Effect of Vitamin D Supplementation on Glucose Homeostasis among Type 2 Diabetic Patients: A Randomized Clinical Trial

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Certification

The thesis entitled **"Effect of Vitamin D Supplementation on Glucose Homeostasis among Type 2 Diabetic Patients: A Randomized Clinical Trial"** has been completed sincerely and satisfactory by Nadia Begum, Session: 2020-21, Re-registration no: 82, enrolled in the Institute of Nutrition and Food Science, University of Dhaka, Bangladesh, for the degree of Doctor of Philosophy (Ph.D.), is an original research work and record and was supervised by us can be submitted to the examination committee for evaluation.

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Nadia Begum
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Dedicated

To

My Beloved Parents

and

My Children

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List of Acronym

ANOVA	-	Analysis of variance
BMI	-	Body mass index
dL	-	deciliter
MDA	-	Malondialdehyde
mg	-	Milligram
SOD	-	Superoxide Dismutase
UV	-	Ultraviolet
WHO	-	World Health Organization
CRP	-	C-reactive protein
µmol	-	Micromole
mmol	-	Millimole
µg	-	Microgram
µL	-	Microliter
mg/L	-	Milligram/liter
ng	-	Nanogram
nmol	-	nanomole
PAL	-	Physical Activity Level
DM	-	Diabetes Mellitus
HbA1c	-	Glycatedhaemoglobulin
IDDM	-	Insulin Dependent Diabetes Mellitus
NIDDM	-	Non- Insulin Dependent Diabetes Mellitus
T2DM	-	Type 2 Diabetes Mellitus
ADA	-	American Diabetic Association
DBP	-	Diastolic Blood Pressure

SBP	-	Systolic Blood Pressure
SD	-	Standard Deviation
Vit D	-	Vitamin D
SPSS	-	Statistical Package for Social Science
HOMA%-β	-	Homeostatic model assessment of βcell function
HOMA% IS	-	Homeostatic model assessment Insulin sensitivity
HOMA% IR	-	Homeostatic model assessment Insulin Resistance
HPLC	-	High-performance Liquid Chromatography
CONSORT	-	Consolidated Standards of Reporting Trials
VDD	-	Vitamin D Deficiency
IDF	-	International Diabetic Federation
TBA	-	Thiobarbituric acid
SEP	-	Socio-economic profile
VDR	-	Vitamin D receptor
BMD	-	Bone Mineral Density
RGL	-	Random Glucose Level
PPAR	-	Peroxisome proliferative-activated receptor
GDM	-	Gestational Diabetes Mellitus
P1	-	Pilot 1
P2	-	Pilot 2
P3	-	Pilot 3
BIRDEM	-	Bangladesh Institute of Research and Rehabilitation of Diabetes, Endocrine, and Metabolic Disorders.

List of Symbols

Symbol		Name
%	-	Percentage
<	-	Less than
>	-	More than
≥	-	More than or equal to
≤	-	Less than or equal to
0_c	-	Degree centigrade
β	-	Regression coefficient
r	-	Spearman's correlation coefficient
p	-	Level of significance

Abstract

Background: Type 2 diabetes mellitus (T2DM) is a major endocrine metabolic disorder, which is highly prevalent in all ages of people in Bangladesh, who are suffering from vitamin D deficiency.

Aims: The purpose of this study was to examine how vitamin D supplementation impacts glucose homeostasis in individuals with Type 2 Diabetes who are deficient in vitamin D.

Materials and Methods: To find the vitamin status in T2DM, a pilot study was conducted among 23 T2DM patients. Vitamin D deficiency (VDD) was prevailing in the 100% T2DM of both sexes, 78.3% were deficient (10-30 ng/ml), and the rest 21.7% were severely deficient (<10 ng/ml). A single-blind prospective randomized clinical trial was conducted among 124 vitamin D deficient Type 2 diabetic patients comprising vitamin D supplementation to 61 T2DM and 63 T2DM patients during the period of 2020 and 2021. Patients were randomly recruited from Z.H Sikder Women's Medical College and Popular Diagnostic Center, Dhaka under defined inclusion and exclusion criteria. The vitamin D deficient T2DM treatment group of patients received 20,000 IU vitamin D capsules (D-Rise, Beximco Pharmaceuticals) and placebo or control patients received 'placebo' at every 5th day for 12 weeks with a follow-up at 6 weeks simultaneously. Analysis of fastingblood glucose (FBG), fasting blood insulin (FBI), glycated hemoglobin (HbA1C), C-

reactive protein (CRP), serum calcium, 25(OH) vitamins D, malondialdehyde (MDA) and superoxide dismutase (SOD) have been estimated at the time of recruitment, at 6th weeks and 12-weeks of vitamin D supplementation (end line). Collection of socio-demographic information, anthropometric data, collection, and laboratory analysis of blood specimens were carried out at the same schedules as of the vitamin D-supplemented subjects. In addition, simple sugar/carbohydrate restriction for foods and performing physical activity were advised and monitored at the timelines. 'Two-way repeated measure ANOVA mixed models' (GLM/general linear model) and 'Fixed-Effect Regression Model using dummy variable' were used in statistical analyses with SPSS, version 26 for measuring the significant changes of different biochemical parameters (as predictor variables) across timelines (denoted as within groups) and between groups (treatment versus placebo) following the 12 weeks vitamin D supplementation. 'Multiple linear Regression' analysis was also used for building multivariate models, with vitamin D as a dependent variable, where necessary.

Results: Present study outlined significant correction of vitamin D status among vitamin D-deficient T2DM patients. As defined by the Endocrine Society (deficient <30 ng/ml, sufficient ≥30 ng/ml) mean vitamin D or 25 (OH)D3 level was increased from deficient (baseline: 14.5±6.1 ng/mL) to a sufficient level (end line 35.8±7.5ng/mL) effectively (P=.000) in the treatment group as compared to placebo. Moreover, end-line 25-hydroxy vitamin D

levels were significantly higher in the treatment group as compared to placebo (treatment: 35.8 ±7.5 ng/mL versus placebo: 20.05±5.2 ng/mL, p=0.001) and baseline to end-line changes in the placebo group was independent (Baseline:19.5 ± 8.8 ng/mL vs end line: 20.05 ± 5.2 ng/mL, p = 0.965). Regarding FBG present study showed after vitamin D supplementation, baseline mean FBG gradually decreased significantly (P<0.001) from 10.9 mmol/L (±3.5) to 8.42 mmol/L (±1.7) at the end line in the treatment group as compared to placebo. Additionally, mean HbA1C gradually decreased (P=0.004) from baseline (8.97±1.9) to the end line (8.5±1.6) only in treatment while changes in placebo were independent (p=0.587). Other biochemical indices such as FBS, Fasting Insulin, HOMA-IR, HOMA-β, Calcium, SOD, MDA, and CRP which also showed significant changes within timelines and between groups (treatment versus placebo) even in short interventions except HbA1C for placebo (P=.587); Calcium and FBI level between treatment and placebo respectively (p=0.08 and P>0.05). However, vitamin D supplementation showed no significant impact on sociodemographic variables, BMI (Kg/m2), co-morbidity, and Stress- related characteristics between treatment and control groups as those variables are somewhat independent (p>0.05) both at baseline and end-line.

Multivariate analysis showed 44.4% variation for different biochemical predictors of vitamin D (overall R2-change=44.4%, F=19.17, P<0.001) and revealed that FBG, CRP, and MDA (P<.05) were inversely associated with

vitamin D levels of T2DM patients. In contrast, SOD and calcium are significant positive predictors of changing Vitamin D levels over 3 months of intervention. However, important glycemic indices -HbA1C, FBI and derived parameters (HOMA β, HOMA-IS, HOMA-IR) were not significant predictors of variation in Vitamin D level analysis in the treatment group as compared to placebo. Surprisingly, after adjusting physical activity level (PAL) multiple regression analysis showed the increment of HOMA-IS and SOD along with reduction of FBG and MDA were changed vitamin D levels over the timelines. Similarly, an increment of vitaminD was also found associated with reduced FBG and MDA while adjusting for total calorie consumption among vitamin D-deficient T2DM patients.

Conclusion and Recommendation: Vitamin D supplementation to vitamin D deficient type 2 diabetic patients had significantly improved their vitamin D levels. It supported the glycemic indices to maintain glucose homeostasis in type 2 diabetic patients. Effective awareness of adequate intake of vitamin D as well as consumption of natural vitamin D-rich foods and physical activity would help Type 2 diabetic mellitus patients to support glucose homeostasis.

Chapter One

Introduction

1.1 Introduction

Now a days diabetes especially Type 2 diabetes mellitus (T2DM)─a non-communicable disease and vitamin D deficiency are ubiquitous and more prevalent among both urban and rural populations of Bangladesh despite having abundant sunshine at this latitude (Mohiuddin,2019). As per the definition of WHO Diabetes is a non -communicable disease that is chronic in nature and responsible for high-rise glucose in the blood causing detonation of the cardiovascular system, renal system, and so on. It is due to insufficient secretion of insulin from β- cells of the pancreas, nonresponse to insulin, and cells are not able to convert glucose into energy (Galiciaa et al., 2020.). Diabetes is one of the four major types of noncommunicable diseases (NCDs) that make the largest contribution to morbidity and mortality worldwide. Bangladesh is a developing country where 75% of the total population lives in rural areas and 7.1 million Adults live with diabetes as per IDF (The International Diabetes Federation) estimated. Among them almost an equal in number of undetected. This number is expected to double by 2025. This number is expected to reach 13% by 2030 (Mohiuddin,2019).

Globally DM is proving to be a global public health burden as this number is expected to rise to another 200 million by 2040. A majority (over 3 in 4) live in low middle-income countries.6.7 million deaths in 2021(1 every 5 seconds) due to diabetes. According to WHO global health days 2016, about 422 million people globally had diabetes, with most living in developing countries, and unfortunately, more than 80% of diabetes deaths occur in low- and middle-income countries like Bangladesh (Mohiuddin,2019).

In Southeast Asian region almost 90 million (1 in 11 adults) reside with diabetes. This number is expected to reach 113 million by 2030 and 2045

respectively. Undiagnosed over 1 in 2 adults. In 2021, 747,000 deaths are due to diabetes. And expenditure in 2021 for diabetes spent about USD 10 billion. **A**ccording to geographical location, there is a variation in Incidence and prevalence of T2DM. A majority (>80%) were living in low-to-middle-income countries, which postulates additional challenges in vigorous treatment (IDF, 2019).

Vitamin D deficiency is a silent and neglected global public health issue. Surprisingly in South Asia, 80% of the apparently healthy population is deficient in vitamin D (<20ng/mL) and up to 40% of the population is severely deficient (<9ng/mL) (Zaman et al., 2017). Vitamin D is fat-soluble and unique because it can synthesize by own self as well as can get through diet and supplements. Technically also acts as a hormone because it controls the amount of calcium and phosphate absorbed from the diet**.** For bone development, Calcium plays an important role. Loss of bone density is a major cause of vitamin D deficiency which may lead to osteoporosis and fractures (broken bones). It is observed in Ghana (2018) that 92.2% (both Deficiency and insufficiency) of diabetic women presented with vitamin D inadequacies (Fondjo et al., 2018)**.** In Qatar estimated that 87% were severely deficient (D level <20ng/L)(Al Thani et al., 2019)**.** Among Sudanese women showed a majority (82.6%) had levels of 25 (OH) D below 20 ng/ml (deficient**) (**Husain et al, 2019)**.**

In Bangladesh in 2019 among 793 respondents (86%) had hypovitaminosis D, 61.4% had a deficiency and 24.1% had insufficiency (Islam et al., 2019)**.** In 2019 among 102 doctors revealed that 66.6% had Vitamin D deficiency (Khan et al, 2019)**.** In 2019 despite having abundant exposure to the sun,

among healthy fishermen, 71% had low vitamin D in terms of levels mentioned in the guideline of the Endocrine Society (Haque et al., 2019). In 2018 among 353 Muslin women, 253(71.67%) had vitamin D deficiency and 80 (22.66%) subjects were insufficient (Shefin et al, 2018). In 2018 there was another study among 264 respondents showed the prevalence of hypovitaminosis was 84.84% (Rahman et al, 2018).

A very recent study (Hossain et al., 2021) elucidated that vitamin D level was lower and the frequency of vitamin D deficiency was higher in among adult Bangladeshi patients with T2DM. There are several studies (Al-Sofiani et al., 20150) supporting this idea that vitamin D is an important nutrient in the control of glucose homeostasis.

1.2 Rationale of the Study

Diabetes mellitus is more prone to develop serious life-threatening micro and macrovascular complications like neuropathy, retinopathy, nephropathy, etc. responsible for higher healthcare costs, reduced quality of life, and increased morbidity as well as mortality. Moreover, hypovitaminosis D is known to be alarmingly high worldwide, estimated to affect over one billion people, even in tropical countries. The prevalence of vitamin D deficiency is also very high in South Asian populations due to intake of inadequate diets, social customs, and living more times in households without exposure to sunlight. In Bangladesh, the prevalence of diabetes is more among the urban population (8.1%) than in the rural population (2.31%) (Diabet Med. 2016). A study in Iran showed that women living in urban areas had higher rates of Vit D deficiency than rural residents (Butt TA,2014). A study reported that people of Bangladesh have 30-100 ng/ml of vitamin D (Islam etal,2019). Chowdhury *et al*. (2018) also reported that above 65% of the population in Bangladesh is vitamin D deficient. Moreover, vitamin D deficiency (VDD) in children is a silent epidemic in a selected rural area of Bangladesh (Ahmed, 2021). More importantly, vitamin D deficiency is common in patients with type 2 diabetes mellitus (T2DM), which may be the etiology for the pathophysiology of this disease and may influence the glycemic control in the patients (Alam et al, 2018). Before that a pilot study has been conducted among 23 type 2 diabetic patients showed 100% vitamin D deficiency.

Vitamin D helps in the regulation of mineral ion homeostasis and bone metabolism; therefore, it maintains bone health by reduction of bone resorption and subsequent bone loss. Furthermore, vitamin D triggers most of the actions through the human vital organs (e.g. heart, brain, livers, bone,

kidney, and urinary system) and several tissues (immune cells, pancreatic cells, cardiomyocytes, endothelial cells, and vascular smooth cells). Vitamin D also controls the regulation of endocrine effects (insulin resistance), immune function, control of cell growth, and renal and muscle function. vital genes related to bone metabolism, oxidative damage, inflammation, and chronic diseases. Therefore, vitamin D plays an important role in the normal functioning of multiple organs of the body and has been linked to a wide spectrum of diseases including osteoporosis, cancer, diabetes, cardiovascular, and immune disorders. This study, thus, is designed to supplement vitamin D (Cholecalciferol, D Rise, Beximco Pharmaceutical) to vitamin D deficient type 2 diabetic patients. The outcome of the investigation would help maintenance and treatment of T2DM.

Chapter Two
Literature Review

2 Literature Review

2.1 Diabetes Mellitus:

Diabetes mellitus is an endocrine gland disorder, which is responsible for the rising of blood glucose levels due to lack of insulin secretion or inadequate insulin action or both (Amirasgari, et al, 2019) So that there is a disturbance of metabolism of carbohydrates, fat, and protein in the body (WHO, 2019) It is the 3rd leading cause of death globally (Amirasgari, et al, 2019). At least 2.8% of the population suffers from diabetes as per statistics from the World Health Organization. Considering the rising rate, it may be expected that by 2030 the prevalence will be double in the world (Mohiuddin, 2019).

2.2 Mechanism of blood glucose equilibrium state:

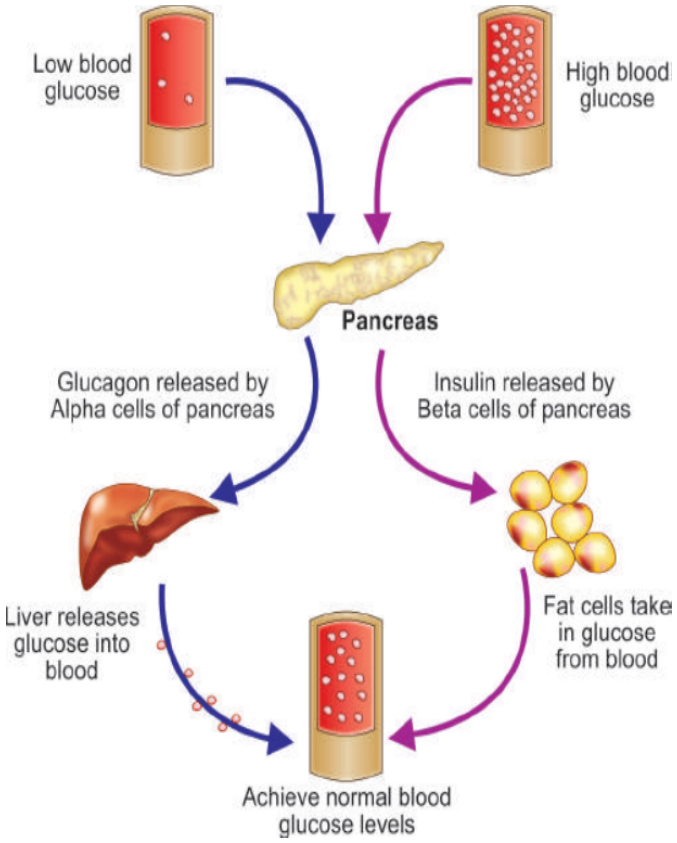

Image 1: Mechanism of blood glucose equilibrium state
Source: www.wisegeek.com/what-is-glucose-homeostasis.htm

2.3 Classification of DM:

According to American Diabetes Association, 2004: Diabetes can be classified as 1. Insulin-dependent (Type I diabetes) is due to the destruction of beta-cells, responsible for insulin deficiency. 2. Non-Insulin dependent (Type II diabetes) is due to defects in insulin secretion and insulin action.3. Unknown diabetes 4. Gestational diabetes mellitus also called diabetes at the time of pregnancy. Classified into two major types: Type I Diabetes and Type II Diabetes (Ullah et al., 2016).

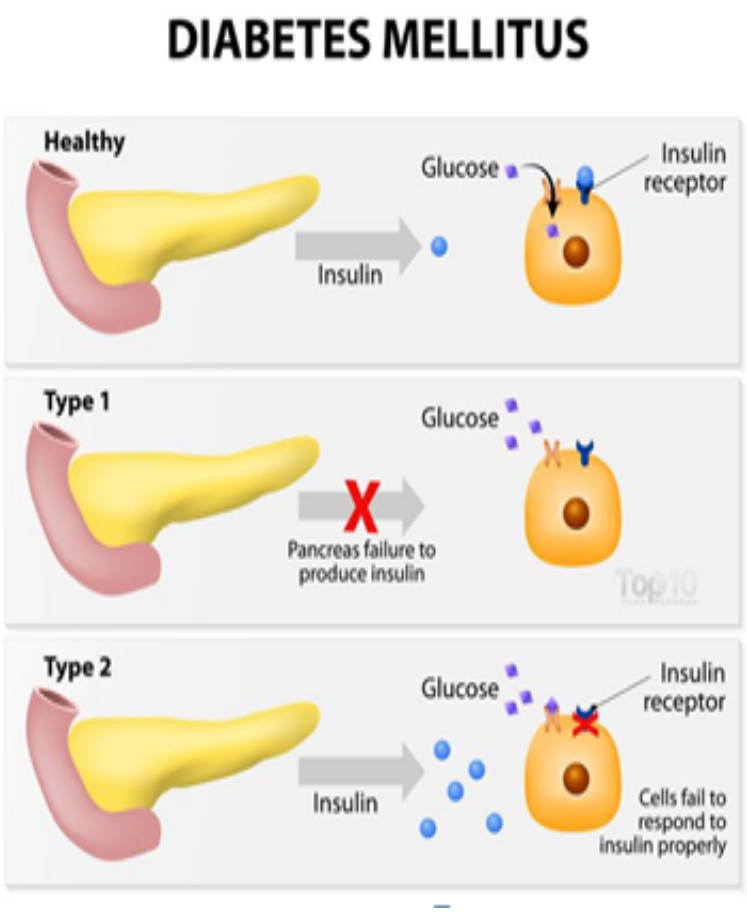

Image 2: Classification of Diabetes Mellitus

2.4 Glucose Homeostasis:

It can be defined as the tendency of organisms by using various physical and biochemical processes to maintain different internal systems equilibrium. When there is process of maintaining blood glucose at the equilibrium state level is known as glucose equilibrium (Szablewski, 2011).

2.5 Criteria for the Diagnosis of Diabetes:

Four diagnostic tests for diabetes are recommended according to WHO.

1) Value of 8 hours fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dl),
2) Value of 2-h after 75gm intake of glucose ≥ 11.1 mmol/L (200 mg/dl)
3) Value of Glycated hemoglobulin HbA1c ≥ 6.5% (48 mmol/mol); or
4) Value of random blood glucose ≥ 11.1 mmol/L (200 mg/ dl) along with the presence of signs and symptoms of diabetes (WHO, 2019).

2.6 Target of Control of Diabetes

Category	Normal	Pre-diabetes	Diabetes
FBS	<5.6 mmol/l or <200 mg/dl	5.6-6.9 mmol/l(IFG) or 100-125 mg/dl	≥7 mmol/l or ≥200 mg/dl
RBS	<11.1 mmol/l Or <200 mg/dl	N/A	≥11.1 mmol/l or ≥200 mg/dl for the first time at presentation without any history of ketoacidosis and clinical features suggesting other types of DM.
HbA1c	< 6.0%	5.7%-6.4%	≥ 6.5% or in a patient with classical symptoms of hyperglycemia or hyperglycemic crisis
OGTT	<7.8 mmol/l or <140 mg/dl	7.8-11.0 mmol/l(IGT) or 140-199 mg/dl	≥11.1 mmol/l or ≥200 mg/dl

Source (According to ADA, 2018 criteria) (Hossain et al, 2021)

2.7 Gold Standard Test for Diabetes

The oral Glucose Tolerance Test (OGTT) is a gold standard test for diagnosis of the Type 2 diabetes. HbA1c value is also considered as the gold standard for monitoring glycemic control in patients with diabetes mellitus. When compare with OGTT, HbA1c was much less sensitive. In essence, the OGTT test is the best to diagnose diabetes. When vulnerable to type 2 diabetes, OGTT shows more accuracy **(**Pajunen, et al, 2011).

2.8 Vitamin D or Calciferol:

Worldwide deficiency of vitamin D becomes a major public health problem. The following categories of 25(OH) D level were considered according to The Endocrine Society:

- Optimal when vitamin D level was >30 ng/mL
- Insufficient when it was 20–30 ng/mL and
- Deficient when it was <20ng/mL (Ghavideldarestani et al., 2020)**.**

2.9 Sources of vitamin D:

There are two forms of vitamin D are available. One is Cholecalciferol (vitamin D3) and the other is Ergocalciferol (D2). Though sunlight is the major source of vitamin D3 so 85-95% of vitamin D can be achieved by endogenous synthesis through direct ultraviolet B-mediated synthesis in the skin (Hossain et al., 2018), and good quantity Ergocalciferol (vitamin D2) we can achieve through few numbers of foods like fatty fish (salmon, tuna, etc.), fish oils (codfish liver oil). There are fewer amounts of vitamin D2 are present in red meats, egg yolk, and other animal food products. It is also available in

fortified (vitamin D is added to some foods to enhance the food quality) like milk and milk products, ghee Dalda, breakfast cereals, etc (Freitas, 2009).

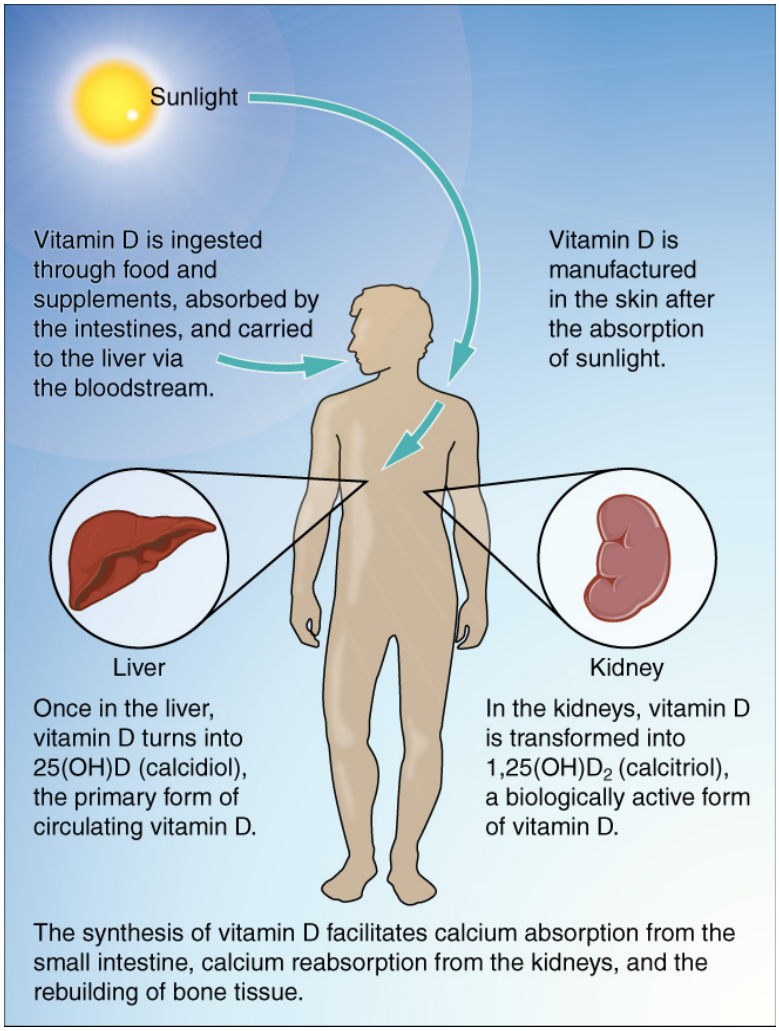

Image 3: Synthesis of vitamin D

Source Web site. http://cnx.org/content/col11496/1.6/,

2.10 Daily requirements:

The requirements of vitamin D vary with the age and physical condition of a person. The age of 50 needs vitamin D 400-800IU/day and above the age of

50 years needs vitamin D 800-1,000IU/day. This guideline was followed by the National Osteoporosis Foundation (NOF) (Shefin et al., 2018).

2.11 Cut of the value of vitamin D:

This study followed the Clinical Practice Guidelines of The Endocrine Society (Holick et al, 2011) for the Evaluation, treatment, and prevention of vitamin D deficiency where cut-off values of vitamin D level were as follows:

- If vitamin D level is <10 nanograms (ng/ml) is labeled severely deficient. (Amrein etal,2020)
- If the vitamin D level is 10 to 12 nanograms (ng/ml) is labeled deficient.
- If the vitamin D level is in between 12-20 ng/ml is labeled as insufficient.
- If the vitamin D level is ≥20 ng/ml is labeled as sufficient. (Hassan etal,2018)
- If vitamin D level is >50 nanograms (ng/ml) is labeled toxic. (Fookes, 2022)

2.12 Diagnostic Evaluation:

25(OH) D is the major circulating form of vitamin D and is the best indicator of vitamin D status because it reflects cutaneous and dietary contributions. It is thought to be a precursor for calcitriol (1,25-dihydroxy vitamin D), which is an active vitamin D metabolite. (Bordelon et al, 2009). This metabolite 1,25-dihydroxy vitamin D should not be used to measure vitamin D levels because of its short half-life and vitamin D level influenced by secondary hyperparathyroidism because it is closely regulated by the Parathyroid gland. (Waterbury, 2018).

2.13 Persistent of Vitamin D after supplementation

After vitamin D supplementation, it may take up to a week for blood levels to rise significantly. Each 1,000 IU of vitamin D3 taken daily is expected to raise blood levels of 25(OD) D by 10 ng/ml after a few weeks. But it may take months to resolve symptoms of severe vitamin D deficiency such as rickets in children. It depends on how low your vitamin D levels were in the first place and some individual factors (Fookes, 2022).

Rise in vitamin D levels in the blood depends on at least 2 factors:

1) Starting time of vitamin D level in blood
2) How much and how frequently one takes vitamin D.(Henriques T)

2.14 Vitamin D and sunlight:

According to the World Health Organization, 5 to 15 minutes of exposure to sunlight on the exposed part of the body like arms, hands, and face 2-3 times a week is enough to get the required amount of vitamin D. Use of sunscreen or use of full clothing will hamper the vitamin D synthesis. Skin penetration is required for effective results. However, we have to remember that excess exposure to sunrays might cause risks to our health like skin cancer. By exposure to sunlight, adequate levels of vitamin D can be achieved. Without applying sunscreen for half an hour (between 1000 and 1400 h) every day skin exposed to sunlight is considered adequate to avoid VDD (Kamboj et al., 2019).

2.15 Metabolic conversion process of 25(OH)D to 1,25(OH)D :

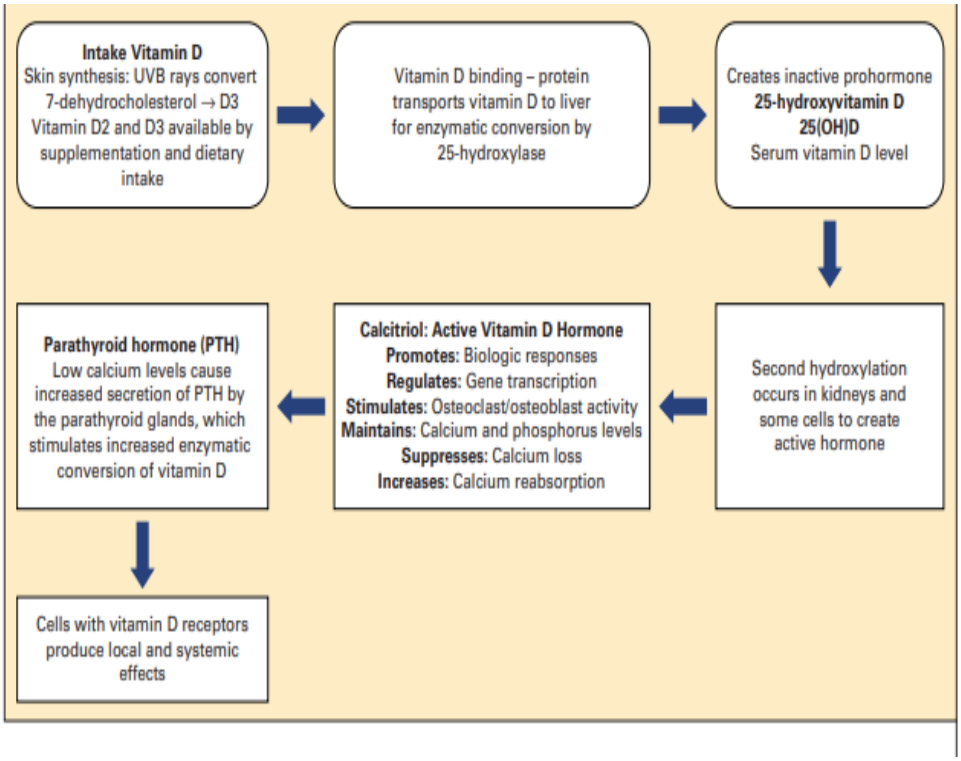

Image 4: Metabolic conversion process of 25(OH) D to 1, 25 (OH)2D (Waterbury, 2018)

2.16 Metabolic functions of vitamin D:

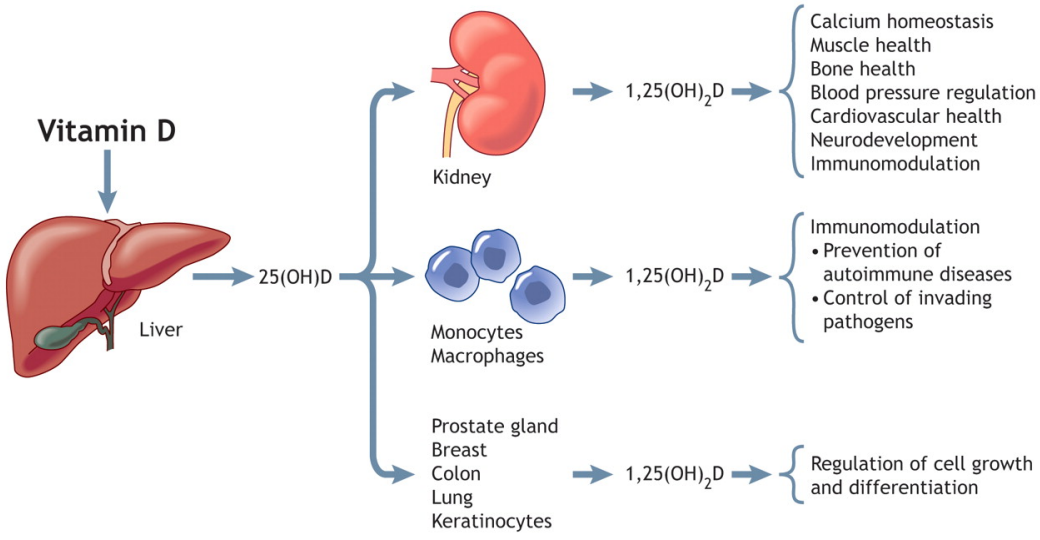

Image 5: Metabolic Functions of Vitamin D

2.17 Mechanism of vitamin D on β cell :

Vitamin D is reported to be insulin mimetic. It also decreased the prevalence of diabetes type 2 in long-time (Yousefi et al, 2014). It directly facilitates insulin secretion from β cell of the pancreas with the interaction of the 1.25(OH)2D3-RXR-vitamin D receptor complex, which is responsible for increasing insulin synthesis as well as decreasing insulin resistance (If a lot of insulin need to be secreted to deposit a certain amount of glucose). Secretion of insulin depends on a calcium-related process and indirectly by alternating calcium flux within the β islet cells it increases the concentration of calcium. Additionally, insulin sensitivity is regulated by vitamin D and calcium (If a small amount of insulin needs to be secreted to deposit a certain amount of glucose) by activating the peroxisome proliferative-activated receptor (PPAR) and inducement the insulin receptor (Husain, 2019).

2.18 Vitamin D & β cell functions/ insulin secretion:

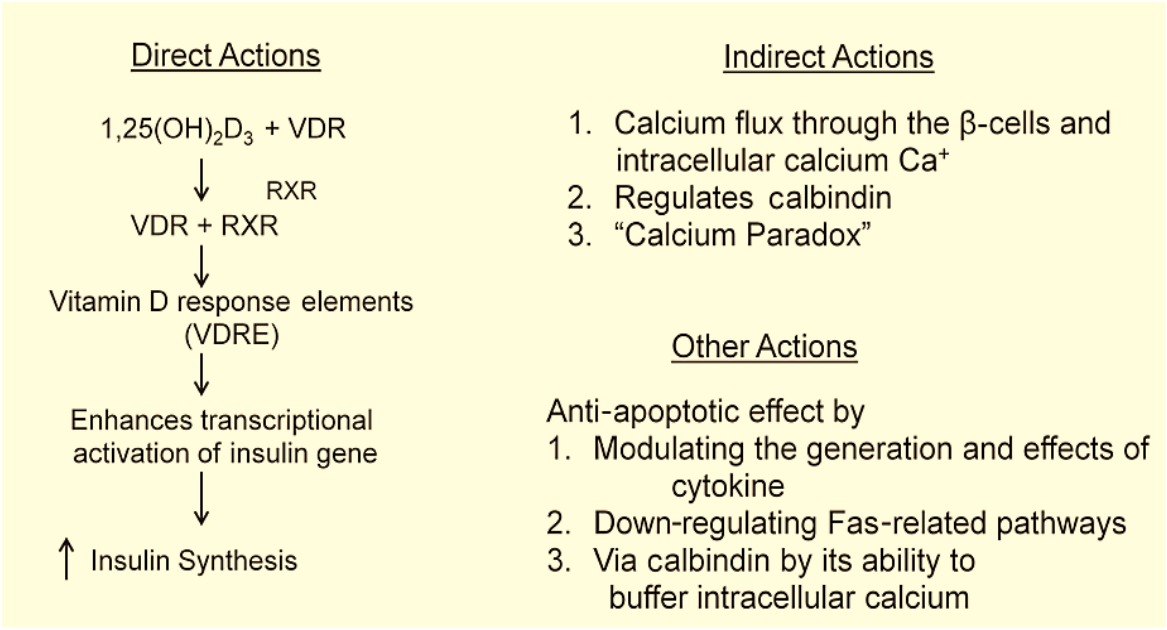

Image 6: Vitamin D & β cell functions/ insulin secretion

2.19 Direct action of Vitamin D and β-cell function

Direct action of vitamin D & β-cell function/ insulin secretion

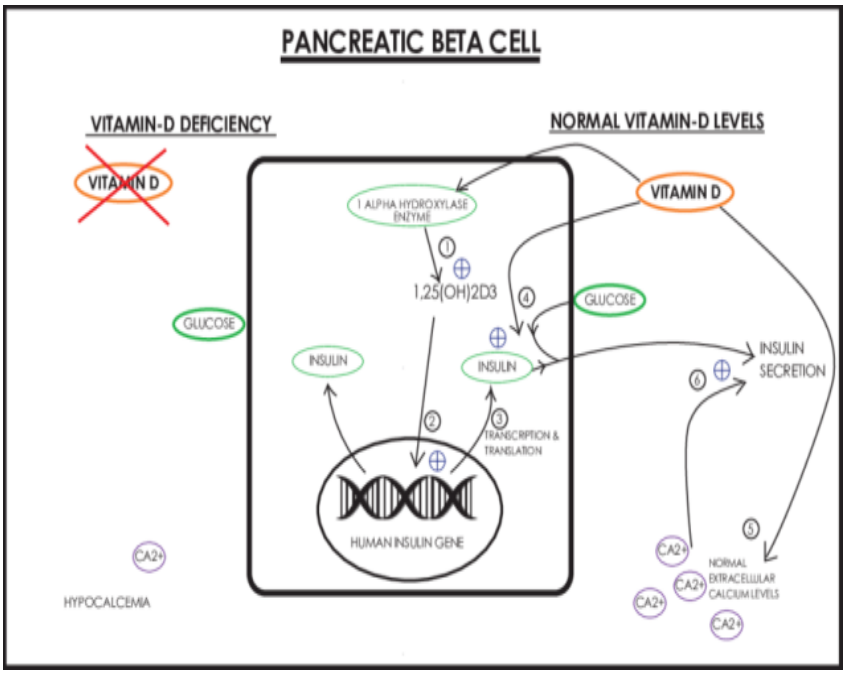

Image 7: Direct action of D and β-cell function

2.20 Factors regulating insulin secretion:

Glucose is a vital nutrient for insulin secretion. GLUT 2 acts as a glucose transporter on β cells (which is not insulin dependent for activation). Via this transporter, the glucose can enter the β cell, and with the help of glucokinase, metabolism takes place. As s result there is a generation of ATP and responsible for the shutdown of the ATP-sensitive K+ channels. Responsible for lowering potassium efflux which causes the degradation of the cell membrane. Responsible for the beginning of voltage-gated calcium channels and fast entry of calcium into the cell. By exocytosis from the granules in the

β cells this increased intracellular calcium triggers the release of insulin into the islet capillaries. Ultimately release of insulin.

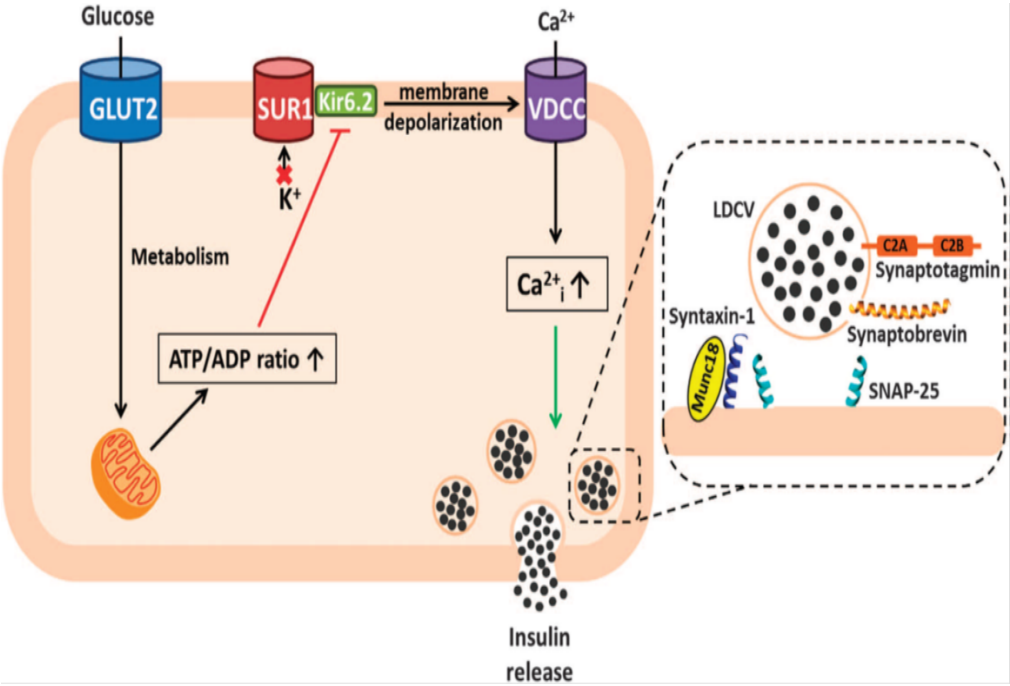

Image 8: Factors regulating insulin secretion (Source: Röder et al., 2016)

2.21 Diabetes and inflammation:

In 1930 C-reactive protein (CRP) was first discovered. It is a protein in nature in an acute phase. During the time of tissue injury, CRP is synthesized by the liver or adipose tissue. It acts as an indicator for diagnosis of clinical purposes and for treatment to some extent of acute and chronic inflammatory conditions as well (Song et al, 2015). The inflammatory process thinks about a part of insulin resistance, also considered prone to develop atherosclerosis and coronary heart diseases in diabetic people (Ali et al, 2015). During starts to develop type 2 diabetes, the body becomes insensitive to insulin and causes evolve insulin resistance also leads to inflammation. A vice-versa contradiction cycle may result, where there is more inflammation and more insulin resistance (Dansinger et al, 2019). High concentrations of

inflammatory biomarkers (like C-reactive protein) have been associated with chronic inflammatory diseases. (Oliveira et al, 2017). The normal reference value of CRP is between 0.8 mg/L and 3.0 mg/L. However, it may increase to 10 mg/L. The concentrations of CRP may be influenced by age, probably due to subclinical conditions. Metabolic inflammation may be considered if CRP concentrations are between 2 and 10 mg/L.. which may cause arteriosclerosis and type II diabetes mellitus. Which significantly increases HbA1c level. (Seo et al.,2020)

2.22 C-reactive protein status

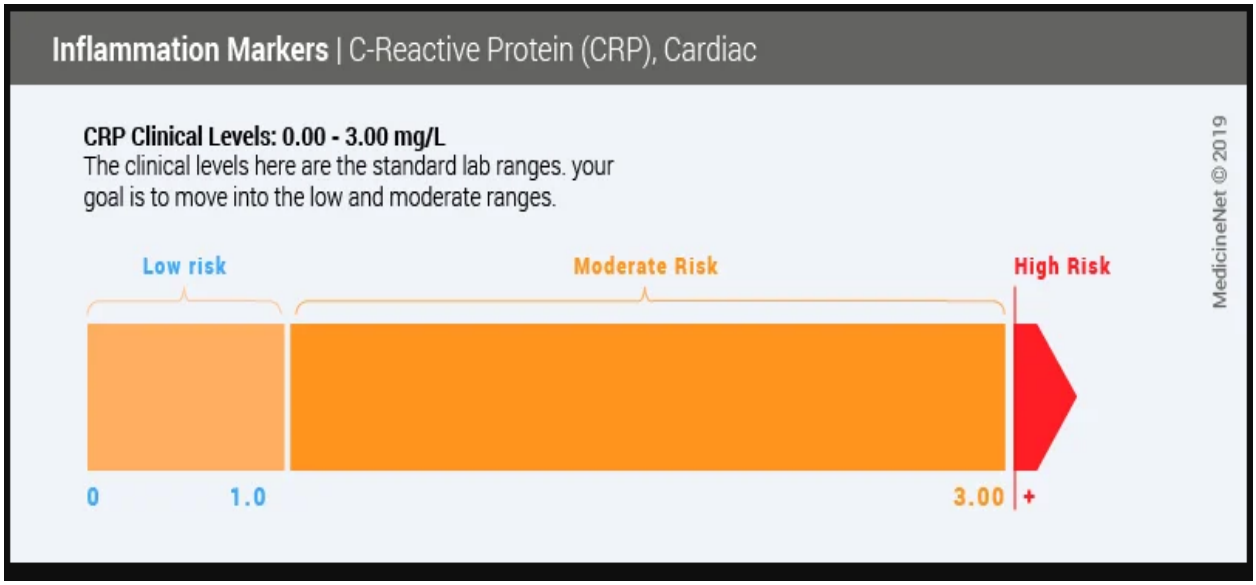

Image 9: C-reactive protein status

(Source: MedicineNet @2019)

2.23 Exercise or Physical activity:

The term physical activity denotes the movement of the body produced by the skeletal muscle that requires energy expenditure. As a prevention of type 2 diabetes physical mobility acts as a cornerstone. Regular exercise has

beneficial effects on overall glycemic control through improved glucose tolerance, lowered insulin requirements, and improved insulin sensitivity. These will help slow down the progress of diabetic complications. Ultimately establishing a quality of life (AADE/American Association of Diabetes Educators, 2015). During and after physical activity insulin sensitivity become increased, so that when muscle cells contract cells can use any available insulin to take up glucose and use it for energy whether insulin is available or not. In this way exercise can help lower blood sugar and as well as lower A1C (Thani et al, 2019).

2.24. **How does insulin work**

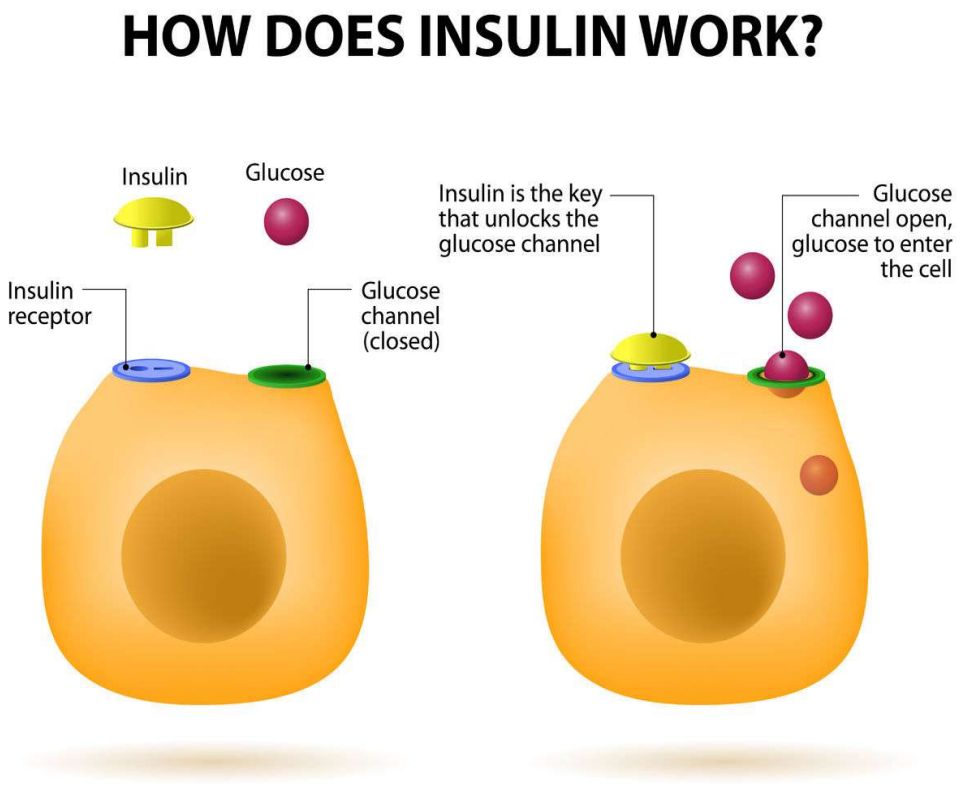

Image 10: How does insulin work

2.25 Oxidative Stress and Diabetes

Oxidative stress is a leading cause of diabetic complications. With the decreased level of SOD, there is an increase in MDA and Hba1c levels**.** It denotes is loss of balance among free radicals and antioxidants within the body, which can initiate cell and tissue destruction. Naturally, it occurs and plays a role in the aging process. By non-enzymatic glycation of proteins, oxidation of glucose, and high level of lipid peroxidation resulting destruction of enzymes, cellular types of machinery are ultimately responsible for insulin resistance Oxidative stress increases when there is high blood sugar in the blood which is considered breakage the main function that fails during diabetes, insulin action, and insulin secretion (Ullah et al., 2015).

2.26 MDA and SOD level during diabetes

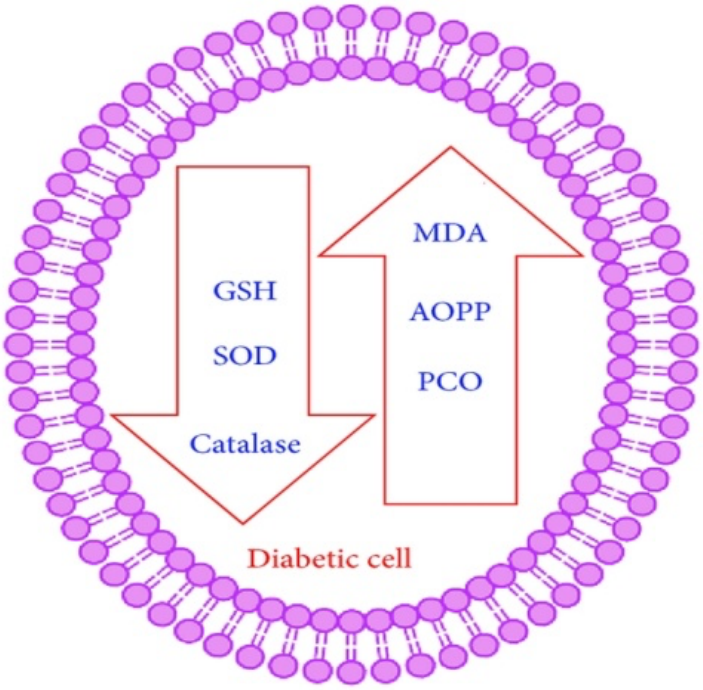

Image 11: MDA and SOD level during diabetes

2.27 Factors Affecting Cutaneous Endogenous Synthesis :

1. Age:

The age factor plays a vital role in diminishing levels of 25(OH) D due to impaired intestinal absorption of vitamin D as well as a decline in the concentration of vitamin D precursors when exposed to UVB radiation coupled with reduced capacity to synthesize vitamin D which is normally stored in the skin (Fondjo et al., 2018).

2. Sun exposure:

The time between 10 am to 2 pm, 30 minutes of exposure of skin to sunlight daily over the exposed part of the body without using any sunscreen is sufficient to fulfill vitamin D requirements (Pawale, et al., 2016). According to the rule of Horlick's, at 9 p.m. for 25 min, 3 times a week sunlight exposure at the site of the face and both arms should maintain adequate vitamin D status (Nimitphong and Holick, 2013).

3. Skin Pigmentation:

Skin pigmentation affects the body's production of vitamin D, which is determined by the concentration of melanin. Among black people vitamin D deficiency is common compared with white individuals, as they are deficient in vitamin D; behind this reason genetic variation is responsible (Waterbury, 2018).

4. Sunscreen:

Sun protective behaviors like the use of a hat or cap, use of sunscreen, use of an umbrella, wearing long sleeves, or staying under the shade interfere with vitamin D status even in sunset areas. Sunscreen acts as an artificial melanin

by absorbing the UVB radiation, even impairing their ability to synthesize vitamin D from sunlight when consistently applied SPF (sun protection factor) of 8. 90% vitamin D3 production is diminished by this process (Nimitphong and Holick, 2013).

5. Obesity—Excess adiposity, is associated with vitamin D deficiency (Fondjo et al., 2017)**.**

2.28 Vitamin D Status:

The serum concentration of 25(OH)D is considered the main indicator of vitamin D status due to its ability to produce vitamin D endogenously and can get from the source food and supplementation. Moreover, it has a long circulating half-life of 15 days than 1,25(OH)D. We should remember one nmol/L is equal to 0.4 ng/mL, and 1 ng/mL is equal to 2.5 nmol/L.

Serum 25(OH)D categories

Category	National Institute of Health	Endocrine Society
Deficiency	<12ng/ml	<20 ng/ml
Insufficiency	12-20 ng/ml	21-29 ng/ml
Insufficiency	≥20 ng/ml	≥30 ng/ml
Excess	>50 ng/ml	>100 ng/ml

Image 12: Demarking Vitamin D status

2.29 Adverse effects of Vitamin D

Vitamin D intoxication may be linked to when serum 25(OH)D levels of more than 150 ng/mL are responsible for increased calcium levels in the blood and potential acute kidney injury. Increased 25(OH)D and calcium levels (above 14 mg/dl) are major diagnostic findings of vitamin D intoxication (Waterbury, 2018) which can cause confusion, disorientation, and problems with heart rhythm. Vitamin D poisoning is not from excess exposure to sunlight, it is due to excess of vitamin D supplements because the body limits the amount of this vitamin.

2.30 Hypothesis

Vitamin D supplementation for 12 weeks would improve glycemic indices of:

a) HbA1C
b) Fasting blood glucose
c) Homeostasis model assessment/HOMA (Beta cell function, Insulin sensitivity/IS, and Insulin resistance/IR)

2.31 Objectives of the Study

General Objective:

To examine the effect of vitamin D supplementation on glucose homeostasis among Type 2 Diabetic patients by comparing treatment and control groups.

Specific Objectives:

1. Assessment of the socio-demographic characteristics
2. Assessment of nutritional status with anthropometric parameters.
3. Advising, monitoring, and controlling the diabetes diet and physical activity.
4. Analyzing different biochemical profiles:
    - Vitamin D,
    - Fasting Blood Glucose (FBG),
    - Fasting blood insulin (FBI),
    - Derived parameter Homeostatic model assessment (HOMA) for β-cell function, Insulin sensitivity (IS), and insulin resistance (IR).
    - HbA1c (Glycated Hemoglobin /A1C),
    - Serum calcium,
    - C-reactive protein (CRP),
    - MDA (malondialdehyde) and
    - SOD (Superoxide dismutase),

5. To find out the influence of vitamin D on:

- Fasting blood Insulin (FBI)
- Fasting blood glucose (FBG)
- Glycated hemoglobin (HbA1c or A1c)
- Malondialdehyde (MDA) and Super-oxide Dismutase level (SOD)
- Serum Calcium and
- Inflammatory-marker like C-reactive protein (CRP).

Chapter Three
Methodology

3. Methodology

3.1 Type 2 Diabetes Mellitus patients

This study was conducted among Type 2 Diabetes Mellitus patients suffering from vitamin D deficiency. The patients were diagnosed by Gold standard test for T2DM- measuring the fasting blood glucose, postprandial blood glucose, and $HBA1_C$ level also, Patients were first confirmed with Type 2 DM, who was assured for vitamin D deficiency. Vitamin D deficiency was investigated with a pilot study among 23 Type2DM subjects. Confirming Type 2 DM with vitamin D deficiency patients were enrolled in the study. During enrolment. each patient was tested again for diabetes with fasting blood glucose, and finally, under defined inclusion and exclusion criteria they were selected for the study.

3.2 Criteria of enrollment population:

3.2.1 Inclusion criteria:

- Diabetic patients (Type 2) aged range between 30 years to 70 years who were in treatment with oral hypoglycemic agent.
- Allotted to sustain their usual dietary habit and exercise during the start of the intervention process.
- Voluntarily willing to participate in this clinical study.

3.2.2 Exclusion principle:

- Diabetic patients (Type 1) of age below 30 years to above 70 years who were in treatment on insulin.
- Continue vitamin D supplements for 3 months before of starting of study
- Women patients who were pregnant or breastfeeding.
- Both written and verbal consent was obtained from each study population. Also made me understand about the aims and objectives of the study. They had full right to refuse to participate in the study.
- The secrecy of acquired information was assured through invisibility.

The study population was randomized into treatment and placebo. According to the advice of the physician, patients were advised to continue their drugs, diet as well as exercise but they were asked to prohibit from taking any other vitamins or minerals as well.

3.3 Conduction of a Pilot Study:

A pilot study has been conducted among 23 Type 2 Diabetic Patients to find out the current prevalence of vitamin D among diabetic patients. Sociodemographic profiles were observed and the following parameters (25(OH)D, Serum calcium, and Random Blood Glucose) has been analyzed at that time.

3.4 Ethical Clearance:

The study was overseen after ethical clearance was approved from the ethical review board ethical review committee of the Faculty of Biological Science, University of Dhaka (**Ref.No. 92/Biol.Scs**).

The study also registered as a clinical trial with "University hospital Medical Information Network (UMIN) Center, Japan" which is the largest and most versatile academic network information center for biomedical sciences in the world. (**UMIN Clinical Trial Registry No: UMIN000048031**).

3.5 Categorization of research work:

Group A: Socio-demographic, anthropometric parameter (BMI), food intake habit (24-hour recall) and exercise (24-hour physical activity) data were acquired and kept record.

Group B: Vitamin D, Plasma FBS, HbA1c, Serum calcium, C-reactive protein, Fasting Insulin, SOD, and MDA levels were measured at the beginnings, each follow up times, and

Group C: Association of --

- Vitamin D with Fasting insulin
- Vitamin D with HbA1c,
- Vitamin D with fasting glucose
- Vitamin D with calcium and CRP
- Vitamin D with Stress marker (superoxide dismutase and malondialdehyde)

3.6 Intervention procedure of type 2 diabetic patients

3.6.1 Treatment group:

- Tab Cholecalciferol (D-Rise) USP 20,000 IU (Beximco Pharmaceuticals) was supplemented to the "Treatment group" for every 5th day.
- All eight parameters (Vitamin D3, FBG, FBI, A1C, CRP, MDA, SOD, calcium) for both groups were analyzed at baseline and end line (after 12th weeks).
- However, a few parameters (e.g. FBG, Insulin, and CRP) were collected and analyzed after the 6th week (1st follow-up) of vitamin D supplementation.
- Moreover, homeostasis model assessments (HOMA-**β**, HOMA-IS, and HOMA-IR) were performed from FBG and FBI for all 3-timelines.

3.6.2 Placebo group:

The placebo group received a placebo (Microcrystalline Cellulose BP) was supplemented Simultaneously along with the treatment group.

3.7 Data collection:

3.7.1 Collection and processing of information

Patients were selected as per clinical history or who were receiving treatment. After taking consent a previously designed structured questionnaire was supplied and filled. The content of the questionnaire was recorded regarding social and economic characteristics, dressing style, sunlight exposure, feeding

habits, knowledge about the importance of sun exposure, BMI & presence of co-morbid conditions like diabetes & hypertension.

3.7.2 Measurement of anthropometric indices

The weight and height of diabetic patients of the treatment and placebo group were measured with bare feet. Adult metric scale (Detector Scale Inc. Brooklyn New York USA) was used to measure height and weight. According to WHO the computation of BMI is body weight in Kg divided by body height in meters^2. It can be categorized as underweight (<18.5kg/m2), normal weight (18.5-24.9kg/m2), and obesity (≥30kg/m2).

3.8 Sample size estimation:

The sample size for a clinical trial (comparing two means; Rashidi F et al., 2016)

Formula: $$\mathbf{n} = \frac{(Z_{\alpha/2} + Z_{\beta})^2 * 2(SD)^2}{D^2}$$

Where,

n = sample size required in each arm/group

D=μ_t- μ_c= minimally significant difference between the treatment and placebo group= 0.42 (*Yousefi Rad et al.*, 2014)

μ_t= Mean change of A1C in treatment group = 0.53

μ_c = Mean change of A1C in placebo/control group = 0.11

SD= Standard deviation = 0.60

$Z_{\alpha/2}$ =Level of significance is 1% (i.e. 2.56)

Z_{β} =Power of the Test is 90% (i.e.1.28)

$$n = \frac{\left(Z_{\frac{\alpha}{2}} + Z_{\beta}\right)^2 \times 2\,(SD)^2}{(\mu_t - \mu_c)^2}$$

$$n = \frac{(2.56 + 1.28)^2 \times 2 \times (.60)^2}{(.42)^2}$$

$$n = \frac{(3.84)^2 \times (2 \times .36)}{.1764}$$

$$n = \frac{14.7456 \times .72}{.1764}$$

$$n = \frac{10.616832}{.1764}$$

n= 60.186

n= 61 for a 1:1 ratio it will be 122 (Treatment and Placebo group)

Based on this formula, for a 1:1 allocation ratio, the required sample size was 61 in each group. Hence total sample size required is 122. Allowing an attrition rate of 20% (the working sample size required was 146).122 subjects are sufficient to explore a clinically important difference of 0.42 and assume a standard deviation of 0.60 by using a two-tailed test of the difference between means with 90% power and at a 1% level of significance.

3.9 Collection of Blood Sample

Ten milliliters (10 ml) venous blood sample was collected aseptically from the antecubital vein from each of the study population into a heparin tube and kept in a cool box. It was immediately processed to obtain plasma, which was then aliquoted into Eppendorf and stored at -20^{o}C for biochemical analysis. The serum sample was transferred in a cool box to the analytical lab with a

special type of container. Insulin analysis was carried out within 7 days of blood collection.

The study was designed to analyze the fasting blood glucose level of every participating patient. It is the simplest and fastest test to diagnose and monitor. Since the patients were not admitted into the hospital, it was difficult to measure the Postprandial Blood Glucose (PPBG). Diagnosed Type 2 diabetes mellitus patients were recruited for this study, who were tested for fasting blood glucose, blood glucose level after taking 75g glucose or meal, and $HBA1_C$ level. After recruitment for the study, the fasting blood glucose, and $HBA1_C$ levels were measured for every patient.

3.10 End-line status of Vitamin D and Placebo

During study time among total patients (n=146) few numbers were missed out at follow up and finally at the end ultimately it stands as

-- Vitamin D group: 61 (Sixty-one)

-- Placebo group: 63 (Sixty three)

In 1st follow-up, there were 7 dropouts in the control group and 8 in the number in the treatment group. But at the end line, there were 4 dropouts in the treatment group whereas 3 drop out in the placebo group.

Follow-up	Treatment	Placebo
Baseline	73	73
1st follow-up (after 6 weeks)	08	07
Endline (after 12 weeks)	04	03

3.11 Patients allocation following CONSORT:

We set up a list of items from existing quality assessment and reporting tools, with Consolidated Standards of Reporting Trials guidelines. CONSORT flow diagram of the progress through the phases of a parallel randomized trial of a few groups (that is, enrolment, intervention allocation, follow-up, and data analysis).

[URL: Consort-statement.org/consort-statement/flow-diagram]

CONSORT-Steps:

- Patients were included according to inclusion and exclusion criteria and assigned serial numbers of regular counting orders from 1 to 157.
- A total of 124 random counting numbers with a minimum value of 1 through a maximum value of 157 are determined (88, 68 …130, 98) from an online random number generator.

  (URL: calculator.com/statistics/random number generator 1-100)
- All those patients (n=61) with their serial number matched with a pre-determined random number are included in Group-A **(Intervention group**)
- The remaining 63 patients are included in Group B (**Placebo group**).

3.12 Diagrammatic presentation of the sampling procedure and technique

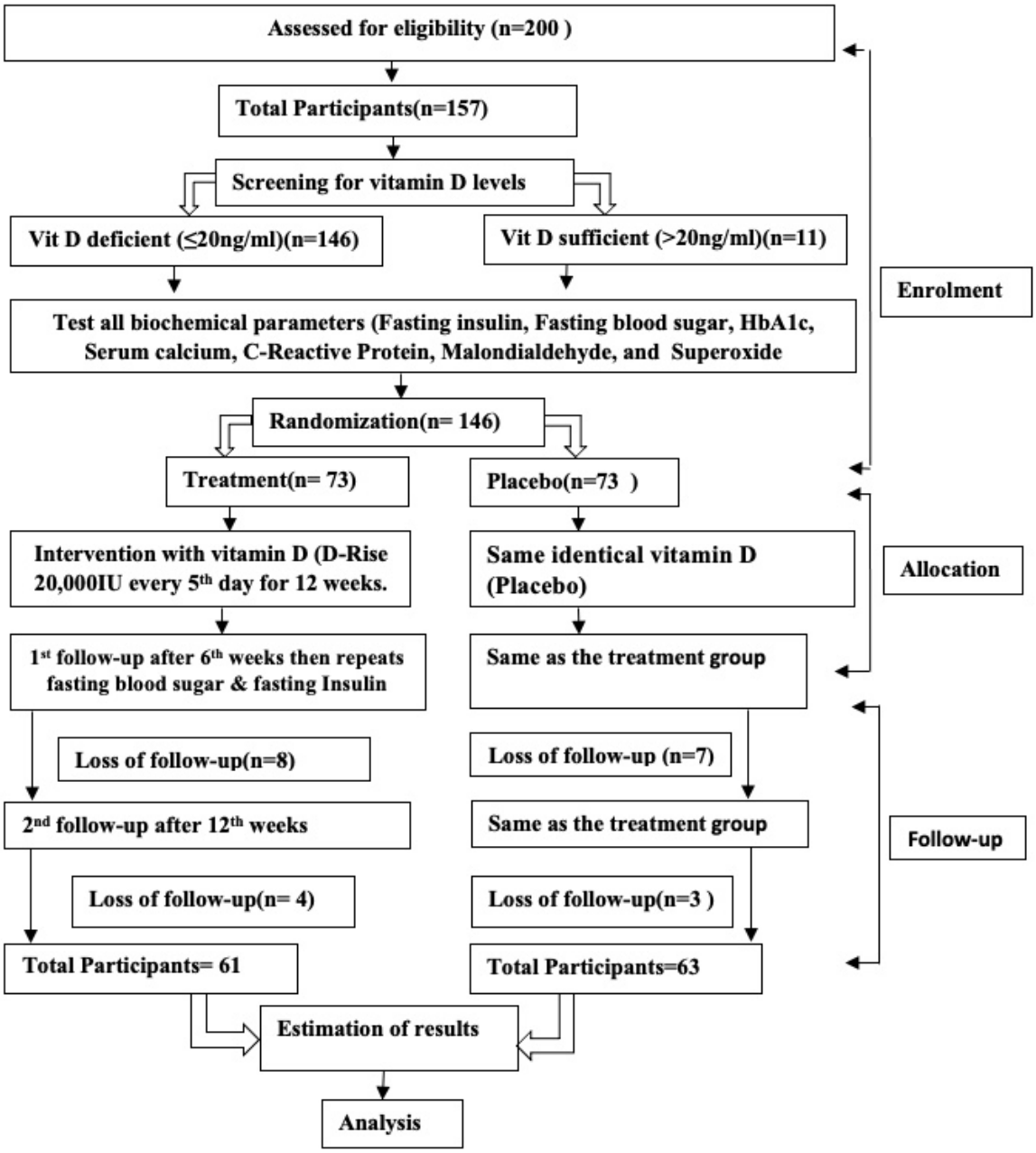

Figure 1 CONSORT Flow diagram

3.13 Investigation Schedule:

Information	Baseline visit	1st follow-up at 6 weeks	2nd follow-up at 12 weeks
Informed consent form	►		►
Collection of demographics	►		
Medical history taking	►		
Medical examination	►		
Physical examination	►		
Dietary habit	►		►
Physical activity	►		►
Fasting Blood sugar	►	►	►
Fasting Insulin	►	►	►
Vitamin D	►		►
HbA1c	►		►
C-reactive protein	►	►	►
Serum Calcium	►		►
Malondialdehyde	►		►
Superoxide dismutase	►		►

3.14 Statistical analysis:

- Data (Socio-demographic, Anthropometric, Dietary, Physical activity, and biochemical) were analyzed by SPSS (Version 26).
- For calculation of **β**-cell function, Insulin sensitivity (IS), and Insulin resistance (IR) used HOMA2-calculator (V2.2.3). (Oxford University, Diabetes trial unit, UK. http/www.dtu.oxiac.UK)
- 'Two-way repeated measure ANOVA Mixed models (GLM/general linear model)' and 'Fixed-Effect Regression Model using dummy variable' etc.
- Statistical methods were used respectively to measure the changes of biochemical indices across different time points and to estimate the biochemical predictors of Vitamin D levels following (3-months) vitamin D supplementation.

3.15 Procedure of 'Fixed-Effect Regression Model using dummy variable'

- All cases/ID i.e., 61 treatment groups and 63 placebo groups converted to long format along with 2 time-lines (thus total cases are 248 (from 124) as each case becomes doubled representing 2 timelines)
- Estimation of 4 dummy variables for "Group" (placebo and treatment) and "Time" (baseline and end line) keeping the first one as "Reference category" (i.e., placebo and baseline) for all models.
- Two times linear regression was performed where Vitamin D was always the dependent variable. Firstly, for model 1 (incorporating only the dummy treatment group itself) and secondly for model 2 (incorporating model-1 with 10 time-varying predictors and the time-dummy variable itself) were performed.

3.16 Analytical Method Employed:

Biochemical assessment of blood samples were collected from all patients (both treatment and placebo group) before treatment (zero time sample) then after 6 weeks and after 12 weeks of treatment to monitor the change in the studied parameter.

Biochemical analysis	Methods	Reagent kits	Machine used	Procedure	Reference
Serum Fasting Insulin	Chemilumine scence Micro particle Immunoassay (CMIA)	8K41 ARCHITECT Insulin Reagent Kit. (Brand Architect TM, Abbott Laboratories, Japan)	Architect 4100	Before centrifugation for 10 minutes of 3000 RCF before testing give proper time for blood samples to clot properly. An RV (Reaction Vessel dispenses 150 µL of a sample. In the reaction mixture, 100 µL Pre-Trigger and 300µL Trigger Solutions are mixed.	Moriyama et al.2006

Roche/Hitachi Cobas c 311/501 Analyzer	Enzymatic method (Hexokinase-mediated reaction)	Hexokinase (Roche Diagnostics, Switzerland)		Specimens must be transferred to a centrifuge tube For 10 minutes of 3000 RCF before testing Dispenses R1: 28 µL + Diluent (H_2O): 141 µL into a Reaction Cuvette, then dispense sample: 2 µL and R2: 10 µL + Diluent (H_2O): After 10 minutes 141 µL Incubation at 37°C.	T. A. Alaidarous. 2020
Serum Calcium	Photometric estimation	The Calcium Gen. 2 test system	Roche/Hitachi Cobas C Analyzer	Before testing specimens must be moved to a centrifuge tube for 10 minutes of 3000 RCF. Dispenses R1: 20 µL + Diluent (H_2O) into a Reaction Cuvette, then dispense sample: 3 µL and R2: 20 µL. After 10 minutes of incubation at 37°C.	W. Alan.2006

Serum Vitamin D	Chemilumine scence Microparticle Immunoassay (CMIA)	ARCHITECT (Abbott Laboratories, Lake Forest, IL, USA)	Architec4 100	Before centrifugation give proper time for blood samples to clot properly. Specimens must be shifted to a centrifuge tube and centrifuged for 10 minutes of 3000 RCF before testing. Dispenses 60 μL of a sample into an RV (Reaction Vessel).	K. Hutchinson. 2017
Serum C-reactive protein (CRP)	Immunoturbi dimetric assay	CRPHS reagent kit (Cat. No. 04628918190, Roche Diagnostics, Switzerland)	Roche/Hit achi Cobas C systems	Specimens were mixed properly, given time to clot to fully form, and centrifuged for 10 minutes at 2000 x g before use. A liquor a minimum of 0.1 mL was taken.	Rao et al.2020

Serum HbA1c	Ion-exchange high-performance liquid chromatographic (HPLC) method	Bio-Rad D-10TM Haemoglobin A1c (Bio-Rad Laboratories, USA	HPLC Analyzer (Fully automate)	At room temperature (15-30°C) samples were stored for 1 day, and 7 days at 2-8°C. Before sample analysis on Variant II Turbo quality control was maintained. After separating haemoglobin then pass through the flow cell of the filter photometer, where changes in the absorbance at 415 nm are monitored. Corrects for background absorbance an additional filter at 690 nm.	Thevarajahe tal.2009
MDA and SOD				Spectrophotometric	

Chapter Four

Results

4. Results

4.1 Result of the Pilot Study

Before performing the randomized controlled trial, a pilot study (Results shown in Table pilot-1 and Table pilot-2) was conducted to find out the current prevalence of vitamin D levels (table pilot-2) among T2DM patients of Dhaka city. Association (Cross tabulation/Chi-square test) of current vitamin D status among respondents of the pilot study was also statistically analyzed with their usual habits of sun exposure, BMIs (Kg/m^2), and random blood sugar (mmol/L) levels.

Table pilot-1 represents the socio-economic and vitamin D-related characteristics of the respondents. Among the respondents most (47.8%) were in between 40-50 years, thus vitamin D deficiency was more prevalent (n=11) in this age range. Both primary and secondary level education rates were 30.4% and only five persons had informal education. Regarding gender and occupation, most of the participants (65.2%) were female (n=15), and of them 47.8% (n=11) were housewives. Most (n=07) of the participants had >50,000 BDT monthly income followed by 26.1% had 30,000-40,000 BDT. A majority (56.5%) had a brown skin tone naturally and more than half of them (56.5%) had a history of diabetes for more than 5 years. Although a majority (n=15) had a history of sun exposure for <1 hour, most of them did not cover (n=13). However, most of the respondents (65.5%) were overweight while only 8.7% (n=2) were obese according to the classification of WHO (2004).

Table pilot-2 showed a biochemical profile of the study participants. According to Table 2, the prevalence of vitamin D deficiency among T2DM patients was 100% (n=23), mostly (78.3%) deficient (10-30 ng/ml) and the rest (21.7%) of them were severely deficient (<10 ng/ml). However, a majority had (91.3%) normal range of calcium level (8.6-10.3 mg/dl). 'Random glucose level' (RGL) among the respondents of the pilot study showed 39.17% had diabetes (RGL ≥11.1mmol/L), 34.7% had pre-diabetes (7.9-11.0 mmol/L) followed by 26.1% had normal RGL (4.4-7.8 mmol/L).

Cross tabulation showed current vitamin D status among respondents of the pilot study was associated with their usual habits of sun exposure (figure P1), BMIs (Kg/m^2) (figure P2), and random blood sugar (mmol/L) levels (figure P3). All Figures (P1-P3) revealed that a higher percentage of severely vitamin D deficient were associated with low sun exposure, overweight/obese, and high blood glucose levels.

Table Pilot-1: Frequency distribution of the pilot study showing socio-economic and vitamin D-related characteristics of Type 2 diabetic patients of Dhaka city (n=23)

Characteristics	Categories	All Respondents(n=23)	
		Number	Percent (%)
Age	40-50	11	47.8
	50-60	9	39.1
	>60	3	13.0
Education	Informal education	5	21.7
	Secondary level	7	30.4
	Higher Secondary	7	30.4
	Graduate	4	17.4
Religion	Muslim	22	95.6
	Hindu	1	4.3
Sex	Male	8	34.8
	Female	15	65.2
Occupation	Housewives	11	47.8
	Service holder	4	17.4
	Business	2	8.7
	Cleaner	4	17.4
	Retired	2	8.7
Monthly Family Income (BDT)	20.000-30,000	5	21.7
	30,000-40,000	6	26.1
	40,000-50,000	5	21.7
	>50,000	7	30.4
Skin tone	Fair	10	43.5
	Brown	13	56.5
Per day Sun exposure time	<1 hour	15	65.2
	1-2 hour	5	21.7
	>2 hour	3	13.0
Covered/worn Hijab	Covered	10	43.5
	Uncovered	13	56.5
History of diabetes	<5years	10	43.5
	≥5 years	13	56.5
Nutritional status BMI (Kg/m^2)	Normal (18.5-24.9)	8	34.7
	Overweight (25-29.9)	13	56.5
	Obese (>30)	2	8.7

Table Pilot-2: Biochemical parameters of the pilot study showing a prevalence of vitamin D deficiency among Type 2 diabetic patients (n=23)

Biochemical parameters	**Characteristics/Values**	**Total (N=23) % (n)**
Prevalence of vitamin D deficiency among T2DM		100.0% (23)
Plasma 25 (OH) D3	Deficient ~ 10-30 ng/ml	78.3 (18)
	Severely deficient <10 ng/ml	21.7 (05)
Serum Calcium	Deficient <8.6 ng/dl	8.6 (02)
	Normal 8.6-10.3mg/dl	91.3 (21)
Plasma Random glucose level (RGL)	Normal 4.4–7.8 mmol/L (or 80–140 mg/dl)	26.1 (06)
	Pre-diabetes 7.9–11.0 mmol/L (or 140-200 mg/dl)	34.7 (08)
	Diabetes ≥11.1mmol/L (or ≥ 200 mg/dl)	39.1 (09)

ng/ml =Nano gram per milliliter; mmol/L= micromole per liter; mg/dl= milligram per deciliter

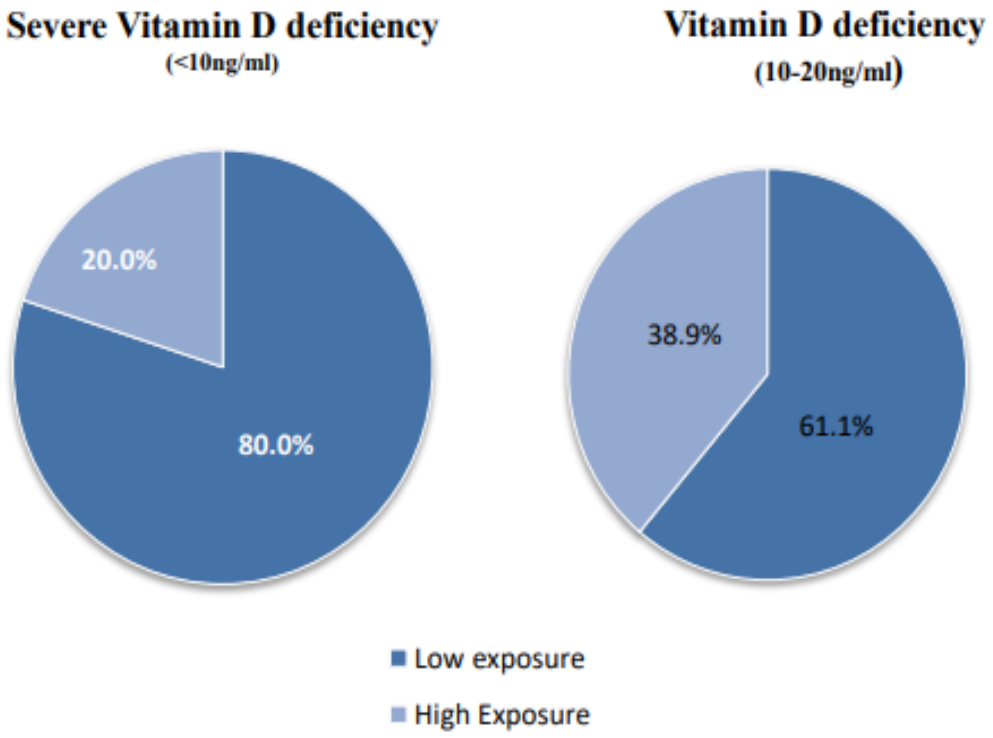

Figure P1: Association of Vitamin D status and sun exposure among respondents of the pilot study

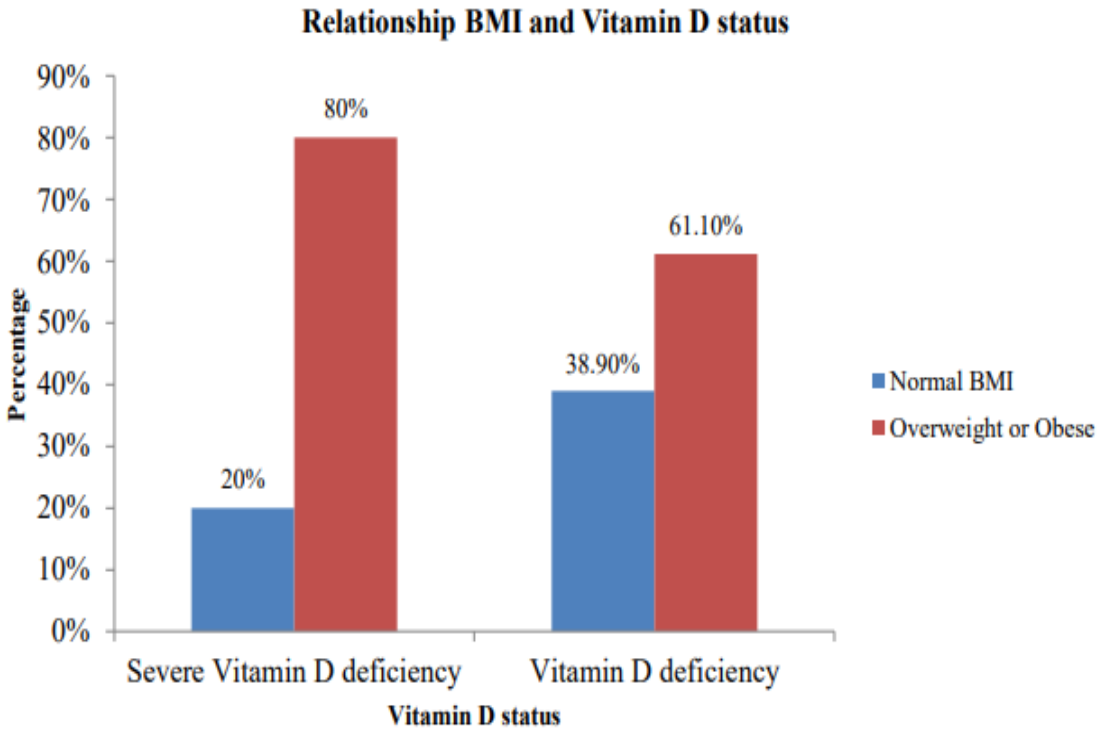

Figure P2: Association of Vitamin D status and BMI (Kg/m^2) among respondents of the pilot study

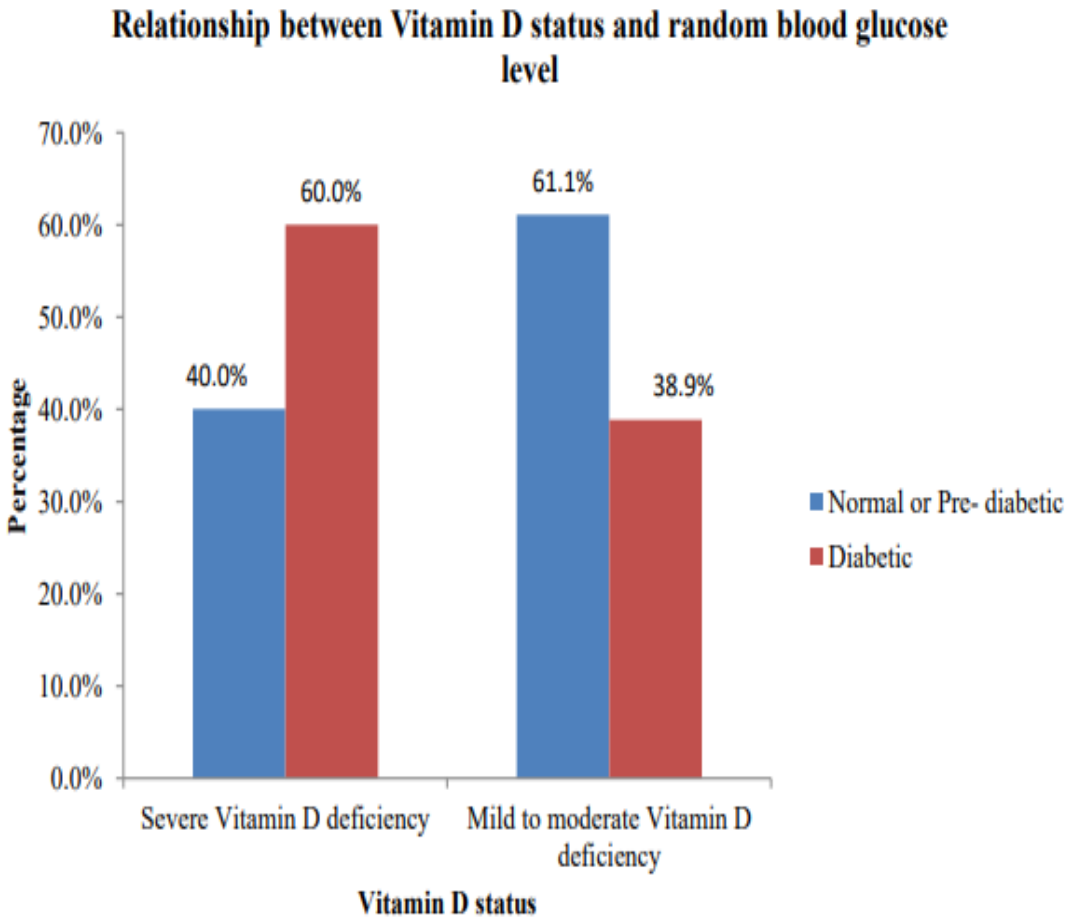

Figure P3: Association of Vitamin D status and Random blood glucose levels (mmol/L) among respondents of the pilot study

4.2 Results of the Study (Randomized Clinical Trial)

4.2.1 Descriptive and bivariate analysis

According to Table 1, no significant (P>0.05) differences were observed between the 'treatment' and 'placebo' groups in terms of socio-economic variables (ages in years, sex, education, occupation, marital status, living area, religion, and monthly income in BDT). Similarly, no significant (P>0.05) differences were also observed in Table 2 regarding BMI (Kg/m^2), co-morbidity, and Stress-related Characteristics between groups except systolic blood pressure was noticed higher among the treatment group (P=.002) (table 2).

Table 3 compares vitamin D-related characteristics (e.g. skin tones, usual exposure to sunlight, sun-exposed time, wearing hijab, areas/skin of the body exposed to sunlight, etc.) for both groups and showed that all these variables were independent (P>0.05) between groups except the variable 'areas/skin of the body exposed to sunlight' (P=.020), it was observed more women (63.5%) of placebo covered face, hand, and legs as compared to treatment group (42.6%). However, among all women (n=68) most (n=64) wear hijab.

Both Table 4 and Table 5 respectively represent (and also Figure 4) 'dietary intake' at baseline and end line between 'treatment' and 'Placebo' groups according to 'food groups' and 'consumption of calories. It is noticed that the amount of calorie consumption according to food groups was independent (P>0.05) between the two groups for both at baseline (2698.4 kcal verses 2743.3 kcal) (table 4) and end line (2549.5 verses 2575.7 kcal) (table 5). Moreover, figure 4 also represents insignificant differences in dietary calorie

consumption between the treatment and placebo groups from baseline to the end line.

Table 6 and Table 7 respectively represent 'physical activity' levels at baseline and the end line between 'treatment' and 'Placebo' groups. According to both tables, no significant differences were observed for physical activity levels (PALs) between the two groups (P>0.05) both at baseline (2429.0 minutes /1440 minutes=1.7 verses 2359.2/1440=1.7) (table 6) and at end line (2488.6/1440=1.7 verses 2364.5/1440=1.7 kcal) (table 7).

Table 1 Socio-economic Profile (SEP) of the Treatment and Placebo Group

SEP Characteristics	Total N=124	Treatment (n=61) %	Placebo (n=63)%	P-value
Age in years				
(Mean± SD)	(46.2±9.9)	(46.4±9.6)	(46.1±10.3)	P=.861
(Minimum-maximum)	(30-72)	(30-68)	(30-72)	
30-40	39 (31.5)	17 (27.9)	22 (34.9)	
41-50	57 (46.0)	28 (45.9)	29 (46.0)	
≥51	28 (22.6)	16 (26.2)	12 (19.0)	
Sex				
Male	56 (45.2)	26 (42.6)	30 (47.6)	P=.576
Female	68 (54.8)	35 (57.4)	33 (52.4)	
Education				
Illiterate	42 (33.9)	21 (34.4)	21 (33.3)	
Primary (1-5 y)	41 (33.1)	18 (29.5)	23 (36.5)	P=.790
Secondary (6-12y)	31 (25.0)	16 (26.2)	15 (23.8)	
Masters	10 (8.0)	06 (9.9)	4 (6.3)	
Occupation				
Services	45 (36.3)	17 (27.9)	28 (44.4)	
Business	23 (18.5)	10 (16.4)	13 (20.6)	
Housewives	29 (23.4)	18 (29.5)	11 (17.5)	P=.129
Others	27 (21.8)	16 (26.2)	11 (17.5)	
Marital status				
Married	121 (97.6)	60 (98.4)	61 (96.8)	
Unmarried	03 (2.4)	01 (1.6)	02 (3.2)	P=.578
Living area				
Urban	113 (91.1)	54 (88.5)	59 (93.5)	P=.359
Rural	11 (8.9)	07 (11.5)	04 (6.3)	
Religion				
Muslim	110 (88.7)	53 (86.9)	57 (90.5)	P=.528
Hindu	14 (11.3)	08 (13.1)	06 (9.5)	
Income (BDT)				
(Mean± SD)	(31,967.7 ±10150.8)	(31,098.4 ±10738.9)	(32,809.5 ±9557.7)	P=.350
(minimum-maximum)	(15,000-60,000)	(15,000-60,000)	(15,000-55,000)	
≤20000	22 (17.7)	11 (18.0)	11 (17.5)	
20001-30000	48 (38.7)	27 (44.3)	21 (33.3)	
≥30001	54 (43.5)	23 (37.7)	31 (49.2)	

Table 2 BMI (Kg/m^2), Co-morbidity, and Stress-related Characteristics between groups

Characteristics	Total N=124	Treatment (n=61) %	Placebo (n=63) %	P-value
BMI (Kg/m^2)				
Normal (18.5-24.9)	45 (36.3)	23 (37.7)	22 (34.9)	
Overweight (25-29.9)	65 (52.4)	32 (52.5)	33 (52.4)	P=.321
Obese (≥30)	14 (11.3)	06 (9.8)	08 (12.7)	
Duration of diabetes				
<5-years	82 (66.1)	39 (63.9)	43 (68.3)	P=.611
≥5-years	42 (33.9)	22 (36.1)	20 (31.7)	
History of family-diabetes				
Yes	77 (62.1)	34 (55.7)	43 (68.3)	P=.151
No	47 (37.9)	27 (44.3)	20 (31.7)	
Blood Pressure(systolic)				
<120 mmHg	45 (36.3)	14 (23.0)	31 (49.2)	P=.002
≥120 mmHg	79 (63.7)	47 (77.0)	32 (50.8)	
Blood Pressure (Diastolic)				
<80 mmHg	64 (51.6)	30 (49.2)	34 (54.0)	P=.594
≥80 mmHg	60 (48.4)	31 (50.8)	29 (46.0)	
Having stress				
Yes	67 (54.0)	38 (62.3)	29 (46.0)	P=.069
No	57 (46.0)	23 (37.7)	34 (54.0)	
Reasons for Stress				
Economic	48 (38.7)	28 (45.9)	20 (31.7)	P=.246
Health	37 (29.8)	17 (27.9)	20 (31.7)	
Sleep Lately at night				
Yes	51 (41.1)	24 (39.3)	27 (42.9)	P=.868
No	36 (29.0)	19 (31.2)	17 (27.0)	
Sometimes	37 (29.8)	18 (29.5)	19 (30.1)	

Table 3 Vitamin D related Characteristics for both groups

Vitamin D-related Characteristics	Total (N=124) %	Treatment (n=61) %	Placebo (n=63) %	P-value
Skin Tone				
White	69 (55.6)	36 (59.0)	33 (52.4)	
Brown	51 (41.1)	25 (41.0)	26 (41.3)	P=.128
Black	04 (3.2)	00 (0.0)	04 (6.3)	
Usual Exposure to sunlight				
Yes	118 (95.2)	58 (95.1)	60 (95.2)	P=.968
No	06 (4.8)	03 (4.9)	03 (4.8)	
Sun-exposed time				
<One-hour	94 (75.8)	51 (83.6)	43 (68.3)	P=.046
≥One-hour	30 (24.2)	10 (16.4)	20 (31.7)	
Wearing Hijab				
Yes*	64 (51.6)	36 (59.0)	28 (44.4)	P=.105
No	60 (48.4)	25 (41.0)	35 (55.6)	
Areas (Skin) of the body exposed to sunlight				
Only face	58 (46.8)	35 (57.4)	23 (36.5)	P=.020
Face, hand, and leg	66 (53.2)	26 (42.6)	40 (63.5)	

*Among 68 females most (94.1%, n=64) wear hijab

Table 4: Dietary intake at baseline by 'treatment' and 'Placebo' according to 'food groups' and 'consumption of calorie'

Food Sources	Treatment (Mean weight) (g)	Kcal	Placebo (Mean weight) (g)	Kcal	P-value
Rice	58.94±16.3	235.8	60.0±17.3	240.0	0.877
Wheat	60.0±30.0	240.0	66.66±36.0	266.6	0.611
Potato	15.53±4.7	62.11	16.11±4.85	64.4	0.763
GLVs	37.89±13.6	151.6	36.66±13.2	146.6	0.595
NLVs	66.84±32.5	267.4	74.44±39.7	297.8	0.929
Fruits	44.21±6.07	176.8	44.44±7.26	177.8	0.737
Total Carbs	134.47±38.3	537.9	142.77±49.6	571.1	0.630
Beef	29.73±8.07	118.9	27.22±3.63	108.9	0.384
Chicken	44.21±15.4	176.8	46.66±15.8	186.6	0.699
Egg	50.0±0.00	200.0	50.00±.00	200.0	0.929
Milk	39.47±14.3	157.9	40.00±15.0	160.0	0.890
Fish	32.63±2.6	130.5	32.77±2.63	131.1	0.828
Lentil	62.63±17.2	250.5	61.11±16.91	244.4	0.823
Total Protein	407.63±38.9	1630.5	413.33±46.7	1653.9	0.756
Plant oil	40.53±15.5	364.74	38.88±14.52	349.9	0.788
Total energy	**-**	**2698.4**	**-**	**2743.3**	**0.933**

Table 5: Dietary intake at end line by 'treatment' and 'Placebo' according to 'food groups' and 'consumption of calorie'

Food Sources	Treatment (Mean weight) (g)	Kcal	Placebo (Mean weight) (g)	Kcal	P-value
Rice	58.02±4.2	232.09	58.59±5.1	234.75	0.575
Wheat	60.6±3.0	242.4	61.13±6.6	245.5	0.635
Potato	15.33±3.6	61.33	16.58±2.9	68.3	0.08
GLVs	38.09±3.4	152.22	38.75±2.9	154.65	0.351
NLVs	65.94±3.7	261.56	66.15±9.9	266.6	0.898
Fruits	45.0±3.1	180.0	45.6±2.7	182.65	0.337
Total Carbs	282.99±5.7	1190.0	286.8±14.6	1139.0	0.109
Beef	30.2±3.2	120.6	29.4±2.2	117.4	0.219
Chicken	45.1±3.1	180.5	45.7±2.4	183.2	0.382
Egg	50.0±3.2	201.1	50.6±2.9	203.4	0.396
Milk	40.0±3.1	160.0	40.9±2.4	164.5	0.123
Fish	32.01±3.2	128.0	34.8±2.41	134.23	0.208
Lentil	62.97±3.0	252.2	63.63±3.06	254.5	0.321
Total Protein	260.3±18.1	1042.5	263.5±10.87	1054.18	0.135
Plant oil	42.0±3.13	378.0	42.5±2.91	382.5	0.449
Total	**-**	**2549.5**	**-**	**2575.7**	**0.333**

No significant change calorie consumption between the placebo and treatment groups

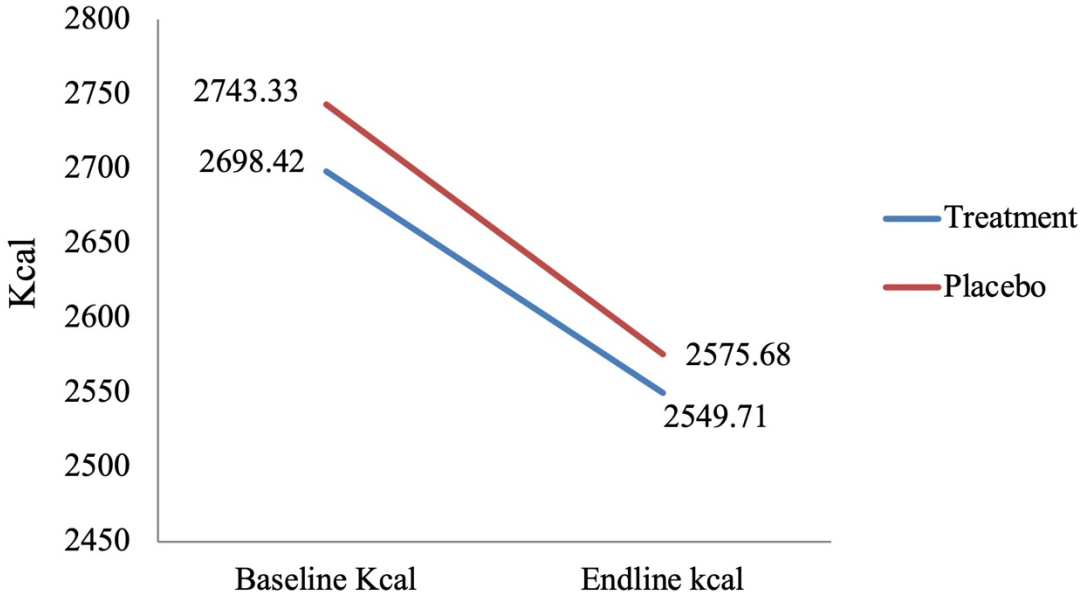

Figure 4: Dietary calorie consumption of the treatment and placebo groups from baseline to end line

Table 6 Physical activity levels at baseline performed by 'treatment' and 'Placebo'

Physical activities at baseline		Treatment (n=61) (Minutes)	values	Minutes* Values	Placebo (n=63) (Minutes)	Values	Minutes* Values
Personal activities	sleeping	500	1	500	600	1	600
	lying	40	1.2	48	30	1.2	36
	eating	90	1.6	144	70	1.6	112
	dressing	40	3.3	132	20	3.3	66
	shower	35	1.5	52.5	45	1.5	67.5
	recreation	65	1.72	111.8	35	1.72	60.2
	walking	50	3	150	40	3	120
	praying/moving/strolling	120	2.5	300	90	2.5	225
Personal activities			940			930	
Household chores	Washing dishes	60	1.7	102	50	1.7	85
	house cleaning	65	3	195	90	3	270
	cooking	120	2	240	115	2	230
	washing clothes	65	3	195	75	3	225
Total Household chores			310			330	
Trips	daily trips	85	1.2	102	75	1.2	90
	occupational activities	105	1.5	157.5	1.5	1.5	172.5
Trips daily trips		85	1.2	102	75	1.2	90
Grand Total		1440	-	2429.8	1440		2359.2
Mean Pal value		**2429/1440= 1.7**			**2359.2/1440=1.7**		

Table 7 Physical activity levels at end line performed by 'treatment' and 'Placebo'

Physical activities at end line		Treatment (Minutes)	Values	Minutes* Values	Placebo (Minutes)	Values	Minutes * Values
Personal Activities	Sleeping	500	1 1	500	600	1	600
	Lying	50	1.2	60	40	1.2	48
	Eating	80	1.6	128	60	1.6	96
	Dressing	40	3.3	132	20	3.3	66
	Showering	45	1.5	67.5	55	1.5	82.5
	Recreation	55	1.72	94.6	25	1.72	43
	Walking	60	3	180	50	3	150
	Praying/ moving /strolling	110	2.5	275	80	2.5	200
Total Personal Activities			940			930	
Household chores	Washing dishes	50	1.7	85	40	1.7	68
	House cleaning	75	3	225	100	3	300
	cooking	110	2	220	105	2	210
	Washing clothes	75	3	225	85	3	255
Total household chores			1250			1260	
Trips	Daily trips	95	1.2	114	80	1.2	96
	Occupational activities	95	1.5	142.5	100	1.5	150
Trips & occupational acts			190			180	
Grand Total (time)		1440		2448.6	1440		2364.5
Mean Pal Value		**2448.6/1440 = 1.7**			**2364.5/1440 = 1.7**		

4.2.1 Descriptive and bivariate analysis (continued)

Tables 8 and 9 respectively represent baseline (table 8) and end line (table 9) influences of sociodemographic factors and nutritional status (BMI Kg/m^2) on biochemical profiles of the 'treatment group' undergoing vitamin D supplementation. Chi-square analysis indicated that, at baseline, HbA1C was significantly related to sex and BMI, and education level was related to CRP level (Table 8). At end line calcium level was found to be significantly related to BMI and CRP was significantly associated with sex and age of the participants (Table 9).

Table 10 outlines the changes of different non-biochemical variables (Weight, BMI, sun-exposed time and Blood pressure, etc.) among 'within groups' (timeline groups of both treatment and placebo) and in 'between groups' (treatment versus placebo) following (3-months) vitamin D supplementation. Here, no significant differences ($P>0.05$) were observed for weight, BMI, having sun exposure, sun-exposed time, and blood pressure levels (both systolic and diastolic) between baseline and end line in the treatment group. Similar results were also found for placebo for all these time-variant variables. Consequently, there were no significant differences ($P>0.05$) for weight in kg, BMI (kg/m^2), having sun exposure, sun-exposed time, and both systolic and diastolic blood Pressure level between the treatment and placebo groups. It is important to notify that both 'sun-exposed time' (in Table 3) and 'systolic blood pressure' (in Table 2) were showed significant differences ($P<0.05$) at baseline between the treatment and placebo group, however at the end line these 2 variables are remained independent ($P>0.05$) (table 10).

Table 8 Influence of sociodemographic factors and nutritional status (BMI Kg/m^2) on Baseline biochemical profiles of 'treatment group' undergoing vitamin D supplementation

Socio-demography and Nutritional status		Biochemical Profiles (Treatment group)				
		HbA1c	**FBS**	**Calcium**	**CRP**	**Fasting**
Ranges		**≤7.8,>7.8**	**≤11.5,>11.5**	**≤9.5,>9.5**	**≤3,>3**	**≤12,>12**
Sex	Female	X^2=4.344	X^2=4.344	X^2=0.072	X^2=0.39	X^2= 0.574
	Male	P=0.03	P=0.03	P=0.78	P=0.42	P=0.45
Age (years)	<40	X^2=0.287	X^2=0.271	X^2=1.151	X^2=0.197	X^2=2.172
	>40	P=0.59	P=0.60	P=0.69	P=0.65	P=0.14
Education	Up to Primary	X^2=0.075	X^2=3.18	X^2=1.405	X^2=5.67	X^2=4.091
	Secondary Above	P=0.78	P=0.2	P=0.49	P=0.05	P=0.13
Income	<20 K	X^2=0.075	X^2=0.300	X^2=0.085	X^2=0.341	X^2=0.00
	>20 K	P=0.78	P=0.58	P=0.77	P=0.56	P=1.00
BMI (kg/m^2)	<25	X^2=4.344	X^2=0.067	X^2=1.099	X^2=3.68	X^2=2.71
	>25	P=0.03	p=0.79	P=0.29	P=0.54	P=0.10

*Type 2 diabetic patients with vitamin D deficiency (<20 ng/ml)

Table 9 Influence of sociodemographic factors and nutritional status (BMI Kg/m^2) on end-line biochemical profiles of 'treatment group' undergoing vitamin D supplementation

Socio-demography and Nutritional status Of the treatment group*		Biochemical Profile				
		HbA1c	**FBS**	**Calcium**	**CRP**	**Fasting Insulin**
		≤7.8,>7.8	**≤11.5,>11.5**	**≤9.5,>9.5**	**≤3,>3**	**≤12,>12**
Sex	Female	X^2=1.033	X^2=0.574	X^2=0.016	X^2=4.474	X^2= 2.391
	Male	P=0.31	P=0.45	P=0.9	P=0.03	P=0.12
Age(years)	<40	X^2=0.889	X^2=2.172	X^2=0.739	X^2=6.27	X^2=1.824
	>40	P=0.24	P=0.14	P=0.39	P=0.01	P=0.18
Education	Up to					
	Primary	X^2=1.292	X^2=1.092	X^2=0.022	X^2=1.292	X^2=0.600
	Secondary	P=0.52	P=0.58	P=0.99	P=0.52	P=0.74
	Above					
Income	<20K	X^2=1.086	X^2=0.938	X^2=1.667	X^2=0.068	X^2=0.287
	>20K	P=0.29	P=0.33	P=0.19	P=0.79	P=0.59
BMI	<25	X^2=1.033	X^2=0.033	X^2=2.917	X^2=0.475	X^2=2.63
(kg/m^2)	>25	P=0.31	p=0.83	P=0.08	P=0.49	P=0.1

*Type 2 diabetic patients with vitamin D deficiency (<20 ng/ml)

Table 10 Changes of different non-biochemical variables (Weight, BMI, sun-exposed time and Blood pressure, etc.) within groups and between groups following (3-months) vitamin D supplementation

Different variables	Treatment (n=61)			Placebo (n=63)			
	Baseline (1)	End- line (3)	P-value for Within-Treatments groups	Baseline (1)	End-line (3)	P-value for Within placebos groups	P-value for Between-groups/ (B-G) + Effect-size
(Mean± SD) Weight (w1 & W3)	59.5.5±6.6	59.3±6.4	P>0.05	59.9±7.6	59.6±7.4	P>0.05	P>0.05 (All-timelines)
(Mean± SD) BMI (BMI1, BMI3)	25.2±3.4	25.1±3.3	P>0.05	24.4±2.7	24.3±2.6	P>0.05	P>0.05 (All-timelines)
Having Sun-exposure (yes, N=118, 95.2%)	58 (95.1)	58 (95.1)	P>0.05	60 (95.1)	60 (95.1)	P>0.05	P>0.05 (All-timelines)
Sun-exposed-time (n %)(base vs. end)							
<1-hour	51 (83.6)	47 (77.0)	P>0.05	43 (68.3)	44 (69.8)	P>0.05	P=.046 in
≥1-hours	10 (16.4)	14 (23.0)		20 (31.7)	19 (30.2)		P=.364 in End-line
Systolic BP							
<120 mmHg	14 (23.0)	22 (36.1)	P>0.05	31 (49.2)	30 (47.6)	P>0.05	P=0.002 in Base-line but
≥120	47 (77.0)	39 (63.9)		32 (50.8)	33 (52.4)		P=.192 in End-line
Diastolic							
<80 mmHg	30 (49.2)	30 (49.2)	P>0.05	34 (54.0)	37 (58.7)	P>0.05	P>0.05
≥80	31 (50.8)	31 (50.8)		29 (46.0)	26 (41.3)		(All-timelines)

Student's t-test was used for Continuous variables and chi-square for categorical variables. 'Within groups' is denoted as a comparison among 2 time periods for both the treatment and placebo groups (Bonferroni-adjusted pairwise comparisons across repeated time levels). 'Between groups' denoted as a comparison between the vitamin D-treated group and Placebo

4.2.2 Multivariate analysis

In this study, two-way repeated measure ANOVA mixed models (GLM/general linear model) and a '**Fixed-Effect Regression Model using dummy variable'** were used for statistical analyses (SPSS, version 26) for the measurement of significant changes of eleven biochemical parameters across timelines (within groups) and between groups (treatment versus placebo) following (3-months) vitamin D supplementation. **'Multiple linear Regression'** analysis was also used for building multivariate models where necessary.

Confounding variables for vitamin D supplementation among T2DM patients were reported. However, as these variables are the risk factors for the disease, and are unequally distributed between exposure groups, so precautionary measures were taken from the data collection such as proper randomization to the analysis procedure (stratification and regression analysis. One study reported (Jager *et al*. 2008) that multivariable analysis allows assessment of the independent effects of many exposures on an outcome while controlling for confounding factors. In this study, "Multivariate linear regression" and "Fixed effect regression models were used in multivariate analyses.

Moreover, Multicollinearity or tolerance was also observed through variance inflation factors (VIF),which was very low, <1. As the multivariate advanced analysis was performed, confounders were substantially minimized.

Therefore, this study did not mention any confounding factors which can influence both the outcome and exposure variables.

The first multivariate model (table 11) showed 3-timelines (baseline, follow-up, and end line) for all glycemic indices (e.g. FBG, FBI, HOMA-**β**, HOMA-IR, HOMA-IS) except vitamin D, HbA1C, CRP, MDA, SOD, and Calcium had two timelines (baseline versus end line). While considering comparison among 'within time variant' treatment groups (baseline versus follow-up, baseline vs. end line, follow-up vs. end line) all biochemical indices like HbA1C, FBG, HOMA-**β**%, HOMA-IS%, HOMA-IR%, vitamin D, CRP, and SOD were significantly different ($P<0.05$) except MDA and calcium. On the other hand, within time variant placebo groups (baseline versus follow-up, baseline vs. end line, follow-up vs. end line) except for HbA1C, vitamin D, calcium, and SOD (all $P>0.05$), other biochemical indices like FBG, HOMA-IR, and CRP significantly increased across 3-timelines (FBG:10.6±2.4, 9.1±3.6, 11.5±2.3, $P<0.001$; HOMA-IR:1.57±0.3, 1.44±0.26, 1.58±0.3, $P<0.05$; CRP: 6.2±1.1, 6.9±1.9, 8.5±2.9, $P<0.05$) and MDA significantly increased from baseline to end line (6.2±1.1 to 8.5±2.9, $P<0.001$). However, FBI, HOMA-**β**% and HOMA-IS% significantly decreased in 3 different timelines (FBI: 10.1±1.5, 9.4±1.0, 9.9±1.4, $P<0.05$; HOMA-**β**: 30.5±11.7, 48.6±26.9, 25.5±8.0, $P<0.05$; HOMA-IS: 65.9±11.7, 71.3±11.6, 64.9±11.4, $P<0.05$). While considering comparison “between groups" (treatment versus placebo) some biochemical variables (vitamin D, A1C, SOD, HOMA-**β**, HOMA-IS) increased ($P<0.05$) and some decreased (FBG, CRP, MDA, HOMA-IR) significantly ($P<0.05$) in the treatment group as compared to placebo except FBI and calcium remain independent ($P>0.05$).

Table 12 is the ‘summary table' of table 11 showing baseline to end line (mainly 2 timelines) changes of eleven biochemical indices by P-trends for within time variant

groups of treatment and placebo and between groups (treatment versus placebo) following (3-months) vitamin D supplementation. While considering comparison 'within time variant treatment groups' (baseline versus end line), all biochemical indices like HbA1C, FBG (all pairs), HOMA-**β**%, HOMA-IS%, HOMA-IR%, vitamin D, CRP, and SOD were significantly different (P<0.05) except MDA. On the other hand, within time variant placebo groups (baseline versus end line), except HbA1C, vitamin D, calcium and SOD (all P>0.05), other biochemical indices like FBG significantly increased from baseline to end line (10.6±2.4 to 11.5±2.3, P<0.001), HOMA-**β**% significantly decreased from baseline to end line (30.5±11.7 to 25.5±8.0, P<0.05), FBI and HOMA-IS% insignificantly decreased from baseline to end line (**FBI:** 10.1±1.5 to 9.9±1.4, P>0.05; HOMA-IS%: 65.9±11.7 to 64.9±11.4, P>0.05), HOMA-IR% insignificantly increased from baseline to end line (1.57±0.3 to 1.58±0.3, P>0.05), CRP significantly increased from baseline to end line (6.2±1.1 to 8.5±2.9, P<0.05), and MDA significantly increased from baseline to end line (6.2±1.1 to 8.5±2.9, P<0.001). Whenever considering between groups (treatment versus placebo) some biochemical variables (vitamin D, A1C, SOD, HOMA-**β**, HOMA-IS) increased (P<0.05) and some decreased (FBG, CRP, MDA, HOMA-IR) significantly (P<0.05) in the treatment group as compared to placebo except FBI and calcium remain independent (P>0.05).

Table 13 describes the association of six glycemic indices (A1C, FBG, FBI, HOMA-**β**, HOMA-IS, HOMA-IR) and other four biochemical indices (CRP, MDA, SOD, and calcium) with vitamin D level by R^2-changes across timelines using 'Fixed-effect regression model using dummy variables and multiple regression'. It outlines that FBG (**β**= -.650, Std. error =.447, p<.05), CRP (**β**= -1.042, Std. error

=.242, p<.001) and MDA (**β** = -3.695, Std. error =.1.368, p<.01) are significant Negative predictors (one unit increase of vitamin D level decreased these variables significantly) of vitamin D status. Inversely, SOD (**β** =1.213, Std. error =.502, p=.016) and Calcium (**β** =1.386, Std. error =.574, p=.017) are significant positive predictors (one unit increase of vitamin D level increased these variables significantly as compared to placebo) of Changing Vitamin D level over 3-months intervention. Moreover, variations in HbA1c, FBI, and different derived parameters (from Insulin and FBG) over time like HOMA-**β**, HOMA-IS, and HOMA-IR were not significant predictors of variation in Vitamin D level over 2 timelines while was significant in bivariate analysis for both within time variant groups (baseline versus end line) and between groups (treatment versus placebo) (shown in table 12). Although regression analysis showed a decrease in insulin sensitivity (HOMA-IR) by a factor of 5.4 due to one unit increase in vitamin D level but it was not significant (P=.263)

Table 14 reveals that FBG (**β**= -.11, 95% CI (.84-.95), p=.000), and MDA (**β** = -3.695, 95% CI (.98-.99), p=.000) are significant Negative predictors (one unit increase of vitamin D level decreased these variables significantly after adjusting total calorie intake as calorie intake of both treatment and place group were independent, P=.933) of Changing Vitamin D level over 3-months intervention where calorie intake was adjusted. Moreover, variations in HbA1c, FBI, CRP, and different derived parameters (from Insulin and FBG) over time like HOMA-IS, and HOMA-IR were not significant (p>0.05) predictors of variation in Vitamin D level over 2 timelines.

Table 15 postulates that FBG (**β**= -.11, 95% CI (.89-.95), p=.000) and MDA (**β**= -.07, 95% CI (0.88–0.99), P=.04) are significant negative predictors of vitamin D status for treatment group while comparing placebo group (one unit increase of vitamin D level decreased these variables significantly after adjusting Physical activity level as PAL value (Both 1.7) of both treatment and place group were independent), whereas SOD (**β** =0.09, 95% CI (0.96-1.22), p=.03) and HOMA-IS (**β**= 1.34, 95% CI (1.84–8.96), p=.023) significant positive predictors (one unit increase of vitamin D level increased these variables significantly after adjusting Physical activity level) of changing Vitamin D level over 3-months intervention when Physical activity level was adjusted. Moreover, variation in HbA1c, FBI, CRP, and HOMA-IR- a derived parameter (from Insulin and FBG) was not a significant predictor of variation in Vitamin D level over 2 timelines (table 15).

Validity tests for different biochemical indices of this study showed that no significant differences exist (P>0.05) if different biochemical parameters like fasting blood insulin (FBI), vitamin D, and CRP were estimated from two different laboratories (i.e. Popular Diagnostic Center and BIRDEM). Thus, test results did not vary for different laboratory diagnoses and thus null hypothesis was accepted (table 16).

Figure 5 showed percent (%) changes in different biochemical parameters among the treatment group from baseline to the end line. The highest changes of variation were observed in Vitamin D (21.3%) followed by HOMA-**β** (14.2%), HOMA-IS (3.0%), and SOD (1.4%).

Table 11 Changes of different biochemical indices across three or two-time points following (3-months) vitamin D supplementation

	Treatment (n=61)				Placebo (n=63)				P-value for Between-groups
Mean Indices	**Baseline (1)**	**6-weeks follow-up (2)**	**End- line (3)**	**P-value for Within-Treatments groups**	**Baseline (1)**	**6-weeks follow-up (2)**	**End-line (3)**	**P-value for Within placebos groups**	
Vitamin D (25-OH)$_2$ (D1, D3) (ng/ml)	14.5±6.1	-	35.8±7.5	P=.000	19.4±8.8	-	20.5±5.2	P=0. 965	P=.001
HbA$_1$C (A1C$_1$ & A1C$_3$) (%)	8.97±1.9	-	8.5±1.6	P=.004	7.9±2.1	-	7.7±0.60	P=.587	P<.01 (All timelines)
Fasting Blood Glucose (FBG$_1$, FBG$_2$, FBG$_3$) (mmol/L)	10.9±3.5	9.98±3.3	8.42±1.7	P<0.001 (All pairs)	10.6±2.4	9.1±3.6	11.5±2.3	P<.001 (All pairs)	P=000 (End-line)
Fasting Plasma Insulin (FPI$_1$, FPI$_2$, FPI$_3$) (µU/ml)	9.8±2.28	9.2±1.0	10.2±1.9	P<0.05 (FPI$_1$ vs. FPI$_2$) (FPI$_2$ vs. FPI$_3$)	10.1±1.5	9.4±1.0	9.9±1.4	P<0.05 (FPI$_1$ vs. FPI$_2$) (FPI$_2$ vs. FPI$_3$)	P>0.05 (All-timelines)
HOMA-**β** (**β**$_1$, **β**$_2$, **β**$_3$) (%)	34.0±21.7	36.9±21.1	48.2±24.7	P<0.05 (**β**$_1$ vs. **β**$_3$) (**β**$_2$ vs. **β**$_3$)	30.5±11.7	48.6±26.9	25.5±8.0	P=.000 (all pairs)	P<.01 (Follow-up, End-line)
HOMA-IS (IS$_1$, IS$_2$, IS$_3$) (%)	66.1±16.4	72.6±10.6	69.7±11.6	P<0.01 (IS$_1$ vs. IS$_2$)	65.9±11.7	71.3±11.6	64.9±11.4	P=0.01(IS$_1$ vs. IS$_2$) (IS$_2$ vs.IS$_3$)	P=.023 (End-line)
HOMA-IR (IR$_1$, IR$_2$, IR$_3$) (%)	1.62±0.45	1.42±0.24	1.48±o.25	P<0.05 (IR$_1$ vs. IR$_2$) (IR$_1$ vs. IR$_3$)	1.57±0.33	1.44±0.26	1.58±0.3	P<0.05 (IR$_1$ vs. IR$_2$) (IR$_2$ vs.IR$_3$))	P=.030 (End-line)
C-reactive protein (mg/L) (CRP$_1$, CRP$_2$, CRP$_3$)	5.8±1.6	5.1±2.2	4.1±1.3	P<0.05 (CRP$_1$ vs. CRP$_3$) (CRP$_2$ vs. CRP$_3$)	6.2±1.1	6.9±1.9	8.5±2.9	P<0.05 (CRP$_1$vs.CRP$_2$) (CRP$_1$vs.CRP$_3$)	P<.001 (Follow-up, End-line)
Malonaldehyde (µm/ml) (MDA1 & MDA3)	2.0±0.41	-	1.93±0.42	P=.270	1.95±0.16	-	2.23±0.4	P=.000	P=.000 (End-line)
Super-Oxide-Dismutase (U/ml) (SOD1 & SOD3)	4.42±0.97	-	5.8±1.17	P=.000	4.6±0.68	-	4.6±0.87	P=.644	P=.000 (End-line)
Serum Calcium (Ca1, Ca3) (mg/dl)	9.7±0.57	-	9.8±0.46	P=0.295	9.8±0.56	-	9.4±1.42	P=.080	P=0.086 (End-line)

Two-way repeated measure ANOVA Mixed models and 'Fixed-Effect Regression Model using a dummy variable. 'Within groups' is denoted as a comparison among 3 time periods for both treatment and placebo (Bonferroni-adjusted pairwise comparisons across repeated time levels). 'Between groups' is denoted as a comparison between the vitamin D-treated group and Placebo (independent sample t-tests for two or three timelines).

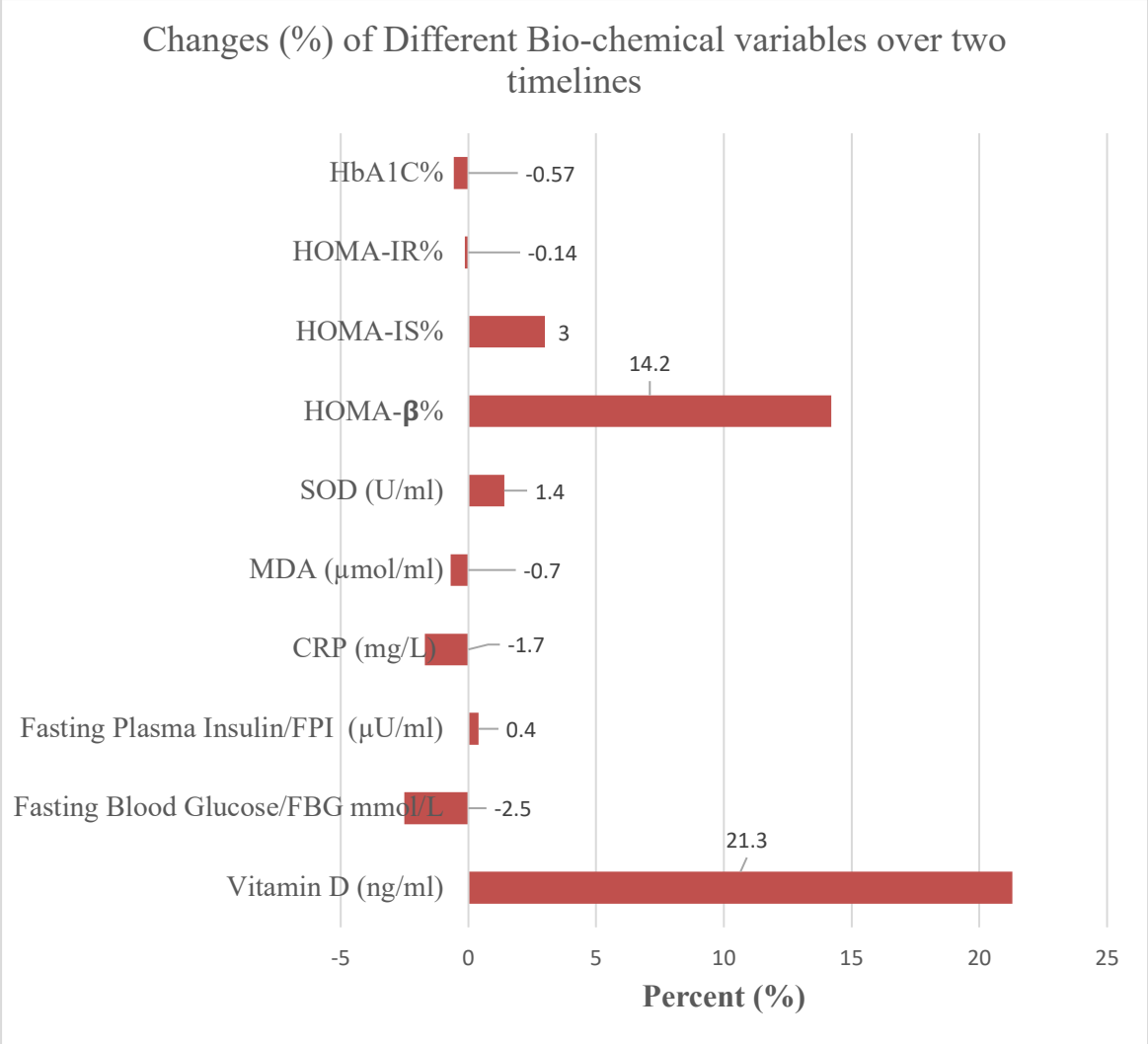

Figure 5: Percent (%) changes of different biochemical parameters among treatment group from baseline to the end line.

Table 12: Changes of different biochemical indices between treatment and placebo group following (3-months) vitamin D supplementation (summary table* by P-trends)

Biochemical Indices	P-trend for within groups (baseline to end line)		Between groups
	Treatment	Placebo	P-trend
1. Vitamin D $(25\text{-}OH)_2$ (ng/ml)	↑P=.000	P=0.965	P=0.001(All timelines)
2. HbA1C (%)	↓ P=.004	P=0.587	P<.01 (All timelines)
3. Fasting Blood Glucose (mmol/L)	↓P<0.001 (all pairs)	↑ P<.001 (all pairs)	P=0.00 (End line)
4. Fasting Plasma Insulin (µU/ml)	↑P<0.05	↓P>0.05	P>0.05 (All timelines)
5. HOMA-**β** ((%)	↑P<0.05	↓ P=.000 (all pairs)	P<.01(Follow-up, End line)
6. HOMA-IS (%)	↑P<0.01	↓ P>0.05	P=.023 (End line)
7. HOMA-IR (%)	↑P<0.05	↑ P>0.05	P=.030 (End line)
8. C-reactive protein (mg/L)	↓P<0.05	↑ P<0.05	P<.001 (Follow-up, End line)
9. Malonaldehyde (MDA, µm/ml)	↓P=.270	↑ P=.000	P=.000 (End line)
10. Superoxide dismutase (SOD, U/ml)	↑P=0.000	P=0.644	P=0.000 (End line)
11. serum calcium (mg/dl)	↑P=0.295	P=0.080	P=.086 (End line)

*Summary table of table 11 by showing P-trends for within-group (treatment and placebo for baseline versus end line) and between groups (treatment versus placebo).

Table 13: "Fixed-Effect multiple Regression Model" describing the association of glycemic indices and other important biochemical parameters with Vitamin D (ng/ml) by R^2-changes across timelines

Bio-chemical Variables	β-Coefficient (Unstandardized)	Standard error	P-value	R^2-change**	Interpretation (One unit change in vitamin D, decreases/Increase)
HbA1C (%)	-0.291	.351	0.408	Onlythe treatment group (model 1)	⇓ HbA1C by 0 .291 unit
FBG (mmol/L)	-0.650	.447	0.010	=6.2%	⇓ FBS by 0 .650 unit
FBI (µIU/mL)	0.232	.623	0.711	Including 10 bio-chemical-parameters	⇑ insulin by 0 .232 unit
HOMA-β (%)	0.034	.056	0.542	(model-2) = 50.6%	⇑ HOMA-βby 0.034 unit
HOMA-IS (%)	0.064	.157	0.683	R^2-change =44.4%	⇑ HOMA-IS by 0.064 unit
HOMA-IR (%)	-5.366	4.778	0.263	(F=19.17, P<0.001)	⇓ HOMA-IR by 5.366
CRP(mg/L)	-1.042	.242	0.000		⇓ CRP by 1.042 unit
MDA(µM/ML)	-3.695	1.368	0.007	Time-varying	⇓ MDA by 3.695 unit
SOD(U/ML)	1.213	.502	0.016	Changes=47.3%	⇑ SOD by 1.213 unit
Calcium	1.386	.574	0.017		⇑ Calcium by1.386 unit

**Model 1 has only a treatment group (R^2 accounted for 6.2%), model-2 has included 10 biochemical predictors (R^2 accounted for 50.6%), and thus time-varying changes accounted for 47.3%. Overall R^2-change =44.4%, (F=19.17, P<0.001).

Table 14: Multiple Linear regression showing the association of Vitamin D with different biochemical parameters after adjusting total kcal intake (mean difference) for treatment group

Variable	β-Coefficient	95% CI [lower-upper]	P- value	Interpretation (One unit increase in vitamin D)
HbA1c	-0.04	0.93 – 0.99	0.214	⇓ HbA1C by 0.04 unit
FBG	-0.11	0.84 – 0.95	0.000	⇓ FBG by 0.11 unit
FBI	0.06	0.97 – 1.16	0.216	⇑ Insulin by 0.06 unit
HOMA-IR	-0.02	0.94 – 1.02	0.420	⇓ Insulin resistance by 0.02
HOMA-IS	1.34	1.69 – 8.56	0.121	⇑ Insulin sensitivity by 1.34 unit
CRP	-0.03	0.92 – 1.02	0.187	⇓ CRP by 0.03 unit
MDA	-0.01	0.98 – 0.99	0.000	⇓ MDA by 0.01 unit

Table 15: Multiple Linear regression showing the association of Vitamin D with biochemical parameters after adjusting physical activities (mean differences) for treatment group

Variable	Coefficient	95% CI [Lower-upper]	P-value	Interpretations (One unit increase in vitamin D)
HbA1c	-0.04	0.93 – 0.99	0.215	↓ HbA1C by 0.04 unit
FBG	-0.11	0.84 – 0.95	0.000	↓ FBS by 0.11 unit
Insulin	0.06	0.97 – 1.16	0.216	↑ Insulin by 0.06 unit
HOM-IR	-0.02	0.94 – 1.02	0.420	↓ Insulin resistance by 0.02 unit
HOMA-IS	1.40	1.84 – 8.96	0.023	↑ Insulin sensitivity by 1.40 unit
CRP	-0.03	0.92 – 1.02	0.197	↓ CRP by 0.03 unit
MDA	-0.07	0.88-0.99	0.04	↓ MDA by0.07 unit
SOD	0.09	0.96-1.22	0.03	↑ SOD by 0.09 unit

Table 16: Validity Test for different biochemical indices of the study

Pairs	Biochemical Parameters	Mean± SD	P value
Pair 1	Fasting blood Insulin (Popular Diagnostic Center)	9.52 ± 4.45	0.415
	Fasting blood Insulin (BIRDEM)	9.98 ± 2.52	
Pair 2	Vitamin D (Popular Diagnostic Center)	16.84 ± 6.94	0.055
	Vitamin D (BIRDEM)	18.24 ±1.47	
Pair 3	CRP (Popular Diagnostic Center)	6.84 ±3.75	0.154
	CRP (BIRDEM)	5.83 ±5.61	

Chapter Five

Discussion

5. Discussion

This 12-week-long randomized clinical trial was conducted following a pilot study (Begum et al, 2021) to find out the current prevalence of vitamin D levels among T2DM patients of Dhaka city. The pilot study showed a 100% (n=23) prevalence rate for both sexes [(mostly 78.3% were deficient (10-30 ng/ml) and the rest (21.7%) were severely deficient (<10 ng/ml)] which is in line with most of the earlier studies conducted in Bangladesh (Hossain et al., 2018). A previous study (Micka, 2009) also informed that over 81% of the Bangladeshi women of reproductive age had vitamin D levels below the cut-off value (30 ng/ml) and the vast majority (90%-95%) of diabetic patients of Bangladesh have Type 2 diabetes with often insidious and asymptomatic onset (Mahtab et al., 2003). A recent study (Hossain et al, 2021) elucidated that vitamin D level was lower and the frequency of vitamin D deficiency was higher in among adult Bangladeshi patients with T2DM. However, vitamin D was not associated with pre-diabetes or its categories also reported in a cross-sectional study (Morshed et al., 2022). Studies also outlined that vitamin D deficiency was associated with low sun exposure, overweight/obesity (Hossain et al., 2018, 2021), and high blood glucose levels among T2DM patients (Nadia et al., 2021).

This study found that supplementation with 20,000 IU 25-hydroxy cholecalciferol (vitamin D3) at every 5th day for 12 weeks effectively (P=.000) increased the 25 (OH) D3 level from deficient (baseline: 14.5±6.1 ng/mL) to sufficient levels (end line 35.8±7.5 ng/mL) (i.e. deficient=< 30 ng/ml, sufficient ≥30 ng/ml) as defined by the Endocrine Society (Holick et al., 2011). Moreover, end-line 25-hydroxyvitamin D levels were significantly higher in the treatment group as compared to placebo (treatment: 35.8 ±7.5

ng/mL versus placebo: 20.05±5.2 ng/mL, p=0.001) and baseline to end-line changes in the placebo group was independent (Baseline: 19.5 ± 8.8 ng/mL vs. end line: 20.05 ± 5.2 ng/mL, p = 0.965). Mazahery & von Hurst (2015) also observed that factors significantly affect 25-Hydroxyvitamin D concentration in response to vitamin D supplementation. Similar results also reported by couple of studies [(Tang et al., 2018) (baseline: 41.16 nmol/L versus end line: 82.22 nmol/L);(Ryu et al., 2014) (35.4 ± 8.5 ng/mL vs. 18.4 ± 7.3 ng/mL, p < 0.001); (Anyanwu et al., 2017)].

Vitamin D and glucose homeostasis: Present randomized single-blind placebo-controlled study postulated significant correction in vitamin D status among the T2DM patients along with changes only in FBG level which has both agreement (Al-Zahrani et al., 2014) and disagreement (Krul-Poel et al., 2015) with existing works of literature. There are several studies (Al-Sofiani et al., 2015) supporting the idea that vitamin D is an important nutrient in the control of glucose homeostasis. However, studies (Al Thani et al., 2019) showed the opposite finding and elucidated that there is no effect of intermittent vitamin D3 supplementation on glycemic control in diabetic patients (including pre-diabetic and T2DM) and also for overweight and obese individuals (Jamka et al., 2015). There is one mechanism of relating vitamin D to diabetes is the action of the insulin receptor in beta cells which can stimulate gene expression of insulin receptor and thereby increases the transport of glucose from the intestine. Another mechanism is that the active form of vitamin D [1, 25 $(OH)_2$ D3] helps calcium absorption from the gut which is responsible for insulin release from beta-cells. It has been cleared that beta-cells have receptors for 1, 25 $(OH)_2$ D3, and these cells can convert 25-hydroxy Cholecalciferol or 25 (OH) D3 (Esmaeil et al., 2014). However,

this short period may not be enough to manifest improvement in HBA1c measurement which is routinely recommended to be measured every 3-4 months, although other important indices associated with some changes even in a short intervention period reported (Anyanwu et al., 2017).

Results from the bivariate analysis of this study revealed that mean FBG gradually decreased ($P<0.001$) from baseline 10.9 ng/ml (±3.5) to the end line 8.42 (±1.7) in the treatment group as compared to placebo ($P>0.05$) due to 12-weeks vitamin D supplementation. Moreover, multiple regression analysis asserted that FBG is negatively associated with vitamin D levels among T2DM patients which is in agreement with a cross-sectional study (Haidari et al., 2016). Similar findings have been also observed in other studies (Anyanwu et al., 2017; Dhillon et al., 2016; Haidari et al., 2016; Shivaprakash & Joseph, 2014). Foroughi et al., (2016) observed that after supplementation of vitamin D caused a marginally significant decrease in FBG levels.

Overall R^2-change ($F=19.17$, $P<0.001$) in multivariate analysis (table 13) showed 44.4% variation for different biochemical predictors of vitamin D and revealed that FBG, CRP, and MDA are ($P<.05$) are inversely associated with vitamin D levels of T2DM patients. In contrast, SOD and calcium have positive associations with changing Vitamin D levels over 3-months of intervention. However, other important glycemic indices like HbA1C, FBI, and derived parameters (HOMA-**β**, HOMA-IS, HOMA-IR) remained significant ($P<0.05$) only in bivariate analysis (table 11, 12) but not in multiple regression analysis (table 13). Non-significant changes in the mean FBS and HbA1c level after 12 weeks of Vitamin D3 supplementation in the treatment group compared to the placebo group were also reported in other studies (Anyanwu et al., 2017; Rashidi et al., 2016).

An increment of vitamin D reduced fasting blood glucose (FBG) while adjusting physical activity level (PAL) and total calorie consumption among T2DM patients was also noticed in the present study. Surprisingly, after adjusting PAL multiple regression analysis showed a rise in insulin sensitivity (derived glycemic index HOMA-IS) (table 15). This might be a positive effect of PAL on insulin sensitivity (Ginszt et al., 2018). A study by Al Thani et al., (2019) showed measures of glucose tolerance or insulin sensitivity, concerning baseline did not differ between the placebo and treatment groups (pre-diabetic and severely vitamin-deficient) following 24-week Vitamin D supplementation. However, in participants with pre-diabetes, 6-month supplementation with vitamin D and calcium may improve insulin sensitivity but not for adults with low vitamin D status at risk of type 2 diabetes also reported (Gagnon et al., 2014). Contrarily, another study by Dhillon *et al*. (2016) showed insulin sensitivity is inversely proportional to insulin resistance in the treatment group and a significant reduction in HOMA-IR levels suggests improved insulin sensitivity in this group of patients compared to placebo due to 12-week vitamin D supplementation. In another study outlined (Anyanwu *et al*., 2017) 12 weeks of vitamin D3 supplementation results in a reduction in insulin resistance, but does not affect pancreatic beta-cell function in T2DM patients. Vitamin D repletion for 12 weeks increased serum vitamin D concentrations and improved ß-cell (the ability of insulin secretion) activity in vitamin D-deficient type II diabetes with no significant changes in HbA1c or insulin sensitivity or insulin resistance also reported (Al-Sofiani et al., 2015). A study by Ryu et al., (2014) observed after supplementation of vitamin D (4,000IU daily) improved insulin sensitivity (HOMA-IS %) and HOMA-IR% and decreased fasting blood insulin (FBI) in insulin-resistant Asian females compared to placebo. Contrarily, studies

reported after vitamin D supplementation no significant differences between treatment and placebo were observed in terms of HOMA-%B (Tang et al., 2018) and HOMA-IR (Mabhala et al., 2017). However, Foroughi et al., (2016) observed that after supplementation of vitamin D caused a marginally significant decrease in HOMA-IR and FBG, but no significant effect on insulin level and HOMA-B.

A recent meta-analysis elucidated that Vitamin D supplementation in T2DM patients can improve HbA1c, insulin resistance, and insulin in a short-term intervention (Hu *et al.*, 2019). HbA1c is representing glycosylated hemoglobin which has around 100 days of lifetime, a favorable follow-up duration is more than three months. The present study showed that during the vitamin D supplementation period mean A1C level (%) decreased (P=0.004) among the treatment group than placebo from baseline **(**8.9±1.9) to the end line (8.5±1.6). However, despite remaining significant (P<0.05) in bivariate analysis (table 11, 12) HbA1C could not remain significant in multiple regression analysis (table 13) which is in line with a recent meta-analysis (Krul-Poel et al., 2017) and a study conducted by Tang and his colleagues (2018) where HbA1c not able to decreased significantly after vitamin D supplementation in the treatment group. However, despite having no effect of vitamin D supplementation on the decrease of HbA1C levels among T2DM patients, it decreases the pathophysiological process of the disease (Al-Sofiani et al., 2015). In contrast to this finding, an Indian study (Dhillon et al., 2016) showed a significant mean percentage reduction in HbA1c and HOMA-IR levels, and the treatment group showed a more favorable effect in terms of mean percentage reduction in HbA1c and HOMA-IR compared to placebo.

Progression and the major pathophysiological abnormalities of type 2 diabetes are insulin resistance and pancreatic Beta-cell dysfunction (Ajabshir, 2018). The functions of vitamin D in glucose homeostasis are to increase the secretion of insulin from β-cells of the pancreas and increase the sensitivity of insulin in the peripheral target tissue. A probable mechanism of the Vitamin D effect on insulin secretion can be obtained by the existence of Vitamin D Receptors and the expression of 1-α-hydroxylase in pancreatic cells. Also, response elements to Vitamin D in human insulin promoters may activate insulin gene transcription (Islam SS et al., 2016). Insulin is a peptide hormone that helps unlock the cells for the absorption of glucose from the bloodstream and inhibits the mechanism of gluconeogenesis by the liver. Beta cells which release from the pancreas are responsible for insulin production as well as secretion. If there is a lack of insulin production and decreased insulin sensitivity (insulin resistance) results from an accumulation of excess glucose in the blood, as a result, cells become starved for glucose, which can lead to multi-organ damage. Vitamin D has a beneficial effect on insulin action because in skeletal muscle there is a receptor for vitamin D which helps to demonstrate increased expression of the insulin receptor and therefore enhance insulin responsiveness for glucose transport. Indirectly vitamin D may also enhance the action of insulin by regulating extracellular calcium and thereby it affects calcium influx through cell membranes and maintaining an adequate intracellular calcium pool (Ajabshir et al, 2018). The most common measures of insulin are a measurement of the Homeostatic Model Assessment (HOMA) for Insulin Resistance (HOMA-IR), insulin sensitivity (HOMA-IS), and beta function (HOMA-**β**).

Socio-demography and vitamin D-related characteristics: In this study vitamin D supplementation showed no significant impact on socio-demography, BMI (Kg/m^2), co-morbidity, vitamin D, and Stress-related Characteristics between treatment and control groups as those variables are somewhat independent ($p>0.05$) both at baseline (table 1, 2) and end line (table 10). Vitamin D status was associated with several sociodemographic variables in vitamin D-deficient subjects in Bangladesh (Hossain *et al*., 2018). However, studies also showed that age, sex, smoking status, BMI, systolic BP, diastolic BP, family history of DM (Alam et al., 2018), sun-exposed time, and barriers to sun exposure due to clothing or covering (Al-Zahrani et al., 2014) were not found to influence vitamin D level independently.

Vitamin D and inflammation marker (CRP): In this study, multiple regression analysis outlines that CRP increased significantly when vitamin D level is decreased. As a sensitive marker of low-grade inflammation, CRP is the most commonly measured best indicator or risk marker for Coronary Heart Disease (CHD) in T2DM patients and by enhancing the release of tissue factors from macrophages and accelerates to get atherosclerosis in diabetic patients, which leads to activate the complement system and by binding with them causes the aggregation of LDL-C and VLDL-C (Ali et al., 2015). However, a study showed no association between CRP and vitamin D (Haidari et al., 2016). Low-grade inflammation is one of the hallmarks of T2DM, which is responsible for a rise in circulating cytokines. When there is an increase amounts of circulating inflammatory cytokines such as tumor necrosis factor-alpha (TNF–α) and interleukin 6 (IL-6) contribute to insulin resistance (IR) in muscle and adipose tissues significantly. Vitamin D3 metabolites [1, 25 $(OH)_2$ D3] cause an increase in insulin sensitivity by

reducing inflammatory cytokines and by increasing insulin receptor gene expression (Haidari et al., 2016;).

Vitamin D and Stress markers (MDA and SOD): The two biomarkers of free radical/oxidative stress are plasma superoxide dismutase (SOD) and malondialdehyde (MDA). In the present study, MDA and SOD showed opposite results both in Bivariate (table 11, 12) and multivariate (table 14) analysis. Bivariate analysis (table 11, 12) showed an insignificant reduction of mean MDA level from baseline (2.0±0.41 µmol/ml) to the end line (1.93±0.42) after vitamin D supplementation while the placebo group had significant ($P<0.001$) increment of MDA level in end line (2.23±0.04 µmol/ml) than in baseline (1.95±0.2). In contrast, the treatment group showed a significant difference ($p=0.000$) in SOD level from baseline to end line (4.42±0.97 U/ml to 5.8±1.17U/ml) compared to placebo. However, both MDA and SOD remained as significant predictors of changing vitamin D levels across timelines in multilevel analysis. Multiple regression analysis outlines that MDA increased significantly when vitamin D levels decreased after adjusting total calorie consumption or physical activity. In contrast, SOD has a significant positive effect in changing Vitamin D levels over 3 months of intervention.

As biological markers of oxidative stress both SOD and MDA have been considered. Systemic inflammation has been linked to oxidative stress. Impaired oxidative balance and increased ROS production result in activation of nuclear factor κB (NFκB) which plays a critical regulatory role in immunity and inflammatory responses (Ajabshir, 2018). Malondialdehyde (MDA) is a stable end product of lipid peroxidation and therefore can be used as an

indirect measure of cumulative lipid peroxidation. Oxidative stress plays a vital role in systemic inflammation, the development of type 2 diabetes, and its related complications (Ajabshir, 2018). Increased activity of SOD was independently associated with lower all-cause mortality in older women but not in men reported in a recent study (Mao et al., 2019) and physical activity significantly reduced MDA levels also reported in an earlier study (Arslan M., et al., 2014).

Vitamin D with Diet and Physical Activities: Present study could not find any significant relation of vitamin D level with BMI (kg/m^2), although more than half of them (52.4%) were overweight, which is one of the risk factors for diabetes. Bivariate analysis (table 4, 5, 6, 7) showed calorie consumption (Treatment vs. placebo baseline: 2698 vs. 2743; end-line Treatment vs. placebo: 2549 vs. 2575, all $P>0.5$) and physical activity level (PAL) of both treatment and placebo group were independent (baseline and end line PAL=1.7, $P>0.05$) for both timelines. However, after adjusting calorie consumption (table 14) and PAL (Table 15) multivariate analysis showed respectively FBG, MDA, and FBG, HOMA-IS, MDA, and SOD are significant predictors of changing Vitamin D levels over 3-months intervention. This might be due to the influential effect of PAL either on glucose homeostasis (Ginszt et al., 2018; Mann et al., 2014) or on stress markers (Arslan et al., 2014; Poblete-Aro et al., 2018). It can be explained by theoretically, when there is a deposition of vitamin D in body lipids (e.g. lower PAL or overweight/over calorie consumption) it reduces the bioavailability of vitamin D3 from the skin resulting in the interruption of vitamin D synthesis (Hidayat et al., 2010). Vitamin D affects directly insulin secretion, insulin sensitivity, and Beta cell function, and hence mediates glucose homeostasis

(Ajabshir, 2018). When there is less intake of vitamin D or lack of exposure to sunlight vitamin D deficiency occurs. Decreasing oxidative stress (Arslan et al., 2014) and increasing the production and secretion of insulin, and improving β-cell function through physical activities among T2DM patients (Ginszt et al., 2018) enable vitamin D and its metabolites to play a role in reducing glucose in T2DM patients. Studies reported physical activities significantly reduced MDA levels (Arslan et al., 2014) and improved insulin sensitivity, preventing impaired glucose tolerance and delaying the onset of diabetes complications through a synergistic effect with insulin by enhancing glucose uptake into the cells and subsequently increasing blood flow in the muscles (Ginszt et al., 2018).

Lack of physical activity and obesity are important risk factors for T2DM. Central adiposity can be directly related to vitamin D status (Islam et al., 2016). However, Safarpour *et al*. (2020) showed non-significant differences in physical activity levels among the treatment group compared to the control. Greater BMI indicates more occurrence of vitamin D deficiency, especially when it is BMI>25 kg/m2 (overweight/obese). A study reported serum 25(OH) D3 level had a significant negative correlation with body mass index ($r= -0.391$, $p=0.017$) and positive correlation ($r=0.334$, $p=0.044$) with fasting plasma glucose in male subjects while no differences were observed for 25(OH) D3 level among normal weight, overweight and obese adults (30.6±6.2, 35.6±9.5 and 24.3±1.7ng/mL respectively) and between newly diagnosed male and female T2DM patients of Comilla district in Bangladesh (Alam et al., 2018).

Vitamin D and bone mineral Calcium: Multivariate analysis (table13) of this study showed calcium is a significant positive predictor of changing Vitamin D level over 3-months intervention while bivariate analyses revealed that calcium level became higher in the treatment group (baseline: 9.7 ±0.57 ng/mL vs. end line: 9.8±0.56 ng/mL, p = 0.295) compared to the placebo group (baseline: 9.8 ± 0.56 ng/mL vs. end line: 9.4 ± 1.52 ng/mL, p = 0.080). A slight increment of serum calcium in the treatment group due to vitamin D supplementation [0.70 mmol/L (95% CI: 0.06, 1.3), P = 0.03] was also observed by Thani et al. (2019). A low plasma level of Vitamin D usually leads to impaired calcium absorption in the bowel which consequently leads to enhanced bone turnover and impaired bone mineral density (BMD) also reported (Vranić et al., 2019).

6. Conclusion and Recommendation

Vitamin D deficiency has become an epidemic worldwide. Vitamin D deficient Type 2 diabetic patients had shown a favorable effect on glycemic control (in terms of reduction in fasting blood glucose, CRP, and MDA) over 12 weeks in the treatment group. Stress markers like MDA and SOD influence on vitamin D change over time in the treatment group while adjusting 'calorie intake' and 'physical activity'. As no variation was noticed over the period, it can be outlined that this effect is caused by only vitamin D supplementation. However, there was a positive effect on HbA1c, FBI, and Derived parameter HOMA -IR in Diabetes Mellitus as compared to the placebo group but these effects were not significantly different. In this study vitamin D supplementation showed no significant impact on socio-demography, BMI (Kg/m2), co-morbidity, vitamin D, and Stress-related Characteristics between treatment and control groups. Based on the findings there is a need to target high-risk groups such as pregnant, children, and any co-morbid patient. Effective educational campaigns would increase awareness about adequate intake of Vitamin D2(ergocalciferol) or D3(cholecalciferol) according to the recommendations for Endocrine Society and calcium-containing food. Though there is abundant sunlight in our country however there is a huge case of vitamin D deficiency. So exposure to sun rays is an effective way of enhancing vitamin D status. Also encouraging people the consumption of natural food sources rich in vitamin D, like eggs will be helpful. Vitamin D fortification or supplementation may also be viable options to improve the vitamin D status of our population. Last but not least routine supplementation and monitoring of serum vitamin D levels should be considered since this may help reduce the risk of glucose metabolism disorders.

7. Strength and Limitations

- Selection of treatment and placebo group unbiased randomization methods was applied.
- Validation of biochemical parameters (e.g. vitamin D, Fasting Insulin, and C-reactive protein) have been done from other laboratories (e.g. BIRDEM) and results between two laboratories were independent (P>0.005).
- Physical activity level (PAL), dietary Intake pattern, and nutrient analysis were performed, and comparisons between groups for both timelines were incorporated in regression analysis to obtain the influence of PAL and calorie intake on vitamin D levels after an intervention.
- Advanced Statistical analysis ('Fixed effect regression analysis using dummy variables') and the latest version of the software were used (SPPSS version 26).
- Difficult to maintain the time framework because the patient's flow was out of control.
- Due to the COVID-19 situation, it was difficult to follow up on the study subjects.
- Vitamin D estimation has been done twice at baseline and end line however, vitamin D estimation in between (1^{st} follow up) baseline and end line can make the results most precise.

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Appendices

Appendix I

তথ্য প্রদানের সম্মতি পত্র

আসসালামু আলাইকুম,

ঢাকা বিশ্ববিদ্যালয়ের পুষ্টি ও খাদ্য বিজ্ঞান ইনস্টিটিউটের পক্ষ থেকে আমি এসেছি। আপনি জানেন যে ঢাকা বিশ্ববিদ্যালয়ের এই পুষ্টি ও খাদ্য বিজ্ঞান ইনস্টিটিউট ছাত্র-ছাত্রীদেও পাঠদান ছাড়াও দেশের বিভিন্নবড়বড়জরীপকাজপরিচালনাকরে থাকে। এর সকল জরীপ কাজের অন্যতম হচ্ছে দেশের পুষ্টি জরীপ। এই জরীপ কাজের মাধ্যমে দেশের জনগণের পুষ্টি, স্বাস্থ্য ও অন্যান্য বিষয় তথ্য উদঘাটিত হবে। এই তথ্য দেশের জন সম্পদ উন্নয়নে সরকারের গৃহীত বিভিন্ন পরিকল্পনার কার্যকারীতা ও ভবিষ্যতের কর্ম পন্থা নিরূপণে ভূমিকা রাখবে বলে আশা করা যায়।

আমি এই জরীপে আপনার অংশ গ্রহণের জন্য আমন্ত্রণ জানাচ্ছি। আমি আপনার মতামতকে প্রাধান্য দিব। এই জরীপে পরিবারের গঠন, ভোগ ও ব্যয়, অর্থনৈতিক অবস্থা, পুষ্টি ও স্বাস্থ্যগত অবস্থা নিরূপণের জন্য শরীরের মাপজোক দিতে যারা রাজী থাকবেন তাদের নিকট থেকে নিরাপদ পদ্ধতি অবলম্বনের মাধ্যমে ৫ সিসি রক্ত গ্রহণ করা হবে।

এই সাক্ষাতকার নিতে এক ঘন্টা সময় লাগবে। এই কাজে আপনার আর্থিক ভাবে কোন ক্ষতি হবে না, শুধু কিছু সময় দিতে হবে। আপনি স্বেচ্ছায় এই জরীপে অংশ গ্রহণ করতে পারেন ও যে কোন সময় নিজেকে প্রত্যাহার করতে পারেন বা কোন বিশেষ প্রশ্নের উত্তর নাও দিতে পারেন। তবে আপনার একাজে অংশ গ্রহণ ও তথ্য প্রদান, মানব সম্পদ উন্নয়ন তথা জাতীয় উন্নয়নে ভবিষ্যৎ পরিকল্পনা গ্রহণ করতে সাহায্য করবে।
আপনার দেয়া তথ্যের সম্পূর্ণ গোপনীয়তা রক্ষা করা হবে। আপনার অংশ গ্রহণকেআমি স্বাগত জানাই। আপনার অংশ গ্রহণ গবেষণাকারী তথা সরকারকে বিভিন্ন নীতি নির্ধারণে সহায়তা করবে।

জরীপকারী আমাকে উক্ত তথ্য পড়ে শুনিয়েছেন ও মৌখিকভাবে এই জরীপের উদ্দেশ্যে ব্যাখ্যা করেছেন। সবকিছু জেনে স্বেচ্ছায় এই জরীপ কাজে অংশ গ্রহণে রাজী হয়েছি।

তথ্যদানকারী স্বাক্ষর **জরীপকারীর স্বাক্ষর**

Appendix II:

Effect of Vitamin D Supplementation on Glucose Homeostasis among Type 2 Diabetic Patients: A Randomized Clinical Trial

স্বাক্ষাতকারের তারিখঃ ক্রমিক নং মোবাঃ

পরিবার পরিচিতি, গঠন ও আর্থসামাজিক তথ্য

১. আপনি কোন ধর্মালম্বি? কোডঃ ১=মুসলিম, ২= হিন্দু, ৩ বৌদ্ধ, ৪= খ্রিষ্টান) ২. পরিবারের সদস্য সংখ্যা–

৩. জরিপ এলাকাঃ (১ = শহর এলাকা, ২= উপজেলা, ৩= গ্রাম)

সাক্ষাতদান কারীর নাম	বয়স		লিঙ্গ কোড –১	বৈবাহিক অবস্থা কোড–২	পেশা কোড –৩	পাশকৃত শ্রেণী শিক্ষা কোড–৪	ওজন (কেজি)	উচ্চতা (সেঃমিঃ)	বি.এম.আই (কেজি/মি^{২})	পরিবারিক ডায়াবেটিকস		আপনার কি কি লক্ষণ ছিল? কোড–৬	আপনি কিকি জটিলতায় ভুগছেন (গত তিনমাসে) কোড–৭	কোথায় চিকিৎসা নিয়েছেন, কোড–৮
	বছর	মাস								হ্যা (কারআছে) কোড–৫	না			

কোড–১ঃ লিঙ্গ
১= পুরুষ
২= মহিলা

কোড–২ঃ বৈবাহিক অবস্থা
১= অবিবাহিত
২= বিবাহিত
৩= বিধবা/বিপত্নীক
৪= তালাক প্রাপ্ত/প্রাপ্তা
৫=অন্যান্য.....

কোড–৩ঃ পেশা কোড
১= চাকুরী
২= ব্যবসা
৩= গৃহস্থলী
৪= এনজিও কর্মী
৫= দিন মজুর
৬= গাড়ি চালক
৭= বেকার
৮= কৃষি
৯= ছাত্র/ছাত্রী
১০= প্রবাসী
১১= মৎস্যজীবি
১২= প্রতিবন্ধি
১৩= অন্যান্য

কোড–৪ঃ শিক্ষা কোড
১= নিরক্ষর
২= সই করতে পারে
৩= স্কুলে যাওয়ার বয়স হয়নি
৪= পড়তে ও লিখতে পারে
৫= পড়তে পারে
৬= মাধ্যমিক
৭= উচ্চ মাধ্যমিক
৮= স্নাতক
৯= স্নাতকত্তোর
১০= অন্যান্য

কোড–৫ঃ পরিবারিক ডায়াবেটিকস
১= পিতা/মাতা
২= পুত্র/কন্যা
৩= দাদা/নানা
৪= দাদী/নানী
৫= অন্যান্য

কোড–৬ঃ আপনারকিকিলক্ষণছিল?
১= অবসাদ এবং দুর্বলতা
২= ঘন ঘনপ্রসাব
৩= পায়ে প্রদাহ
৪= মাংসপেশীতে পানি
৫= মাথা ঘোরা
৬= পানির পিপাসা পাওয়া
৭= ঘন ঘনক্ষিদে পাওয়া
৮= বমিবমিভাব
৯= অন্যান্য

কোড–৭ঃ আপনিকিকিজটিলতায়ভুগছেন (গত তিনমাসে)
১= দ্রুত ওজন কমে যাওয়া
২= উচ্চ মাত্রায় কোলস্টারাল
৩= উচ্চ রক্তচাপ
৪= দ্রুত শ্বাস প্রশ্বাস
৫= দৃষ্টি শক্তি কমা
৬= রক্তে গ্লুকোজ কমে যাওয়া
৭= স্নায়ু বৈকল
৮= চামড়া সংক্রামন
৯= কিডনি ফেলিওয়
১০= পাকস্থলি প্রদাহ
১১= মানসিক সমস্যা
১২= মাংসপেশীতে পানি
১৩= চোখে ছানি
১৪= অন্ধত
১৫= অন্যান্য

কোড–৮ঃ কোথায় চিকিৎসানিয়েছেন?
১= সরকারি
২= প্রাভেট
৩= ব্যক্তিগত
৪= কবিরাজি
৫= অন্যান্য

পরিবারের মাসিক আয়-ব্যয় তথ্যঃ

৪। পরিবারের মসিক আয় কত?..... টাকা

৫। পরিবারের মাসিক ব্যয় (নিম্নের খাত ওয়ারী)

কোড	ব্যয়ের খাত	টাকার পরিমান	কোড	ব্যয়ের খাত	টাকার পরিমান	কোড	ব্যয়ের খাত	টাকার পরিমান
১	খাওয়া		৪	যাতায়াত		৭	কৃষি কাজ	
২	শিক্ষা		৫	ডায়াবেটিস চিকিৎসা		৮	বসবাস	
৩	ঔষধ		৬	পোষাক-পরিচ্ছদ		৯	অন্যান্য	

সূর্যালোকে অবস্থান সম্পর্কিত তথ্যাবলী :

৬। আপনি কি জানেন, কখন সূর্যালোকের সর্বোচ্চ প্রখরতা সময়?

(কোডঃ ১=সূর্যদোয় থেকে সকাল ১০ টা, ২= সকাল ১০ টা থেকে বিকাল ৩ টা, ৩= বিকাল ৩ টার পর)

৭। দৈনিক কত ঘন্টা আপনি বাইরে সময় কাটান? (যানবাহনে অবস্থান ব্যাতীত)

(কোডঃ ১= ১ঘন্টার কম, ২= ১-২ ঘন্টা, ৩= ২-৩ ঘন্টা, ৪= ৩-৪ ঘন্টা, ৫= ৪-৫ ঘন্টা, ৬= ৫ ঘন্টা, ৭= দরকার হয় না)

৮। সূর্যলোকে অবস্থানের সময় (কোডঃ ১= সকাল ১০ টা, ২= সকাল ১০টা থেকে বিকাল ৩টা, ৩= বিকাল ৩টা, ৪= মাসে, ১ থেকে ৩ বার, ৫= কখনো না

৯। আপনি সূর্যলোকে অবস্থানকালীন সময়ে কি শরীর পুরোপুরি আবৃত রাখেন? (কোডঃ ১=হ্যাঁ, ২=না)

১০। যদি না হয়, তবে শরীরের কোন অংশগুলো অনাবৃত থাকে? (কোডঃ ১= শুধু মুখমন্ডল, ২= মুখ ও হাত, ৩= অন্যান্য)

১১। আপনি যখন সূর্যলোকে যান তখন কি সানস্কীন ব্যবহার করেন? (কোডঃ ১= হ্যাঁ, ২= না)

১২। গায়ের রং? (কোডঃ ১= বাদামী, ২= ফর্সা, ৩= অন্যান্য)

পারিবারিক খাদ্যাভ্যাস ও খাদ্য গ্রহণ :

১৩। গত ২৪ ঘন্টায় পরিবারে গৃহীত খাদ্য (রিকল পদ্ধতি)ঃ এ.ই ☐☐ ☐ সি.ইউ ☐☐ ☐ পরিবার নং ☐☐☐☐

খাদ্য গ্রহণের সময় এবং গৃহীত খাবার		খাদ্যের নাম	খাদ্য তৈরীতে ব্যবহৃত উপকরণের নাম	গৃহীত খাবারের পরিমাণ (গ্রাম)		অফিসে এসে পূরণ করতে হবে	
সকালে গৃহীত খাবার	মেনু কোড			রান্না ওজন	বাজার থেকে ক্রয়কৃত ওজন	খাদ্যেও কোড (এইচ কে আই)	অরাধা কোটা বাছার পরের ওজন
দুপুরে গৃহীত খাবার							
দিনের অন্যান্য সময় গৃহীত খাবার							
রাতের উদ্বৃত্ত খাবার							

১৪। বিগত সাত দিন নিম্নলিখিত খাদ্য গুলো সকাল, দুপুর ও রাত্রে কতবার খেয়েছেন? (না খেলে সেখানে (–) চিহ্ন দিন)

খাদ্যের গ্রুপ	শক্তিদানকারী খাদ্য				শরীর গঠন ও বৃদ্ধিসাধনকারী খাদ্য				রোগ প্রতিরোধক খাদ্য			
বেলা	ভাত	ভূট্টা	রুটি	ডাল	মাছ	মাংস	ডিম	দুধ	দুদ্ধ জাতীয় খাদ্য	শাক	সবজি	ফল
সকাল												
দুপুর												
রাত্রি												

দৈনিককার্যক্রম (গত ২৪ ঘন্টার)

১৫। বিগত ২৪ ঘন্টায় শারীরিক কার্যাবলীর বিবরণ ঃ ☐ **পরিবার নং** ☐☐☐☐

বিভিন্ন সময়	কার্যাবলীর ধরণ	কার্য শুরুর সময়	কার্য শেষের সময়	কার্যাবলীর কোড	মোট সময় (মিনিট)
ফযরের আযানের সময়					
ঘুম থেকে ওঠা					
জোহরের আযানের সময়...					
আছরের আযানের সময়					
মাগরিবের আযানের সময়					
রাতের খাবারের সময়					
ঘুমানোর সময়					

ডায়াবেটিস সংক্রান্ত তথ্যাবলিঃ

১৬। আপনি কত দিন যাবত ডায়াবেটিসে ভুগছেন? কোডঃ ১= < ১ বছর, ২= ১ থেকে ৫ বছর, ৩= ৫ থেকে ১০ বছর, ৪= > ১০ বছর

১৭। আপনি কি কখন ডায়াবেটিসের চিকিৎসা নিয়েছেন? (কোডঃ ১=হ্যাঁ, ২=না)

১৮। যদি হ্যাঁ হয়ে থাকে তবে কি কি ঔষধ নিয়েছেন? ----------

১৯। ডায়াবেটিস সনাক্ত হওয়ার পর আপনার জীবনে কোন পরিবর্তন হয়েছে কি? (কোডঃ ১=হ্যাঁ, ২=না)

২০। যদি হ্যাঁ হয় তবে কোন ধরনের পরিবর্তন হয়েছে? (কোডঃ ১= আগে থেকে ভাল ঘুম হয়, ২= সুস্থতা অনুভব করি, ৩= অন্যান্য)

21| Blood Pressure (mm of Hg) –

22. Biochemical Tests:

Sl No	Biochemical parameters	Reference Value	Baseline	After 6 weeks	After 12 weeks
1	Fasting Insulin Level				
2	25 (OH)vitamin D				
3	Fasting Blood Sugar (FBS)				
4	Serum Calcium				
5	HbA_1C (Glycated Haemoglobulin)				
6	MDA (Malon-dialdehyde)				
7	SOD (Superoxide -Dismutase)				
8	C-reactive protein (CRP)				
9	HOMA-IR(Homeostatic Model Assessment of Insulin Resistant				
10	HOMA-B (Homeostatic Model Assessment of β-cell function).				

তথ্যদানকারী স্বাক্ষর

জরীপকারীর স্বাক্ষর

Appendix III

Work Plan

Activities	2019												2020												2021												2022											
	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
selection of co-supervisor																																															
Collection of Literature																																																
Literature Review																																																
Proposal writing																																															
Synopsis presentation to Supervisor																																															
Acceptance of proposal																																															
Developing a research questionnaire																																															
Pilot Study and Analysis																																															
Data collection and Analysis																																															
Final Write-up & Thesis Submission																																															
Submitted Thesis given to Supervisor for review																																															
Thesis given to the reviewers																																															
Thesis submitted to the Institution																																															
Final Examination (Defense)																																															

Appendix IV

Clinical Record form

Title: Effect of Vitamin D3 Supplement on the Cognitive Status in Patient with Systemic Lupus Erythematosus with Neuropsychiatric Phenomena

Base Line: Follow-up:

General Information

Date form completed *(dd/mm/yyyy)*	
Name/ID of Patient	
Patient Address	
Contact Details	
Referred by	
Date of 1st appointment :	
Clinical Diagnosis:	

Study eligibility

Study Characteristics	Eligibility criteria	Eligibility criteria met?	Note
	1. Confirmed diagnosed cases of SLE having a neuropsychiatric manifestation of both sexes at least 18 years of age along with hypo-vitamin D3 status. 2. Participants must be literate and sufficiently fluent in the language in	Yes No Unclear	

	which cognitive tests will be administered.		
Type of Study	Randomised Controlled Trial	☐ ☐ ☐	
Participants	ID : Baseline : Follow-up :	☐ ☐ ☐	
Types of intervention	Vitamin D supplement	☐ ☐ ☐	
Types of comparison	Group A: Group B (A received vitamin D): (B without Vitamin D supplement)	☐ ☐ ☐	
Types of outcome measures		☐ ☐ ☐	

Characteristics of included studies

Methods

	Descriptions as stated in the report/paper	**Remarks**
Aim of study *(e.g. efficacy, equivalence, pragmatic)*	The study aims to evaluate the effect of Vitamin D3 supplementation on the cognitive status of patients with systemic lupus erythematosus with neuropsychiatric phenomena.	

Design	Randomized controlled parallel arm trail, comparing Vitamin D supplementation to control (1:1 allocation ratio).	
Unit of allocation	Individual	
Start date		
End date		
Duration of participation *(from recruitment to the last follow-up)*		
Ethical approval needed/ obtained for the study	☐ Yes ☐ No ☐ Unclear	
Notes:		

Participant Demographic

	Description	**Remarks**
	Verbal / written invitation of study participants with trial information Baseline evaluation by physical examination, laboratory investigations, NP tests (MMSE score), and Brain SPECT with Z scores; Analysis of vitamin D, anti-ds-DNA antibody, and anti-phospholipid antibody will be performed Intervention group: Participants of the intervention group will be assigned to receive vitamin (40,000 IU Cholecalciferol) per week. Subjects will be provided with 3 months of study medication and will be asked to take the tablet on a particular fixed day with a major meal. To optimize adherence, participants will be sent weekly reminders via text message, email, or via telephone.	
Population description *(from which study participants are drawn)*	Random selection of NPSLE, as per sampling frame (n= n1+ n2)	

Setting *(including location and social context)*	National Institute of Nuclear Medicine & Allied Sciences (NINMAS) and Microbiology and immunology department of Bangabandhu Sheikh Mujib Medical University, Dhaka.	
Inclusion criteria	1. Confirmed diagnosed cases of SLE having a neuropsychiatric manifestation of both sexes at least 18 years of age along with hypo-vitamin D3 status. 2. Participants must be literate and sufficiently fluent in the language in which cognitive tests will be administered.	
Exclusion criteria	1. Current use of vitamin D or calcium supplement. 2. Current or past diagnosis of cognitive impairment or dementia due to known brain disorders or systemic conditions 3. Use of certain drugs such as corticosteroids, hormone replacement, psychoactive drugs	
Method of recruitment of participants *(e.g. phone, mail, clinic patients)*	Referral from a Specialist physician	
Informed consent obtained	☐ Yes ☐ No ☐ Unclear	

Total no. randomized *(or total pop. at the start of the study for NRCTs)*	100	
Age		
Sex		
Weight	Height	
Education Level	☐ primary ☐ HSC ☐ Grad ☐ PG	
Severity of illness		
Co-morbidities		
BMI and Nutritional Status	1. Underweight <18.15 ☐ 2. Normal 18.5-24.9 ☐ 3. Overweight 25-29.9 ☐ 4. Obese >30 ☐	
Other relevant sociodemographic		
Notes:		

Investigational Profile

	Description as stated in report/paper	Note
Blood Routine		
Hb		

ESR		
TSH		
FT4		
Serum Ca		
Anti Ds DNA Antibody		
Anti Phospholipid antibody (Optional)		
IgM		
IgG		
Vitamin D Profile	1. Baseline ☐ 2. Follow-up ☐	
Vitamin D Status	1. Sufficient ☐ 2. Insufficient ☐ 3. Deficient ☐	
Notes:		

Cognitive Function Test

	Description as stated in report/paper	Remarks
	Mini-mental state Examination Test (ref: https://en.wikipedia.org/w/index.php?title=Mini%E2%80%93Mental_State_Examination&oldid=991258855_	

Cognitive Function Test *(Score: Total 30)*	1. >26/30 Normal 2. 25-30 Questionable Significant (Mild Deficit) 3. 20-25 Mild (Significant effect may require some supervision and support) 4. 10-20 Moderate 5. 0-10 Severe (Clear impairment)	☐
Outcome definition *(with diagnostic criteria if relevant)*		
The person measuring/ reporting		
Is the outcome/tool validated?	☐ Yes ☐ No ☐ Unclear	
Notes:		

Neuroimaging Profile

	Description as stated in report/paper	Remarks

Method	After administration of 1m Ci dose of Tc^{99m} ECD dynamic sequential SPECT images were taken from vertex through skull base for 30 minutes in 20 seconds 128IX 128 per frame. Images were analyzed in easy Z-score imaging system (e-ZIS) software.	
Technical Section	Test Done Completely: ☐ Yes ☐ No Use of Sedation: Yes ☐ No ☐	
Imaging Result	Normal ☐ Abnormal ☐	
Area of Perfusing Detected	1. Frontal Lobe ☐ 2. Parietal Lobe ☐ 3. Temporal Lobe ☐ 4. Praecuneus ☐ 5. Basal Ganglia ☐ 6. Cerebellum ☐	
Z Score Severity of Cognitive Impairment	Normal ☐ Mild ☐ Moderate ☐ Significant ☐ (0-1) (1-2) (2-3) (>3)	
Is the outcome/tool validated?	☐ ☐ ☐ Yes No Unclear	
Notes:		

Any Remarks:

Dr. Nadia Begum Date :

Picture: Place of Data collection and analysis

Picture: Place of Data collection and analysis

Appendix V

Estimation of Fasting Blood Glucose Level

Biochemical Analytes: Fasting Blood Glucose

Automated Analyzer: Roche Cobas c501

Manufacturer: Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim

Method: Enzymatic reference method with hexokinase

Reagent Composition: R1: MES buffer: 5.0 mmol/L, pH 6.0; Mg2+: 24 mmol/L; ATP: ≥ 4.5 mmol/L; NADP: ≥ 7.0 mmol/L; preservative

R2: HEPES buffer: 200 mmol/L, pH 8.0; Mg2+: 4 mmol/L; HK (yeast):≥ 300 µkat/L;G-6-PDH (E. coli): ≥ 300 µkat/L; preservative

Assay type, Accepted Specimen, and Procedure:

Assay Type: Two-point end

Accepted Specimen: Serum, plasma, urine, CSF

Procedure: Serum+ **R1 reagent 28 µL** and H2O 141 µL+ **R2 reagent** 10 µL and 20 µL H2O, 10min at 37^0C incubation, photometric result measured automatically at 700/340 nm wavelength.

Calibration and Quality Control *:*

Calibration: Linear, Two-point

Standardization: The method has been standardized against ID/MS.

Quality Control: Two-level (normal and abnormal) Bio-Rad assayed chemistry control tested for test run validation.

Picture: Roche Cobas 6000 System

Appendix VI

Estimation of Serum Calcium level

Biochemical Analytes: Serum Calcium

Automated Analyzer: Roche Cobas c501

Manufacturer: Roche Diagnostics GmbH, Sandhofer Strasse 116, D- 68305 Mannheim

Method: NM-BAPTA method

Reagent Composition:

R1: CAPSO:a 557 mmol/L; NM-BAPTA: 2 mmol/L; pH 10.0; non-reactive surfactant; preservative

R2: EDTA: 7.5 mmol/L; pH 7.3; non-reactive surfactant, preservative

a) 3-[cyclohexylamino]-2-hydroxy-1-propane sulfonic acid

Assay type, Accepted Specimen, and Procedure:

Assay Type: Two-point end

Accepted Specimen: Serum, plasma, and Urine

Procedure: 3 µl Serum + **R1 reagent 20 µl** and 160 H2O +**20 µL R2 reagent**, 10min incubation at 37^0C, photometric result measured automatically at 376/340 nm wavelength.

. **Calibration and Quality Control:**

Calibration: Linear, Two-point

Standardization: This method has been standardized against the SRM 956 c Level 2 reference material.

Quality Control: Two-level (normal and abnormal) Bio-Rad assayed chemistry control tested for test run validation.

Picture: Roche Cobas 501 System

Appendix VII

Estimation of Serum C-reactive Protein level

Biochemical Analytes: Serum C-Reactive Protein

Automated Analyzer: Roche Cobas c501

Manufacturer: Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim

Method: Particle-enhanced immunoturbidimetric assay

Reagent Composition: R1: TRIS buffer with bovine serum albumin and immunoglobulins (mouse); preservative

R2: Latex particles coated with anti-CRP (mouse) in glycine buffer; Preservative

Assay type, Accepted Specimen, and Procedure:

Assay Type: *2*-Point end

Acceptable Specimen: Serum. Plasma

Procedure: 2µl Serum + R1 reagent 150 µl and +R2 reagent 48 µl and 24 µl H2O, 10 min incubation at 37°C , photometric result measured automatically at 800/570 nm wavelength.

Calibration and Quality Control:

Calibration: Non-linear

Standardization: This method has been standardized against the certified reference the material in the human serum of the IRMM (Institute for Reference Materials and Measurements) ERM-DA474/IFCC.

Quality Control: Two-level (normal and abnormal) Bio-Rad assayed chemistry control tested for test run validation.

Picture: Roche Cobas 8800 System

Appendix VIII

Estimation of Serum fasting insulin level

Biochemical Analytes: Serum Fasting Insulin

Automated Analyzer: Abbott, Architect, i1000SR

Method : Chemiluminescence Microparticle Immunoassay(CMIA)

Reagent Composition:

***Microparticles*:** Antibody to human insulin (mouse, monoclonal) coated microparticles in MOPS buffer with protein (bovine) stabilizer

***Conjugate*:** Acridinium-labeled antibody to human insulin (mouse, monoclonal) conjugate in MES buffer with protein (bovine) stabilizer.

Assay type, Accepted Specimen, and Procedure:

Assay Type: One-Step Immunoassay:

Acceptable Specimen: Serum, Plasma

Procedure: 150 µL Serum+anti-insulin coated paramagnetic microparticles+ anti-insulin acridinium-labeled conjugate is incubated for 25 min. Then washing with wash buffer. 100 µL Pre-Trigger and 300µL Trigger Solutions are added to the reaction mixture; the resulting chemiluminescent reaction is measured as relative light units (RLUs) by a photomultiplier tube.

Calibration and Quality Control *:*

Calibration: Six-point calibration

Assay Standardization: WHO 1st International standard.

Quality Control: Tri-level

Appendix IX

Estimation of 25(OH) -Vitamin D level

Biochemical Analytes: 25(OH) -Vitamin D

Automated Analyzer: Abbott, Architect, i1000SR

Method: Chemiluminescence Microparticle Immunoassay(CMIA)

Reagent Composition:

***Microparticles*:** Anti-vitamin D IgG (rabbit monoclonal) coated microparticles in MES Buffer.

Conjugate: Acridinium-labeled vitamin D in MES Buffer and surfactant.

***Assay Diluent*:** Citrate buffer with EDTA, Methanol, 8-aniline-1- naphthalene sulfonic acid (ANSA), and surfactant.

Assay type, Accepted Specimen, and Procedure:

***Assay Type*:** One-Step Immunoassay

Acceptable specimen: Serum, plasma

Procedure : 60 µL serum+ assay diluent+paramagnetic anti-vitamin D coated microparticles+incubation+ acridinium-labeled vitamin D conjugate+25min incubation +wash with wash buffer+100 µL Pre-Trigger and 300µL Trigger Solutions are added to the reaction mixture. The resulting chemiluminescent the reaction is measured as relative light units (RLU) by a photomultiplier tube.

. **Calibration and Quality Control *Calibration:***

Calibration: Six-point calibration

Standardization*:* NIST, 2972

Quality Control: Tri-level

Picture: Abbott, Architect, i1000SR

Appendix X

Determination of HbA1c level

Biochemical Analytes: HbA1c

Automated Analyzer: Bio-Rad, Variant II Turbo Bio-Rad Laboratories, USA)

Method: High-Performance Liquid Chromatography(HPLC)

Reagent Composition:

Whole blood primer: Lyophilized human red blood cell hemolysate with gentamicin, tobramycin, and EDTA as preservatives

Elution Buffer A: sodium perchlorate buffer. Contains <0.05% sodium aside as a preservative

Elution Buffer B: sodium perchlorate buffer. Contains <0.05% sodium aside as a preservative

Analytical Cartridge: Cation exchange cartridge,4.6 mm ID x 27.5 mm. 5 prefilters (500 tests each) are included with the cartridge.

Assay type, Accepted Specimen, and Procedure:

Assay Type*:* Ion Exchange Chromatography

Acceptable Specimen*:* Whole blood

Procedure: 5 ul whole blood sample automatically diluted 1.5 ml on the instrument sampling station (VSS) and pushed into an analytical cartridge. To increase the ionic strength of the cartridge, the instrument chromatographic station dual pumps deliver a programmed buffer gradient where based on their ionic interactions with the cartridge material the hemoglobin were separated. The separated hemoglobin then passes through the flow cell of the filter photometer, where changes in the

absorbance at 415 nm are measured. An additional filter at 690 nm corrects for background absorbance.

Calibration and Quality Control *Calibration:*

Calibration: 3 points

Standardization: Glycohemoglobin Standardization Program (NGSP) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

Quality Control: Two-level Biorad Diabetes control.

Picture : Bio-Rad, Vairant II Turbo

Appendix XI

Determination of Plasma MDA and SOD level

Biochemical Analytes: MDA

Method: Spectrophotometric

Reagent: Reagent

Procedure:

According to the standard method (Ohkawa et al., 1979), plasma MDA was measured. This was level was determined by thiobarbituric acid (TBA) reactive substances (TBARS) in plasma, based on the reaction between MDA and TBA. When TBA was permitted aerobically to react with MDA formed a colored complex [MDA-(TBA)2 complex] which was measured by the spectrophotometer.

1ml plasma + saline 0.6ml+2ml reagent + boiling for 15mins at 1000c, centrifuged at 3000rmp for 10 mins, red absorbance at 550nm & calculation concentration.