia Malhotra- Women's Health in Women's Hands Community Health Centre

**Sponsors**: Mazen J Hamadeh- York University

**Purpose of the Research:** To examine the effects of vitamin D and calcium supplementation on markers of T2DM (fasting plasma insulin and glucose concentration, insulin resistance, etc.). The study also endeavours to strengthen the existing positive correlation between calcium and bone health status (parathyroid hormone) and glycemic control (HbA1c, fasting plasma glucose and insulin, insulin resistance). The dose of 1,800 IU of vitamin D has never been studied in men and women with type 2 diabetes mellitus and is considered experimental.

**Treatment Groups:** We expect a total of 120 individuals to participate in the study. The probability of being assigned to either the vitamin D & calcium group or the placebo group is 50% or less. Your chances of group assignment do not change if you are considered to be insufficient in vitamin D.

**What You Will Be Asked to Do in the Research**: Participants will be required to 1) have anthropometric measurements taken (height, weight and waist circumference, etc.), 2) take a daily supplement containing either 1,800 IU

vitamin D and 720 mg of calcium or a placebo for 36 months, 3) have blood drawn for analysis of biomarkers (insulin, glucose, parathyroid hormone, 25-hydroxyvitamin $D_3$, calcium, etc.) at baseline, 6, 12, 18, 24, 30 and 36 months. Insulin resistance will be calculated at the above time points using the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR). You will also be asked to complete a 3-7 day diet record (a measure of current dietary intake), a Diet History Questionnaire (DHQ; a measure of dietary intake over the past 12 months), and a physical activity log at the same time points mentioned above.

**Risks and Discomforts**: We do not foresee any risks or discomfort from your participation in the research.

**Benefits of the Research and Benefits to You**: The results obtained from this clinical study will provide
us with some insight into the relationship between vitamin D, calcium and T2DM, as well as the relationship between T2DM markers and calcium/bone health. If what we hypothesize is indeed true, then those supplemented with vitamin D and calcium will have a decrease in specific biomarkers (plasma insulin and glucose, tartrate resistant acid phosphatase-maker of bone breakdown, parathyroid hormone) and decreased insulin resistance which are desired changes for individuals with T2DM and can improve the metabolic dysregulation that occurs in these individuals. In other words, patients with T2DM who are supplemented with vitamin D and calcium will have a decrease in the severity of the disease.

**Voluntary Participation**: Your participation in the study is completely voluntary and you may choose to stop participating at any time. Your decision not to volunteer will not influence the treatment you may be receiving, or the nature of

the ongoing relationship you may have with the researchers or study staff, or the nature of your relationship with York University or your Community Health Centre either now, or in the future.

**Withdrawal from the Study**: You can stop participating in the study at any time, for any reason, if you so decide. Your decision to stop participating, or to refuse to answer particular questions, will not affect your relationship with the researchers, York University, or any other group associated with this project. In the event you withdraw from the study, all associated data collected will be immediately destroyed unless consent is given to include your partial data in the study or further analysis.

**Confidentiality**: All information you supply during the research will be held in confidence and unless you specifically indicate your consent, your name will not appear in any report or publication of the research. Your data will be safely stored in a locked facility and only research staff will have access to this information. All data will be stored for the duration of the study plus 25 years post-study completion (as per Health Canada's guidelines) and will be archived in computer databases with limited access to study collaborators. The monitor(s), the auditor(s), the IRB/IEC, and the regulatory authority(ies) will be granted direct access to the subject's original medical records for verification of clinical trial procedures and/or data, without violating your confidentiality, which will be protected to the fullest extent possible by law.

**Compensation**: There will be no compensation for adverse events.

**Questions About the Research?** If you have questions about the research in general or about your role in the study, please feel free to contact Dr. Mazen J

Hamadeh either by telephone (416-736-2100 ext. 33552) or e-mail (hamadeh@yorku.ca), Shahd Abdulkader (416-520-0191) or email (shahda@yorku.ca) or your specific Community Health Centre contact Michelle Westin (416 249 8000 x 2258) Lisa Martin RD, CDE (416-249-8000), or the qualified investigator Dr. Malhotra (416-593-7655 x7). This research has been reviewed and approved by the Human Participants Review Sub-Committee, York University's Ethics Review Board and conforms to the standards of the Canadian Tri-Council Research Ethics guidelines. If you have any questions about this process, or about your rights as a participant in the study, please contact Ms. Alison Collins-Mrakas, Manager, Research Ethics, 309 York Lanes, York University (telephone 416-736-5914 or e-mail acollins@yorku.ca).

**Legal Rights and Signatures**:

I_____, consent to participate in, *The role of vitamin D and calcium supplementation in modulating the pathogenesis of type 2 diabetes mellitus (T2DM),* a clinical study conducted by Dr. Mazen J Hamadeh. I have understood the nature of this project and wish to participate. I am not waiving any of my legal rights by signing this form. My signature below indicates my consent.

**Signature**_____ **Date**_____

Participant

**Signature**_____ **Date**_____

Dr. Sonia Malhotra Qualified Investigator

**Signature**_____ **Date**_____

Principal Investigator