IMPLICATION OF HOMOCYSTEINE, RESISTIN AND VITAMIN D IN DEVELOPMENT OF TYPE 2 DIABETES

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by

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Abstract

Introduction: Type 2 diabetes mellitus (T2DM) is a global health problem and its complications are a significant cause of morbidity and mortality worldwide. T2DM is the common type of diabetes and it affects mainly adults. Insulin resistance is considered as a main factor contributing to T2DM. Different substances are implicated in development of insulin resistance, among them vitamin D, resistin and homocysteine which can interrupt the insulin signalling pathway leading to insulin resistance. Elevated levels of homocysteine have been observed in patients with T2DM and metabolic syndrome, while resistin (a hormone) was suggested to play a role in the pathogenesis of T2DM. Increased levels of resistin have been linked with adiposity/inflammation and insulin resistance in T2DM patients. Vitamin D deficiency is another global problem and elevated levels of blood glucose and vitamin D deficiency have been linked to complications associated with T2DM. Recently studies conducted in ethnic groups in Asia and Europe suggest that vitamin D deficiency, resistin and homocysteine may also be implicated in the pathogenesis of T2DM. However, limited numbers of studies were from African subjects and none investigated vitamin D, resistin and homocysteine together in association with T2DM. T2DM is a common disease in Sudan and it causes considerable morbidity and mortality. Few studies have been conducted in Sudan to explain the increase in prevalence of diabetes but no studies looked at the role of homocysteine, resistin and vitamin D. Methyl tetrahydrofolate reductase (MTHFR) is an enzyme that contribute to the formation of methionine from homocysteine and it has taken all these attention because the present of the genetic mutation which led to hyperhomocysteinemia.

Objective: The aim of this study was to investigate possible associations between homocysteine, resistin and vitamin D with T2DM in a representative study population from Sudan. In addition, associations between the Methyl tetrahydrofolate reductase (MTHFR) gene polymorphisms (C677T and A1298C) with homocysteine levels and with T2DM, as well as, the prevalence of VDR gene polymorphism (T56058C) in patients with T2DM and their healthy controls were investigated.

Material and Methods: This is a case-control hospital-based study conducted at the Diabetes clinic with samples obtained from the Military hospital, Omdurman, Sudan. Two hundred patients with T2DM were consecutively enrolled during 2013-2014. The included patients were known to have T2DM for a minimum of one year and were under-regular follow-up at the diabetes clinic. Patients with chronic illness (especially heart and kidney diseases) and

other types of diabetes (including type 1 diabetes) were excluded. The control group (n=195) consisted of healthy individuals with no family history of diabetes. A questionnaire was used to acquire demographic data (age, gender), anthropometric measurements (weight/height and waist circumference), basic biochemical tests (HbA1c, fasting blood glucose (BG), lipid profile) and blood pressure (systolic/diastolic). Plasma levels of resistin and homocysteine were measured using ELISA-based methods, while total Vitamin D (D2/D3) levels were measured using an UHPLC method. The gene variants MTHFR C677T, MTHFR A1298C and VDR T56058C were typed using real time PCR methods. Associations between plasma levels of resistin, homocysteine and total vitamin D with T2DM, and their associations with different variables (age, gender, BMI, HbA1c, fasting blood glucose, lipid profile) were statistically analysed. This was in addition to the associations between the MTHFR gene polymorphisms and homocysteine levels, and the associations between VDR gene polymorphism and T2DM.

Results: Resistin levels were significantly higher in the healthy controls compared to patients with T2DM ($p<0.0001$). Levels were significantly higher in healthy males compared to healthy females ($p=0.007$) as well as in males in the study population in general compared to females in the study population ($p=0.002$). However, no significant difference in resistin levels was found between male and female in individuals with T2DM ($p=0.09$). My results showed also that healthy controls had significantly higher resistin levels compared to patients with T2DM in groups with BMI>25 and BMI<25 ($p=0.03$, for both comparisons). In addition, the results also showed significantly higher levels of resistin in male healthy controls compared with male patients with T2DM in groups with BMI>25 and BMI<25 ($p<0.001$ and $p=0.007$, respectively), and there was no significant difference in resistin levels between female healthy controls and female patients with T2DM in groups with BMI>25 and BMI<25 (($p=0.45$ and $p=0.16$, respectively). Resistin levels were significantly higher in patients with T2DM not using vitamin D supplementation compared with those using it ($p=0.0039$). There is a positive correlation between resistin levels and 2hBG and HbA1c% in the study population (all participants) (r=0.121; $p=0.035$ and r= 0.237$p<0.0001$, respectively) and also with FBG, BMI (log), homocysteine levels in individuals with T2DM (r=0.188; $p=0.008$; r=0.140; $p=0.048$ and r=0.335; $p<0.0001$, respectively), while there is a negative correlation between resistin levels and vitamin D in patients with T2DM (r= -0.261; $p<0.0001$). Homocysteine levels were significantly higher in patients with T2DM compared to healthy controls ($p<0.0001$). In addition, its levels were significantly higher in females compared with males ($p<0.0001$) and there was a positive correlation between plasma homocysteine

levels and HbA1c% (r=0.298; p<0.0001) and 2hBG (r=0.361; p<0.001) in all participants. The percentage of patients carrying the MTHFR C677T genotypes CC, TT and CT is 85.5%, 0.5% and 14.0% respectively, while among healthy controls it is 88.7%, 0.0% and 11.3%. There is no significant difference in the genotype (CC, CT and TT) and allele (C and T) frequencies between patients and healthy controls (χ^2= 0.909, p=0.340; χ^2= 0.660, p=417; χ^2= N/A, p=N/A for CC, CT and TT genotypes, respectively; and χ^2= 1.110, p=0.292 for the C and T alleles, the % of patients carrying the MTHFR A1298C genotypes AA, CC and AC is 65.0%, 6.0% and 29.0% respectively, while among healthy controls it is 65.1%, 5.1% and 29.8%. There is no statistically significant difference in the genotype (AA, CC and AC) and allele (A and C) frequencies between patients and healthy controls (χ^2=0.001 p=0.098; χ^2= 0.26, p=0.87; χ^2= 0.143, p=0.71 for the AA, CC and AC genotypes, respectively; and χ^2= 1.031, p=0.86 for the A and T alleles). Our results showed that the levels of homocysteine are significance higher in patients with T2DM carrying the three genotypes, 677CT, 1298AC and 1298CC, and carrying the heterozygous haplotype C677T/A1298C compared to the healthy controls carrying the same genotypes/haplotype.

Vitamin D levels was significantly higher in patients with T2DM compared to healthy controls (p<0.0001), and its levels have negative correlation with FBG in patients with T2DM (r= -0.177; p=0.013). The % of patients carrying the VDR T56058C genotypes CC, TC and TT is 36.5%, 46% and 17.5% respectively, while among healthy controls it is 28.7%, 52.8% and 18.5%. There are no significant differences in the VDR T56058C genotype (CC, TC and TT) and allele (C and T) frequencies between the patients and their healthy controls (χ^2= 2.719, p=0.099; χ^2= 1.838, p=0.175; χ^2= 0.062, p=0.803 for CC, TC and TT genotypes; and χ^2= 2.084, p=0.149 for the C and T allele, respectively).

Conclusion: Previous studies have suggested a role for Vitamin D deficiency and elevated levels of plasma resistin and homocysteine levels in the pathogenesis of insulin resistance and T2DM. Our results confirmed previously reported high levels of homocysteine in patients with T2DM. A positive correlation was observed between homocysteine and FBG, HbA1c and resistin, as well as a positive correlation between resistin and FBG and HbA1c, and a negative correlation between vitamin D and resistin, homocysteine and FBG levels in patients with T2DM. The negative association between vitamin D and resistin/blood glucose levels suggest that vitamin D might have an impact on resistin levels in patients with T2DM and improved insulin action/blood glucose levels and T2DM. Taken together, our results suggest a possible role for vitamin D, resistin and homocysteine in the development of insulin resistance and T2DM.

Dedication

I dedicate this work: In memory of father and mother

To my family

In memory of Prof. Elfadil, Rabia Abdel Karim

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25-(OH) D2	25-vitamin D2
1, 25 $(OH)_2 D_3$	1, 25 dihydroxy vitamin D3
25-(OH) D	25-hydroxyvitamin D
2hBG	2 hour blood glucose
ADA	American Diabetes Association
ADP	Adenosine diphosphate
AGC	cAMP-dependent, cGMP-dependent protein kinase C
AKT	Protein kinase B
ATGL	Adipose triglycerids lipase
ATP	Adenosine triphosphate
B12	Cobalamin
B2	Riboflavin
B6	Pyridoxal phosphate
BHM	Betaine –homocysteine methyltransferase enzyme
BMG	Omega microplate reader
BMI	Body mass index
BSA	Bovine Serum Albumin
C2	Carbon number 2
C25	Carbon number 25
CAD	Coronary artery disease
CAL1-CAL	Calibrators, S-adenosyl-L.homocysteine
cAMP	Cyclic Adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CHOL	Cholesterol
CVD	Cardiovascular disease
CYP24A1	24-hydroxylase
CYP27B1	1-hydroxylase enzyme
CYP27A1	25-hydroxylase
CβS	Cystathionine β- synthase

D3	Vitamin D3
DAG	Diglycerides
DBP	Vitamin D binding protein
DBP	Diastolic blood pressure
DCCT	Diabetes Control and Complications Trial
DCCT	Diabetes Control and Complications Trial
DDT	Dithiothreitol
df	Degrees of freedom
DM	Diabetes mellitus
DNA	Deoxy ribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DR	Diabetic retinopathy
EDTA	Ethylenediamine tetraacetic acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESRD	End- stange renal disease
FAD	Flavin adenine dinucleotide
FBG	Fasting blood glucose
FIZZ 1	Found in inflammatory zone 1
FOX	Forkhead
FOX	Forkhead
GLM	General Linear Model
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GSK3	Glycogen synthase kinase 3
GTT	Glucose tolerance test
HbA1c	Glycated haemoglobin
Hcy	homocysteine
Hcy-SS-Hcy	homocysteine-SS-homocysteine
HDL	High density lipoprotein
HMM	High molecular mass
HRP	Horse radishperoxidase

HSL	Hormone sensitive lipase
HTC-IR	Homocysteine thiolactone in Rat
IHD	Ischemic heart disease
IRS	Insulin receptor substrate
IRS -1	Insulin receptor substrate -1
IRS -2	Insulin receptor substrate -2
IDDM	Insulin dependent diabetes mellitus
IF	Interinic factor
IGF1	Insulin-like growth factor 1
IKK	I kappa B kinase
IL-2	Interleukin -2
IQR	Interqartile range
IS	Internal standard
JNK	Jun N-terminal Kinase
LDL	Low density lipoprotein
LLOD	Lower limit of detection
LLOQ	Lower limit of quantitation
LMM	Low molecular mass
MAG	Monoglycerids
MAP K	Mitogen-actived protein kinase
Met	Methionine
MGB	Minor groove-binding
mTORC2	Mammalian target of rapamycin complex 2
mRNA	Massener ribonucleic acid
MS	Methionine synthase
MTHFR	Methyl tetrahydrofolate reductase
MW	Mann whitney test
N/A	Not applicable
NC	Negative control
NIDDM	Non insulin dependent diabetes mellitus
NTC	Negative template control
OGTT	Oral glucose tolerance test
PBS	Phosphate Buffers Saline

PCR	Polymerase chain reaction
PDK1	Phosphoinositide –dependent kinase-1
PEPCK	Phosphoenolpyruvate carboxykinase
PH	Pleckstrin- homology
PI3K	phosphatidylinositol -3'-kinase
PIP2	phosphatidylinositol-4, 5- bisphosphate
PIP3	phosphatidylinositol-3, 4, 5-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLP	Pyridoxal phosphate
Prot-SS-Hcy	Protein-SS-homocysteine
PTB	Phosphotyrosine binding domains
PTEN	Phosphatase and tensin homologue
PTH	Parathyroid hormone
PTP1B	Protein tyrosine phosphatase -1B
R1-ss-Hcy	Any other thiol-residue -SS-homocysteine
RBG	Random blood glucose
RBP	Rretinol binding protein
RD	R&D systems (name of the company)
RD1-19	Assay diluent
RD5K	Calibrator diluent
Real-time PCR	Real-time Polymerase chain reaction
REC	Recovery rate
RELMs	Resistin- like molecules
RELM-α1	Resistin- like molecule-α 1
RELM-β1	Resistin- like molecule-β 1
RNA	Ribonucleic acid
ROX	Rhodamine X
RTK	Receptor tyrose kinase
S6K1	Ribosomal S6 kinase 1
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SBP	Systolic blood pressure

SD	Standard Deviation
SE	Standard error
SH2	Src-homology 2
SHIP2	SH2-domaincontaining inositol 5-phosphate-2
SHMT	Serine hydroxymethyltransferase
SNP	Single nucleotide polymorphism
SNP	Single nucleotide polymorphism
SOCS	Suppressors of cytokine signalling
SP1/SP3	Transcriptional factors
TFs	Transcription factors
T1DM	Type 1 Diabetes
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TC	Transcobalamin
TE Buffer	Tris–HCl and EDTA buffer
TFs	Transcription factors
Th 1	T helper
UHPLC	Ultra high performance liguid chromatography
UV	Ultraviolet light
VDR	Vitamin D receptor
W	Waist circumference
WHO	Word health organization

Chapter 1

1. Introduction

1.1. Background

Diabetes is a global health problem and the World Health Organisation (WHO) estimates the number of diabetics to be around 171 million and it is expected that the number will increase to reach 360 million by 2030 (Shaw *et al.,* 2010). Type 2 diabetes melitus (T2DM) is a polygenic disease associated with defects either in insulin secretion or its action on the target tissues (Abdelgadir *et al.,* 2002). In general, 80% of individuals with T2DM die from thrombosis and 75% from cardiovascular diseases (Carr *et al.,* 2001). In Sudan, a relatively poor country with a multi-ethnic population, T2DM is a major health problem. The prevalence of diabetes (in general) is estimated to be around 3.4%, 75% of the diabetics having T2DM, and the disease is a leading cause of morbidity and mortality.Adipose tissue has the ability to produce and secrete different types of the adipokines (Abdelgadir *et al.,* 2013) among them resistin, which was shown to be linked with insulin resistance and adiposity (Rajkovic *et al.,* 2014). Furthermore, several studies reported a positive relation between resistin concentrations and inflammation in diabetes and its complications (El-Tahir *et al.,* 2016; Burnett *et al.,* 2006 and Osawa *et al.,* 2005). Recently, it was reported that resistin is a main factor in the pathogenesis of insulin resistance and T2DM via obesity (Kusminski *et al.,* 2005). A study from Sudan found that about 40% of patients with T2DM were prone to be obese, and obese subjects were found to exhibit high levels of adipokines (Elmahadi *et al.,* 1991).

Homocysteine is an amino acid that contains a sulphur hydral group (-SH). It is produced during the metabolism of methionine. Methyl tetrahydrofolate reductase (MTHFR) is an enzyme that catalyses the conversion of 5, 10-methylene-tetrahydrofolate to 5-methyl-tetrahydrofolate (Frosst *et al.,* 1995). It is an important reaction in the conversion of homocysteine to methionine (Toffoli *et al.,* 2003). The MTHFR gene is found on chromosome 1 at 1p36.6 (Qutinen *et al.,* 1998). A genetic polymorphism in the MTHFR gene can affect the enzymatic activity leading to an increase in the level of homocysteine in the blood (Kluijtmans *et al.,* 1996). Increased levels of homocysteine (Hcy) in the blood is a condition known as hyperhomocysteinemia, which is considered as an independent risk

factor for atherosclerosis in both diabetic and non-diabetic patients (Glueck *et al.*, 1995 and Perry *et al.*, 1995). Several studies investigated the possible association between levels of homocysteine in plasma and T2DM but results were inconsistent (Masuda *et al.*, 2008; Huang *et al.*, 2006 and Shaikh *et al.*, 2012). Wouters *et al* found that there is no significant association between homocysteine levels and glycaemic control in females. On the other hand, there is a significant association between homocysteine levels and glycaemic control in males, suggesting a role for difference in sex hormones, as estrogen inhibits the progression of atherosclerosis (Wouters *et al.*, 1995).

Vitamin D can be produced by the skin as a result of exposure to sunlight or obtained from food (Basit, 2013). Vitamin D receptor (VDR) has been shown to be expressed by several tissues involved in glucose metabolism (e.g. muscles and pancreatic beta cells) (Bischoff *et al.*, 2001 and Johnson *et al.*, 1994). To produce the hormonally active form 1,25 dihydroxy vitamin D3 (1, 25 $(OH)_2 D_3$), vitamin D3 (D3) undergoes two hydroxylation steps, one in the liver (on C2) and the second in the kidney (on C25). The biological actions of vitamin D in the body are mediated by VDR (Tuoresmaki *et al.*, 2014). Genetic polymorphisms in the VDR gene may affect vitamin D actions, and might be associated with diseases like cancer, metabolic syndrome and T2DM (Rezende *et al.*, 2007). The main form of vitamin D that reflects its status is 25-hydroxyvitamin D (25-(OH) D). Low levels of 25-(OH) D have been reported in obese subjects and in patients with diabetes (Scragg, 2008).

Studies suggested that a deficiency of vitamin D can contribute to the impairment of secretion of insulin leading to glucose intolerance in humans and animal models (Mathieu *et al.*, 2005). Vitamin D deficiency can impair insulin secretion by direct effect on the beta cells (β-cells) of the pancreas. The effects of the active form of vitamin D (1, 25 (OH) 2 D3) on the tissues occurs after the concentration exceeds the optimal level (10^{-10} mol/l) needed for maintenance of calcium and phosphate levels (Mathieu *et al.*, 2005).

In a healthy study population, Vilarrasa *et al* showed no significant association between resistin levels and plasma vitamin D concentration (Vilarrasa *et al.*, 2010) while El-Tahir *et al* observed a negative correlation between resistin levels and vitamin D levels in patients with T2DM (El-Tahir *et al.*, 2016). High levels of homocysteine in the blood promoted insulin resistance via induce production of resistin hormone and finally lead to T2DM. (Li *et al.*, 2008).

T2DM is a common disease in Sudan and it causes several complications. To my knowledge no studies have explored the interaction between resistin, vitamin D and homocysteine in Sudanese individuals with diabetes. In the present study, we investigated possible

associations between resistin, homocysteine, and vitamin D levels with T2DM; this is in addition to their associations with different variables (BMI, blood glucose levels, HbA1c% and lipid profile). We also investigated possible associations between vitamin D receptor (VDR) and MTHFR gene polymorphisms and T2DM; and possible association between MTHFR gene mutations and hyperhomocysteinemia.

1.2. Diabetes Mellitus

Diabetes is not a single disease entity but a group of metabolic illnesses that exhibit high glucose levels in the blood (hyperglycaemia) due to defects in secretion and/or action of insulin (Maori *et al.,* 2012). Continued elevation of blood glucose is the characteristic sign of diabetes and hyperglycaemia can cause long-term damage and dysfunction of different organs including the eyes, bloods vessels, nerves kidney and heart. Diabetes can develop through different processes, ranging from autoimmune destruction of the beta cells (β-cells) of pancreas (that lead to absolute deficiency of insulin) to abnormalities which can lead to resistance of insulin action (Gillett, 2009).

1.2.1. Pathophysiology

The basic concept of the pathophysiology of diabetes is based on our understanding of metabolism of carbohydrate, lipid and protein and action of insulin. After food (containing carbohydrate, sugars and starch) ingestion and digestion, monosaccharides are absorbed and enter the bloodstream (Bjorck *et al*., 1994). In the liver, they are converted to glucose. Blood glucose stimulates β-cells of the pancreas to secrete insulin which in-turn facilitates uptake of glucose by muscle and adipose tissues (Shulman, 2000). In general, when blood glucose levels increase, this leads to stimulation of insulin secretion from the β-cells of the pancreas and subsequent increase in cellular utilization of glucose which leads to a decrease in blood glucose levels and lower insulin secretion.

Insulin resistance and T2DM can be associated with significant alteration in the release of insulin from the pancreas. In case of hyperglycaemia there is decrease in insulin production and subsequently reduced entry of glucose to tissues. Moreover, hyperglycemia occurs also when there is no proper response of target tissue to insulin. On the other hand, hypoglycemia happens when there is overproduction and secretion of insulin and entry of glucose to tissues resulting in lowered blood glucose (Brunzell *et al.,* 1976).

Counter regulatory hormones, especially adrenaline and glucagon, work against insulin action and prevent hypoglycemia by increasing blood glucose levels via stimulating production of

glucose from non-carbohydrate sources (gluconeogenesis) and liver glycogen breakdown (Meley *et al.*, 2006).

1.2.2. Complications

Complications of diabetes could affect different parts of the body. Several dangerous health problems increase in patients with diabetes. Cardiovascular diseases are the major complication of diabetes (Shah *et al.,* 2015). In addition, several parts and organs of the body are affected by diabetes including the eye (retinopathy), kidney (nephropathy) and nerves (neuropathy), and feet. Foot complication is due to vascular as well as neurological problems (Aalto, 1997). Diabetic complications can finally lead to complete disability, reduced quality of life, and death.

1.2.3. Diagnosis

Diabetes is easy to diagnose especially if the patient has classic symptoms of high blood glucose level and has a random blood glucose (RBG) levels of 200 mg/l (11.1mmol/l) or higher. Oral glucose tolerance test (OGTT) can be used to confirm the diagnosis of diabetes. To do the OGTT, patients come to the clinic after over-night fasting for at least 8 hours. In diabetic patients, fasting blood glucose is initially higher than 7.0 mmol/l (126mg/dl) and raises to concentrations greater than 11.1 mmol/l (200mg/dl) after 2 hours following glucose load (Alberti *et al.,* 1998). In general, fasting blood glucose (FBG) test is considered suitable for to diabetes because of its low cost and convenience. Nevertheless, it is not accurate as it may miss some cases of diabetes or prediabetes. OGTT test is highly sensitive compared to the FBG test in diagnosing prediabetes, but it is less convenient to administer. Random blood glucose (RBG) is used for a person who has already been diagnosed with diabetes and it is not suitable for prediabetes subjects. A second test is performed on a different day to confirm that this person has diabetes (Alberti et al., 1998).

According to the WHO, the diagnostic criteria for diabetes include: fasting plasma glucose ≥ 7.0mmol/l (126mg/dl) or 2-h plasma glucose ≥ 11.1mmol/l (200mg/dl) (Report of a WHO Consultation, 1999). Glucose Tolerance Test (GTT) is conducted to confirm the diagnosis of diabetes. Fasting blood glucose is initially higher than 7.0 mmol/l (126mg/dl) in diabetic patients and rises to concentrations greater than 11.0 mmol/l (200mg/dl) after 2 hours glucose load (Alberti *et al.*, 1998; Report of a WHO Consultation, 1999).

1.2.4. Types of diabetes mellitus

WHO published the first classification of diabetes which was widely accepted (WHO, 1980). Diabetic patients are classified into two major groups according to their clinical symptoms: insulin dependent diabetes mellitus (IDDM) (type1 diabetes mellitus) and non-insulin dependent diabetes mellitus (NIDDM) (type 2 diabetes mellitus) (T2DM) (WHO, 1985). There are other types of diabetes including gestational diabetes. This classification, “insulin-dependent diabetes mellitus” and “non-insulin-dependent diabetes mellitus” was based on the treatment rather than pathogenesis. Stogdale recommended using the term "Type 1” because it describes a pancreatic islet β-cell destruction and Type 2 which is a result of a defect in insulin secretion/action (Stogdale, 1986). This classification was agreed upon after several attempts from the National Diabetes Data Group (WHO, 1980). Signs and symptoms used in the diagnosis of diabetes include polyuria, polydipsia and polyphagia. The emergence of these symptoms is usually accompanied by the appearance of other signs such as weakness, fatigue and weight loss.

1.2.4.1. Type 1 Diabetes (T1DM)

T1DM constitutes around 5-10% of all diabetic cases. Its incidence is on the increase worldwide. T1DM is associated with destruction of β-cells of the pancreas requiring the need for insulin injection to prevent patients from developing ketoacidosis, coma and death (WHO, 1999). Vascular complications are considered as the main factors contributing to morbidity and mortality associated with T1DM (Daneman, 2006).

1.2.4.2. Type 2 Diabetes (T2DM)

T2DM is considered as the most common type of diabetes. In T2DM, the β-cells of the pancreas are unable to produce enough insulin or there is resistance to insulin action on the target tissue leading to diabetes and its complications. The more dangerous complications in T2DM include heart attack and stroke. All serious complications occur when diabetes is left undiagnosed or uncontrolled. Serious complications include blindness, foot and leg amputation due to damage to the nerves and blood vessels, and renal failure requiring dialysis or transplantation (Pasinetti, 2011). DePaula showed that there is long-term damage in the body, especially to the heart and circulatory system which occur mainly in the pre-diabetic period (DePaula, 2008).

1.2.5. Type 2 Diabetes in Sudan

Diabetes mellitus is a major health problem in Sudan (Awadalla, et al., 2018). A recent study from an urban area in the north part of Sudan showed a 19.1% prevalence for T2DM (Elmadhoun et al., 2016) with a significant increase in the prevalence of T2DM and prediabetes over the last years (Eltom et al., 2018). Among Sudanese population with diabetes, 24.5% of them are obese and 39.9% are overweight (Ali et al., 2017). Obesity and central obesity were more prevalent in Sudanese females (Ahmed et al. 2017). It has been suggested that lifestyle factors including intake high amount of sugar and low physical activity led to obesity and diabetes, and reduced physical activity is common among women compared with men (El-Sayed et al., 2018 & Khalil et al., 2017). The prevalence rate of uncontrolled T2DM was higher among Sudanese individuals with T2DM (Noor, et al., 2017). Diabetic complications are common among Sudanese patients and were observed in those with poor glycaemic control. Poor glycaemic control (HbA1c>7%), high cholesterol (76.2%), triglyceride (27.5%), LDL (48.8%) and low HDL (33.8%) and long-term complications (like peripheral neuropathy, visual impairment, diabetic foot, and myocardial infarction) were seen in Sudanese diabetics (Almobarak, et al., 2018). In Sudanese population, longer duration of diabetes and poor control of blood glucose have been contributed in increase prevalence of complications in T2DM (Awadalla et al., 2017). Omer et al. 2018 showed that uncontrolled T2DM is more prevalent among unmarried individuals and those adding sugar to the drinks (Omar et al., 2018). Two thirds of the North Sudan population with diabetes had low levels of HDL, while that over one quarter have elevated cholesterol and triglyceride. Coronary heart disease (CHD) is a common complication among Sudanese patients, and it is characterised by an increased level of triglycerides in the blood, especially non-fasting triglyceridemia and decreased the levels of HDL in the blood (Awadalla et al., 2018). About 34% of smokers are individuals with T2DM and an association between smoking and ischemic heart disease (IHD), high triglyceride and HbA1c was observed. It was suggested to the health authorities in Sudan should call on Sudanese to quit smoking (Awadalla et al., 2018). Foot ulcer is one of the diabetic complications with a prevalence of 18.1% in a representative group from the Sudanese community and, its risk increased with duration of diabetes >10 years (Almobarak, et al., 2017). Retinopathy is one of the major complications in Sudanese patients with T2DM with high prevalence among patients attending Makkah Eye hospital in Khartoum (Elwali, et al., 2017). To avoid these complications urgent strategies are needed to monitor and treat hypertension and optimize diabetes control in individuals with diabetes (Elwali, et al., 2017).

Patients attending private clinics had a higher income and cost of diabetes control than those attending public clinics. However, both groups had similar proportion of poor glycaemic control, which reflects the insufficient care given to diabetic patients, mainly due to deficient resources and inefficient utilization of what is scarcely available (Elrayah-Eliadarous, et al., 2010). To reduce the financial burden and adverse social effects of diabetes on diabetics and their families in Sudan, an awareness program should be adopted to protect the population from diabetes (Elrayah-Eliadarous, et al., 2017).

1.3. Insulin and regulation of blood glucose

1.3.1. Glucose

Blood glucose is the sugar that is carried by the bloodstream to all tissues in the body to supply energy. In healthy humans, the levels of blood glucose must be kept within a tight range between 4 and 7 mmol/l. When its level exceeds the normal levels, this is a pathological condition known as hyperglycaemia. On the other hand, when the levels become under the normal range, it is also considered a pathological condition known as hypoglycaemia. Several factors are involved in controlling the blood levels within normal range. To perform its function, insulin binds to specific cellular receptors that facilitate glucose entry into the cells. Induction of secretion of insulin from the β-cells of the pancreas leads to a decrease in the level of glucose in the blood and by facilitating the entry of glucose into cells. Lower glucose levels then result in a decrease in the secretion of insulin. If insulin production and secretion are altered by disease, blood glucose dynamics will also change. If insulin production is decreased, glucose entry into cells will be inhibited, resulting in hyperglycemia. The same effect will happen if the secreted insulin is not taken up and used properly by the target tissues (Brunzell *et al.*, 1976). If the levels of insulin exceed the normal levels due to either overproduction of insulin from the pancreas or overdose of the treatment this leads to hypoglycemia (American Diabetes Association, 2005). Counterregulatory hormones, principally adrenaline and glucagon, prevent the falling of blood glucose levels to extreme levels by working against the effect of insulin action during hypoglycaemia and induce gluconeogenesis and glycogen breakdown.

Following a meal, the amount of glucose obtained from carbohydrates after digestion and absorption often exceeds the cellular need for glucose. Excess glucose is stored in the liver and muscle in the form of glycogen (Fery et *al.*, 2001) as well as in the form of fat in adipose tissues (Bulow *et al.*, 1999; Fig 1.1). Glycogen is a reservoir for glucose and energy. When

needed, the stored glucagon in the liver is converted into glucose via glycogenolysis leading to elevation of blood glucose levels and providing the needed organic energy sources (Liu *et al.*, 2011). In addition, the liver has the ability to produce glucose from non-carbohydrate sources (lactate, glycerol and amino acids) via the gluconeogenesis pathway (Liu *et al.*, 2011). Therefore, glycogenolysis and gluconeogenesis both serve to increase blood glucose levels. Thus, the pancreas is a main organ to control glycaemia because it produces hormones (insulin and glucagon) that control glucose metabolism in the liver (Adkins *et al.*, 2003). Patients with diabetes usually have an imbalance in secretion of these hormones (Fig1.1).

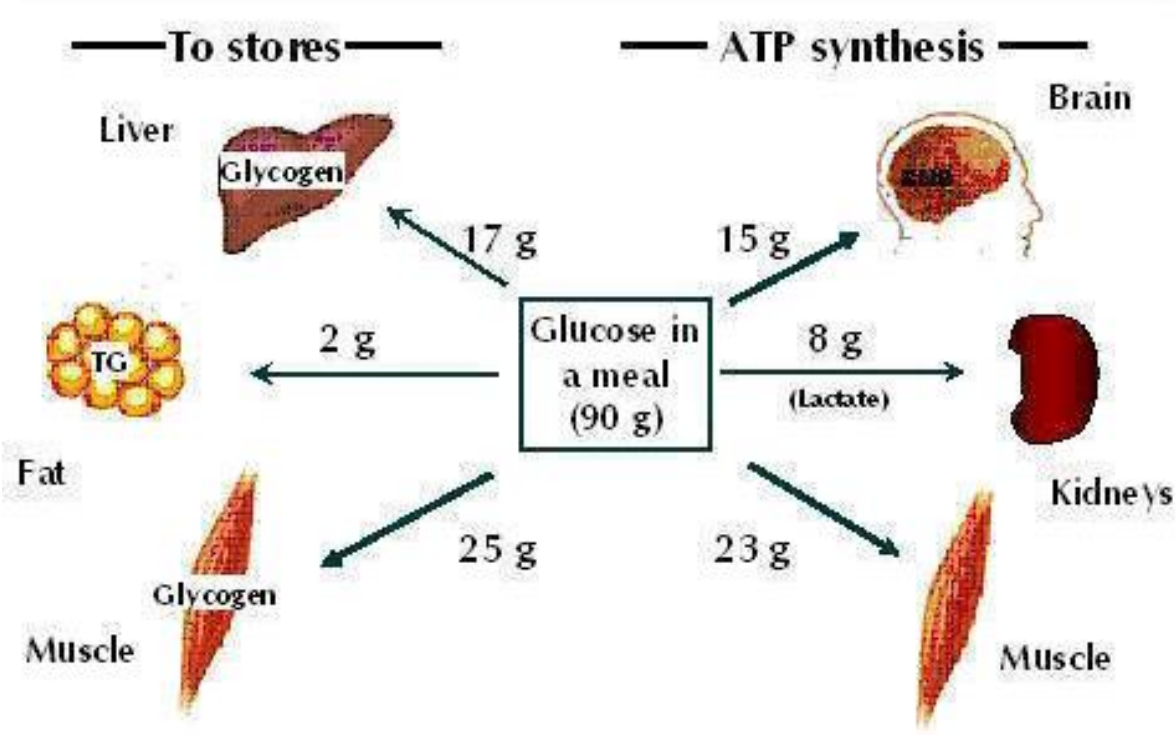


Fig 1.1 distribution of glucose after a meal:
in the anabolic pathways, glucose can be stored either in the form of glycogen (in liver and muscle) or in the form of TAG (in the adipose tissue). While in the catabolic pathways, glucose is degraded, either to lactate in kidney and muscle (by anaerobic pathway) or to CO_2 and H_2O in brain (by the aerobic pathway), to produce energy as a form of ATP. The diagram is taken from this website (www.medbio.info).

1.3.2. Effect of insulin on metabolic pathways

Insulin was discovered in 1923 by Banting and Macleod and it was shown to restore blood glucose in patients with T1DM (Raju, 1998). Human insulin is an anabolic hormone composed of two polypeptide chains. The A-chain contains 21 amino acids and B-chain

contains 30 amino acids, and the two chains are linked together by a disulphide bridge (Fig1.2). It is secreted from the β-cells of the Islets of Langerhans in the pancreas. It is responsible for the regulation of carbohydrate, lipid and protein metabolisms. The main function of insulin is the stimulation or inhibition of several genes that produce proteins that affect various metabolic pathways. Insulin regulates the uptake of glucose from the blood into muscle, and adipose tissue as well as regulates the metabolism of glucose in the liver. Therefore, it was suggested that a deficiency of insulin or reduced sensitivity of its receptor play essential roles in all types of diabetes (American Diabetes Association, 2014).

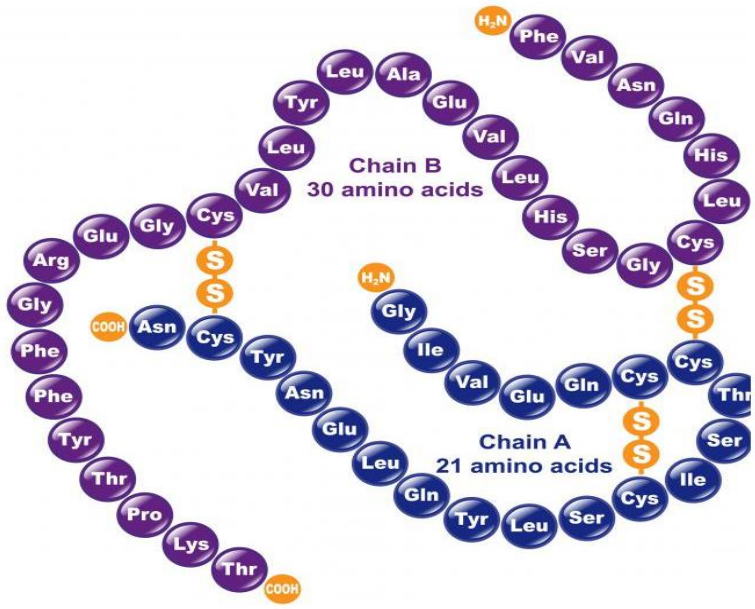


Fig 1.2 The structure of the active form of insulin:
its composed of two chains (A and B) Linked together by Disulphide Bridges between cysteine amino acids. Figure was taken from DeWitt and Hirsch, (2003)

1.3.3. Insulin secretion

In the normal pancreas there are around one million Islets of Langerhans, each of them have endocrine cells, 60-80% of which are β-cells that are responsible for insulin secretion (Marchetti *et al.*, 2008). The pathophysiology of diabetes mellitus includes defect in insulin secretion and/or action. Secretion of insulin starts when blood glucose levels increase and enters the β-cells in the pancreas by way of a glucose transporter 2 (GLUT2). In cells, glucose is phosphorylated by glucokinase and is broken down to produce adenosine triphosphate (ATP). One of the main functions of the ATP is to close potassium channels and depolarize the cell. Depolarisation leads to an opening of the calcium channels and insulin

secretion (Mahler *et al.*, 1999; Fig 1.3). Although glucose is considered the main factor responsible for insulin secretion, other factors, including other hormones, neurotransmitters, drugs and nutrients, also contribute to insulin secretion. Insulin is needed by adipocytes and muscle cells to allow glucose entry, as well as glucose oxidation (Shulman, 2000).

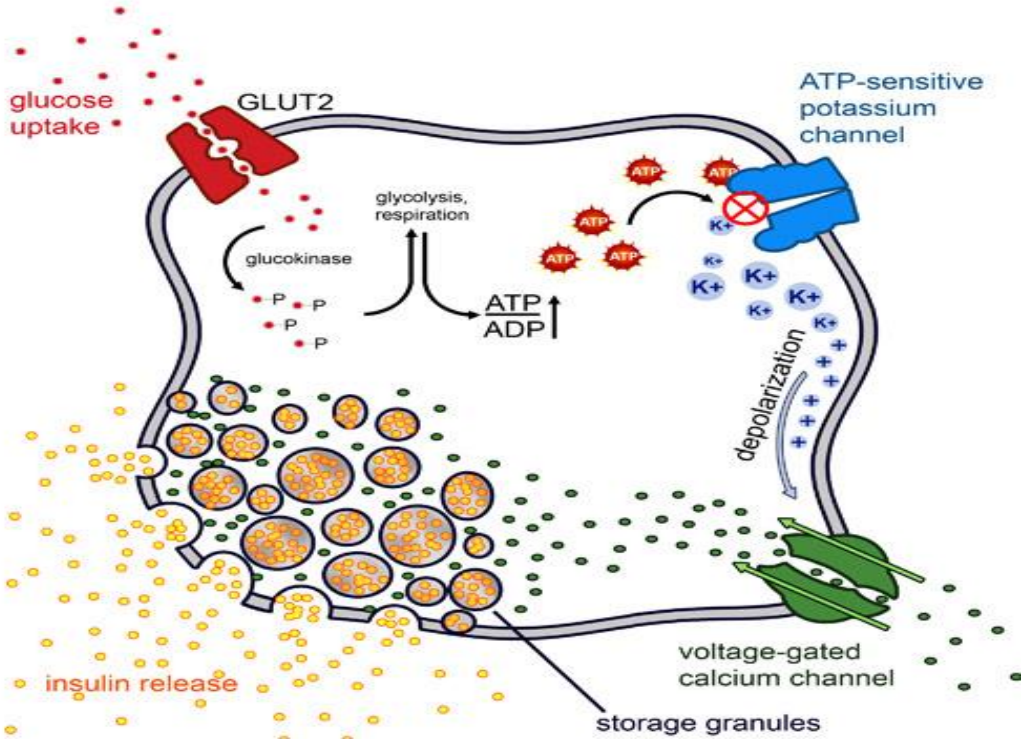


Fig 1.3 Glucose uptake and insulin secretion:

A rise in blood glucose levels triggers secretion of insulin from b Islet cells. The process starts by the uptake of glucose by the GLUT2 transporter. Following entry, glucose is phosphorylated and broken down to produce ATP. As a result, ATP depolarises the cell leading to calcium channel opening and insulin secretion. The figure was reproduced from Henquin, (2009).

1.3.4. Insulin action

To perform its function, insulin binds to its receptor and stimulates intracellular signalling cascades to downstream effectors of insulin's metabolic and mitogenic effects (Fig 1.4). Insulin regulates glucose homeostasis at many sites. Insulin activates the formation of glucose transporter 4 (GLUT 4) in muscle and adipose tissues, glycogen synthase in the liver and muscle tissues and inhibits glycogen phosphorylase in the liver and muscle tissues and also inhibits gluconeogenesis enzymes in liver (Pessin *et al.*, 2000; Fig 1.4). Insulin stimulates the conversion of glucose to fat by producing triacylglycerol (TAG) (lipogenesis) in liver and adipose tissues, and at the same time inhibits release of fatty acids from TAG (lipolysis) in muscle and adipose tissues. Accordingly, several metabolic pathways are

affected in patients with T2DM due to the impairment of insulin secretion/action leading to metabolic abnormalities like hyperglycaemia and dyslipidaemia. Increased levels of blood glucose and fats lead to the disruption of insulin secretion and /or action and finally cause tissue damage.

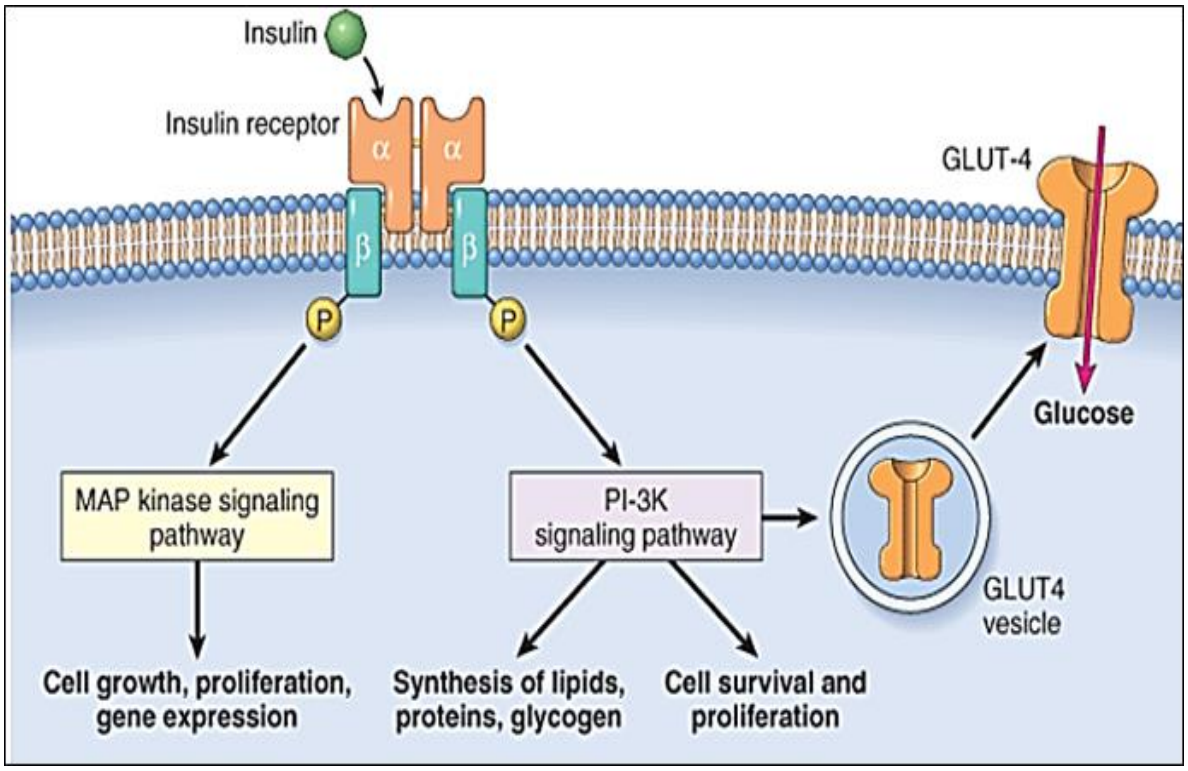


Fig 1.4 Insulin and glucose uptake by tissues:

Insulin receptor consists of 2 alpha and 2 beta subunits linked by disulphide bridges. Activation of the phosphatidylinositol -3'-kinase (PI3K) pathway stimulates translocation of glucose transporter 4 (GLUT-4) to the cell surface. This leads to glucose uptake by muscle and adipose tissue, in addition to stimulation of protein synthesis, glycogenesis and lipid synthesis. Activation of the Mitogen-activated protein kinases (MAPKs) pathway induces cellular proliferation and growth as well as regulation of gene expression in insulin response tissues. The diagram is reproduced from Osman *et al.*, (2000).

1.3.5. Action and secretion of insulin in patients with T2DM

When normal levels of insulin render it unable to perform its function (action) in tissues such as liver, muscle and adipose tissue, this stimulate β-cell to secrete more insulin to compensate for this defect leading to hyperinsulinemia. Insulin resistance and defect in β-cell function lead to insulin deficiency and hyperglycaemia, and therefore development of T2DM (Fig 1.5). A reduction in β-cell mass and function in patients with T2DM has been demonstrated by

several studies (Butler *et al.*, 2003; Del Guerra *et al.*, 2005; Marchetti *et al.*, 2004; Rahier *et al.*, 1983; Sakuraba *et al.*, 2002; and Yoon *et al.*, 2003).

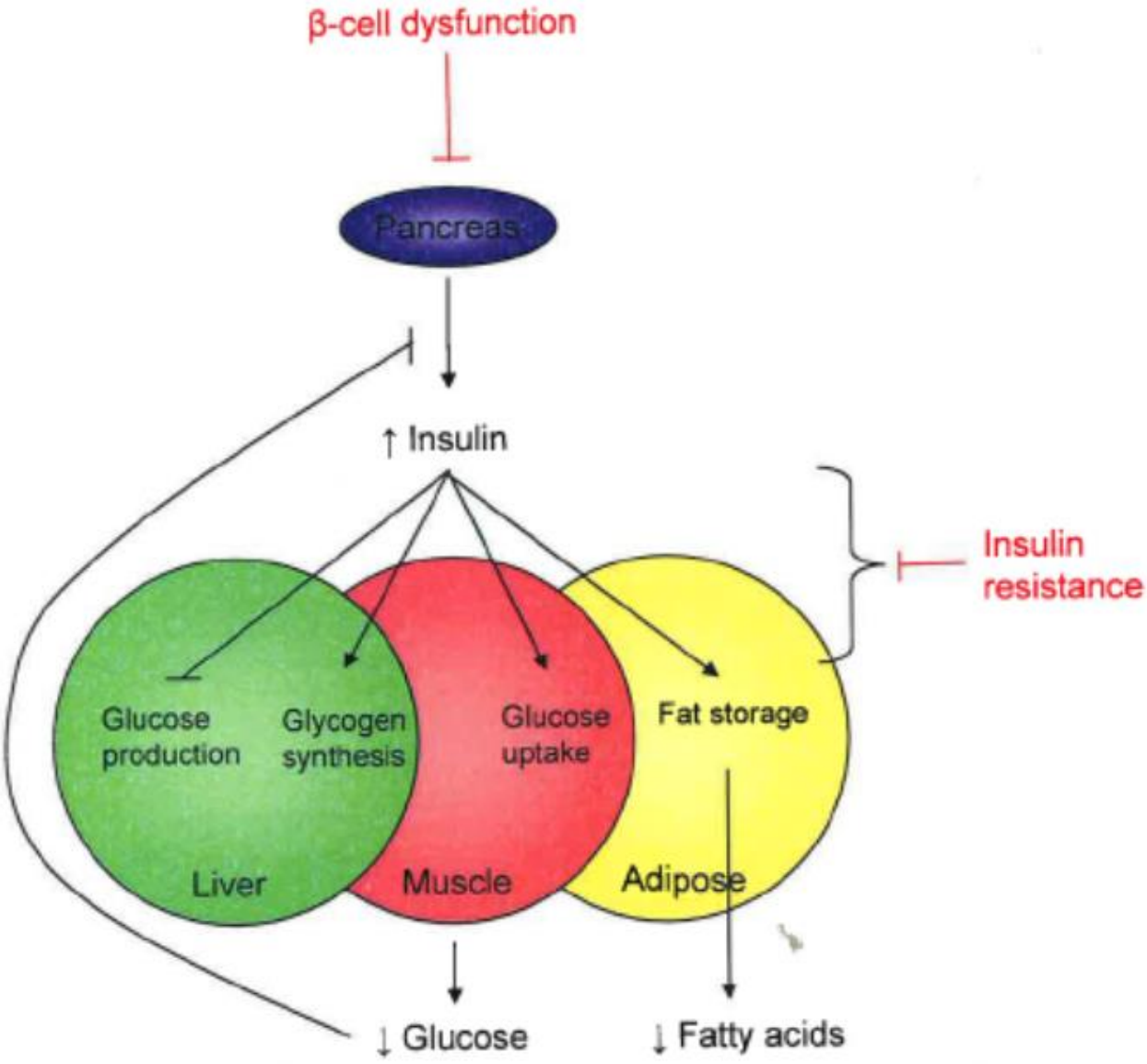


Fig 1.5 Causes of T2DM: Insulin resistance and dysfunction in the beta cell.

High blood glucose stimulates insulin secretion. Insulin activates all anabolic pathways such as glycogen synthesis in liver and muscle and lipogenesis (synthesis of TAG) in Adipose tissue, as well as stimulates glucose uptake by muscle and adipose tissue, on the other hand inhibiting production of glucose (gluconeogenesis) in the liver; these processes lead to reduced blood glucose and therefore reduction in insulin secretion. T2DM occurs when the target tissues do not respond properly to secreted insulin (insulin resistance) and dysfunction in the beta cells which leads to reduced insulin secretion. The diagram is reproduced from Cerf, (2013)

1.4. Insulin signal transduction

After the discovery of insulin and its role in the regulation of metabolic pathways, scientists concentrated on studying the mechanism of insulin action in target tissues (Fig 1.6).

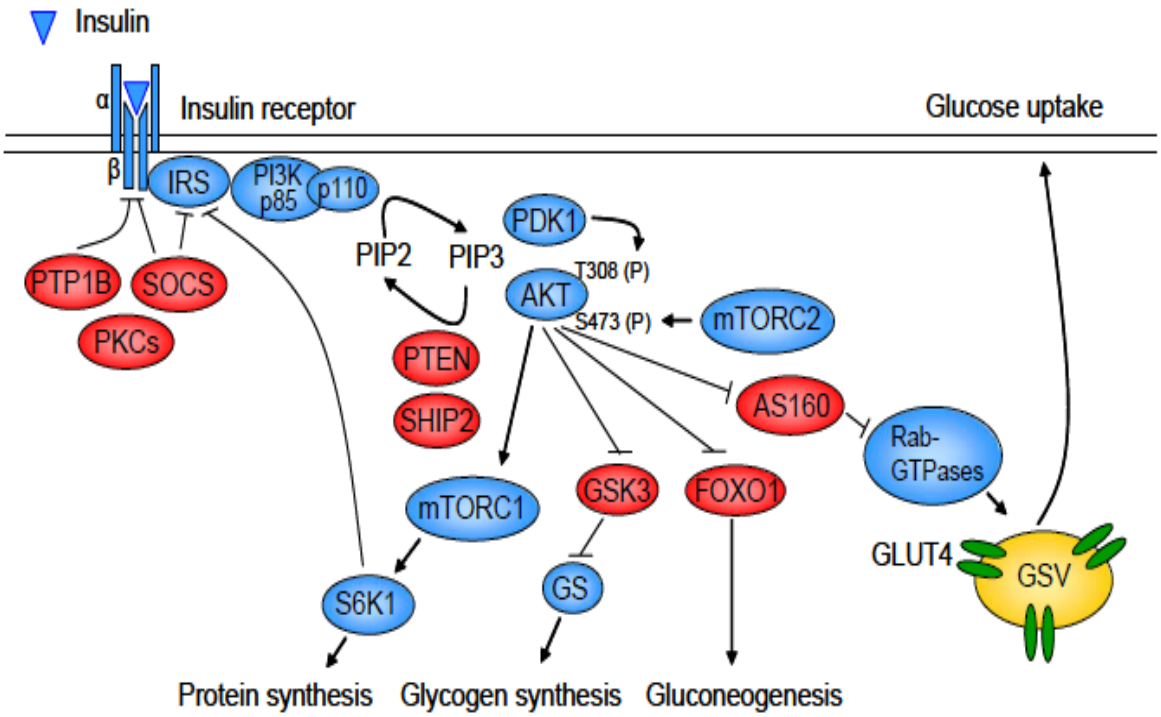


Fig 1.6 Insulin signalling pathway, its regulation and metabolic effects

Insulin binds with its receptor in a target tissue at the α-subunit and this leads to the phosphorylation of the β-subunit and activation of IRS protein. The signal from insulin is then transferred from molecule to another (in cascade) to its action such as protein synthesis, glucose production (gluconeogenesis), glycogenesis (production of glycogen), glucose uptake (GLUT4) and cell growth. The figure is taken from Chakraborty *et al.*, (2011).

1.4.1. The insulin receptor

Insulin receptor consists of two alpha (2α) and two beta (2β) subunits. The α subunit is located outside the cell where it binds to insulin while the β subunit is immersed in the membrane and extends inside the cell. The insulin receptor is a family of ligand-activated receptor tyrosine kinases (RTK) and receptors of growth factors (Ullrich *et al.*, 1985). The intracellular end of the receptor contains a kinase enzyme which is activated when insulin binds to its receptor. This activation leads to autophosphorylation of specific tyrosine residues on each β subunit (Jacobs *et al.*, 1980). Phosphorylation of the β subunit

activates some enzymes and inactivates others. The insulin receptor plays an important role in the metabolic actions of insulin in target tissues. It was shown in murine models that disruption of the insulin receptor gene (Insr) leads to inhibition of its functions (Joshi *et al.,* 1996). Studies have shown that mice with homozygous *Insr* null allele rapidly develop diabetic ketoacidosis after birth and die within few days after birth (Accili *et al.,* 1996; Joshi *et al.,* 1996). In addition, hyperglycamia and hyperinsulinemia were shown to be accompanied by elevated levels of fatty acids and fatty liver. Experiments on disrupted *Insr* gene using murine models enabled scientists to know the details about the metabolic role of insulin and its receptor in different insulin-responsive tissues. Following a carbohydrate rich meal, the majority of glucose is taken-up by muscle cells as facilitated by insulin. Insulin resistance (restricted up-take of glucose) was observed in the early stage of the T2DM (Bruning *et al.,* 1998).

1.4.2. Signalling downstream of the insulin receptor

Following binding of insulin to its receptor, the insulin receptor can phosphorylate a number of proteins named insulin receptor substrate (IRS). IRS acts as an intermediate product in cascades between the tyrosine kinase and the phosphatidylinositol 3-kinase (PI 3-kinase) in the mitogen-activated protein kinase (MAPK) pathways. The role of the IRS is to transfer the signal of insulin to the PI 3-kinase and MAPK. (Fig 1.4)

1.4.2.1. Insulin Receptor substrate (IRS) proteins

IRS is a protein of molecular weight of 131 k Da. There are two types of IRS (IRS -1 and IRS-2) encoded by two genes *IRS-1* and *IRS-2*. They work as signalling adapter proteins (Sun *et al.,* 1991). IRS contains phosphotyrosine-binding (PTB) domains and N-terminal pleckstrin-homology (PH) and about 40 potential tyrosine phosphorylation sites that can bind effector molecules containing Src-homology 2 (SH2) domains (Copps and White, 2012). IRS-1 and IRS-2 play important and overlapping roles in the regulation of blood glucose. knockdown of IRS-1 in mice led to insulin resistance and retardation of growth because of the resistance of Insulin-like growth factor 1 (IGF1) (Araki *et al.,* 1994; Tamemoto *et al.,* 1994), as well as increased serum TAG (due to impaired lipolysis) and hypertension (Abe *et al.,* 1998). Also removal of the IRS-2 of the mice led them to develop T2DM Due to the reduction in pancreatic ß-cell mass and failure to compensate for hepatic insulin resistance (Kubota *et al.,* 2000; Withers *et al.,* 1998) and are also dyslipidaemic and hypertensive (Kubota *et al.,* 2003). Knockdown of IRS-2 from mice led to altered growth has been

observed in a few tissues such as certain neuronal cells (Withers *et al.,* 1998). IRS-1 plays an important role in transmitting the insulin receptor signals to the intracellur pathways PI3K / Akt and Erk MAP kinase pathways. Knock-out of both IRS-1 and IRS-2 from mice hepatocytes led to insulin resistance, glucose intolerance and fatty liver (Taniguchi *et al.,* 2005). There are other IRS proteins (IRS3 and IRS4) but they have limited roles in insulin signal transduction.

1.4.2.2. Phosphatidylinositol 3-kinase (PI3K)

Phosphatidylinositol 3-kinase (PI3K) is an enzyme that has two units, a catalytic subunit and a regulatory subunit. It interacts with specific phosphotyrosine motifs of the insulin receptor substrates (IRS) through the SH2 domains on the regulatory subunit enabling recruitment of PI3K to the cell membrane and release of the catalytic subunit from the regulatory subunit (Yu *et al.,* 1998). Following release of the catalytic subunit of PI3K, it mediates conversion of phosphatidylinositol-4, 5- bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP3) (Whitman *et al.,* 1988). PIP3 interacts with proteins of PH domains, resulting in their activation at the cell membrane (Fig.1.6). Studies showed that inhibitors of PI3K hinder many metabolic actions of insulin including stimulation of glucose transport, glycogen and lipid synthesis, and fat cell differentiation (Kanai, 1993; Cheatham, 1994; Clarke, 1994). Improvement in insulin sensitivity was observed in mice with knockdown of the regulatory unit of PI3K (Terauchi *et al.,* 1999; Ueki, *et al.,* 2002), rescuing the diabetic phenotype and reductions in insulin receptor and IRS (Mauvais-Jarvis *et al.,* 2002). On the other hand, knocking-down the catalytic subunit induces glucose intolerance and elevated insulin levels (Brachmann *et al.,* 2005). These findings suggest that insulin sensitivity is maintained by balancing the two subunits, p85 regulatory and p110 catalytic subunits.

1.4.2.3. AKT/Protein Kinase B (PKB)

PIP3 has several targets, including among others, Phosphoinositide-dependent kinase-1 (PDK1), AKT (also known as protein kinase B (PKB) and some atypical forms of protein kinase C (PKC)) and cAMP-dependent, cGMP-dependent protein kinase C (AGC). AKT interacts with PIP3 via the PH domain and the activation of insulin to AKT requires phosphorylation at Thr308 (phosphorylated by PDK1) and Ser473 (phosphorylated by a mammalian target of rapamycin 2-mTORC2) (Alessi *et al.,* 1996 and 1997; Ali and Sabatini, 2005; Hresko and Mueckler, 2005; Sarbassov *et al.,* 2005). Upon activation of AKT, it can mediate several of the PI3K mediated insulin metabolic actions via phosphorylation of many

substrates. AKT was shown to phosphorylate and deactivate glycogen synthase kinase 3 (GSK3) resulting in the promotion of glycogen synthesis (Shepherd *et al.,* 1995). AKT was shown also to phosphorylate members of the forkhead (FOX) class of transcription factors (TFs) resulting in the prevent on of these TFs from activating genes encoding enzymes involved in gluconeogenesis (Zhang *et al.,* 2006). In addition, AKT enhances insulin-stimulated glucose uptake by phosphorylating and inhibiting a Rab-GTPase-activating protein (Sano *et al.,* 2003), triggering the activation of Rab small GTPases and therefore translocation of the glucose transporter GLUT4 to the cell membrane (Fig 1.6). AKT has three different forms encoded by three different genes (AKT1, 2 and 3). Studies showed that knocking-out AKT1 and AKT3 genes had no effect on glucose homeostasis (Cho *et al.,* 2001), unlike AKT2 gene (expressed in pancreatic ß-cells and skeletal muscle) (Easton *et al.,* 2005) knock-out which impaired insulin-stimulated glucose uptake by tissues (e.g. muscles) and inhibition of glucose production by liver (Cho *et al.,* 2001). AKT2 gene knock-out mice are also glucose intolerant and are hyperinsulinaemic and hypertriglyceridaemic with reduction in pancreatic ß-cell and adipose tissue masses (Garofalo *et al.,* 2003). In addition to the above, AKT2 (unlike AKT1 and AKT3) was found to localise together with GLUT4-containing vesicles and it phosphorylates proteins involved in transport of these vesicles (Calera *et al.,* 1998; Yamada *et al.,* 2005).

1.4.2.4. mTOR signalling pathway

The mammalian target of rapamycin (mTOR) has two components, mTORC1 and mTORC2. They are downstream components of the insulin signalling pathway. As mentioned above, mTORC2 is responsible for phosphorylation of AKT Ser473 (Ali and Sabatini, 2005; Hresko and Mueckler, 2005; Sarbassov *et al.,* 2005b) and is also involved in the actin cytoskeleton organisation (Jacinto *et al.,* 2004; Sarbassov *et al.,* 2004), while mTORC1 is activated by insulin signalling via AKT (Manning and Cantley, 2003; Tee *et al.,* 2003). mTORC1 regulates cell growth by modulating biological processes like translation, ribosome biogenesis and nutrient metabolism (Wullschleger *et al.,* 2006). In addition, activation of mTORC1 signalling and its p70 ribosomal S6 kinase (S6K1) was shown to inhibit insulin signalling through IRS proteins (negative feedback).

1.4.3. Attenuation of the insulin signalling cascade

Insulin signalling can be regulated by different mechanisms (Fig 1.6). Protein tyrosine phosphatase-1B (PTP1B) expression was shown to reverse the autophosphorylation of the

insulin receptor and elevated expression of PTP1B was observed in insulin-resistant humans and PTPB1 knockout mice have increased insulin sensitivity (Elchebly *et al.,* 1999). The tyrosine kinase activity of the insulin receptor was shown to be inhibited by beta-subunit threonine/serine phosphorylation. In addition, serine phosphorylation on IRS proteins was shown to oppose tyrosine phosphorylation and activation of IRS proteins. The actions of insulin, as well as others including free fatty acids, cytokines, factors that induce cell stress, and amino acids (through mTORC1), can result in activation of serine kinases like protein kinase C (PKC), suppressors of cytokine signalling (SOCS) proteins and S6K1. This could also act as a negative regulatory mechanism for the insulin signalling pathway. Finally, phosphatases, PTEN (phosphatase and tensin homologue on chromosome 10) and SHIP2 (SH2-domaincontaining inositol 5-phosphate-2) can reverse the conversion of PIP2 to PIP3.

1.5. Mechanisms of insulin resistance and pancreatic β-cell dysfunction

1.5.1. Obesity

Obesity is a medical condition associated with excess fat deposition, not only around the body but also in the tissues. This has a negative affect on the health leading to decreased life expectancy (Haslam *et al.,* 2005). Individuals are considered obese when the body mass index (BMI) exceeds 30 Kg/m^2, overweight when the (BMI) range is between 25-29.99 Kg/m^2 and normal when the (BMI) range is between 18-25 Kg/m^2 (Flegal *et al.,* 2002) Table 1.1., Fig1.9. In addition, obesity increases the probability of infections in patients with T2DM and heart disease and certain types of cancer (Haslam *et al.,* 2005). The major health risk of obesity depends on the distribution of fat around the body and excess fat located in the central abdominal area of the body is called upper body obesity (apple-shape) Fig 1.9. This type of obesity is a risk factor for cardiovascular diseases, insulin resistance, and diabetes (Ibrahim, 2010). Several studies in humans have reported that expression of resistin increases in adipose tissue (Degawa-Yamauchi *et al.,* 2003) particularly in those with abdominal obesity (McTernan, 2002).

1.5.2. Adipocyte dysfunction

The fat, derived from the excess food intake more than the body needs, in the body is stored in the adipocytes inside adipose tissue in the form of triglycerides (TAG), mostly in a single large lipid droplet. When energy needed, TAG, diglycerides (DAG) and monoglycerides

(MAG) are hydrolysed by adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) (Fig 1.7), and monoglyceride lipase respectively (lipolysis) to produce glycerol and three fatty acids, the free fatty acid can be oxidised by mitochondria in other tissues to generate energy, while the glycerol are transferred to the liver to convert it to glucose by gluconeogenesis pathway. Increased adipose tissue mass due to obesity are associated with insulin resistance. In this scenario, the capacity of adipocytes to store excess lipids becomes saturated, resulting in lipid deposition in non-adipose tissue such as muscle, liver, and pancreas. Furthermore, obesity can cause aberrant secretion of adipokines, the collective term for the large number of hormones, cytokines, and growth factors secreted by adipose tissue to influence whole-body energy balance and nutrient metabolism.

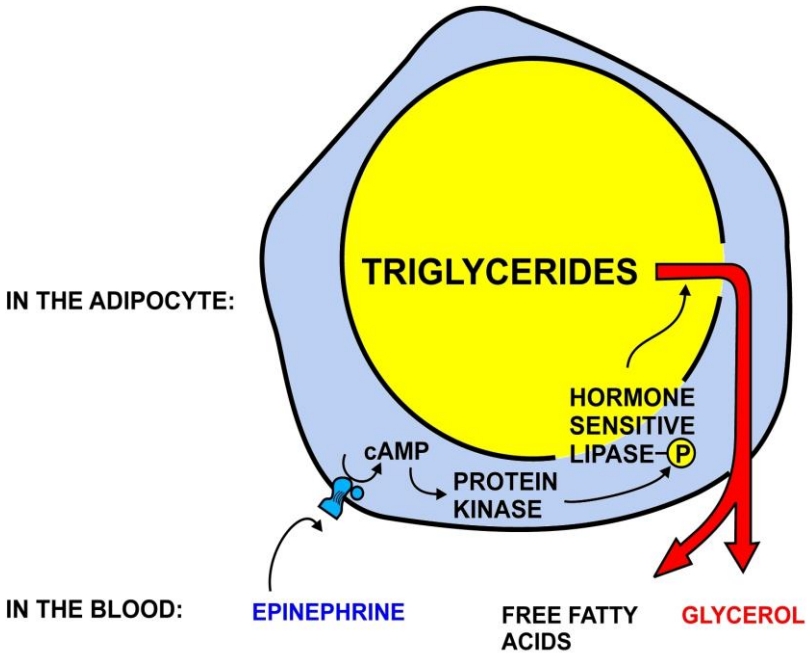


Fig 1.7 Lipolysis.

It is activated by HSL enzyme which its stimulated by epinephrine and cAMP. First epinephrine binds with beta-adrenergic receptors on the cell membrane of the fat cell and therefore generate cAMP inside the cell, which in-turn activated the protein kinase which it phosphorlates and activates HSL.The diagram is provided from Jocken and Black, (2008).

1.5.3. Ectopic accumulation of lipids

Increased free fatty acids in the blood (as seen in obesity) lead to the accumulation of large amounts of lipid (in the form of TAG) in liver causing fatty liver or hepatic steatosis and in

muscle causes muscle steatosis. When activated free fatty acid increases than the capacity of the fatty acid oxidation (the process of breaking down fatty acids to produce energy) the excess of these fatty acids can be used to build up monoglycerides (MAG) and diacylglycerol (DAG). Lipid intermediates are then thought to induce serine phosphorylation on IRS proteins through the activation of serine kinases such as PKC isoforms, I kappa B kinase (IKK), and JNK (Gao *et al.*, 2004; Itani *et al.*, 2002). Insulin resistance in humans and animal models has been shown to correlate with fat content within cells (muscle cells) (Pan *et al.*, 1997; Perseghin *et al.*, 1999; Phillips *et al.*, 1996; Storlien *et al.*, 1991) and lipid content in hepatocytes (liver cells) (Kotronen *et al.*, 2008; Ryysy *et al.*, 2000; Seppala-Lindroos *et al.*, 2002). However, it remains controversial whether lipid accumulation in muscle and liver causes whole-body insulin resistance, or whether it is in fact merely a marker for insulin resistance. The lipogenic effects of insulin may cause, or at least exacerbate, lipid accumulation in ectopic tissues in states of hyperinsulinaemia (Savage *et al.*, 2007).

High circulating fatty acids can also impact negatively on pancreatic β-function. Elevated fatty acid concentrations have been shown to increase basal insulin release and decrease glucose-stimulated insulin secretion *in vitro* (Sako and Grill, 1990), in rats (Mason *et al.*, 1999), and in humans (Paolisso *et al.*, 1995). Fatty acids have also been shown to inhibit insulin gene expression in vitro (Kelpe *et al.*, 2003). Fatty acids in concert with high glucose also promote β-cell death (El-Assaad *et al.*, 2003). Many mechanisms have been suggested to mediate this effect including production of lipid intermediates and oxidative stress. More recently it has been shown that fatty acids can induce markers of (Endoplasmic reticulum) ER stress and changes in ER morphology, leading to β-cell death (Laybutt *et al.*, 2007). There is strong evidence that the detrimental effects of fatty acids on β-cell function only occur in the presence of elevated glucose, which directs fatty acid partitioning away from oxidation and towards storage (Prentki and Corkey, 1996).

1.5.4. Insulin Resistance

Insulin resistance is a pathological condition in which cells fail to respond properly to insulin, thus blood glucose fails to enter the cells resulting in high blood glucose (hyperglycaemia). If not treated this may result in the development of T2DM. Several other factors can also contribute to the development of insulin resistance including obesity, resistin, homocysteine and deficiency of vitamin D.

1.5.5. Adipokines

Adipose tissue has the ability to produce and secrete different types of adipokines. Several adipokines are known to be involved in the regulation of the action of insulin e.g. adiponectin and various inflammatory factors (interleukins, cytokines and tumor necrosis factors), leptins, retinol binding protein (RBP) and resistin. Several studies in experimental models identified genetic mutations in the leptin gene and its receptor gene that were associated with obesity and diabetes (Chen *et al.,* 1996; *Lee et al.,* 1996; Tartaglia *et al.,* 1995; Zhang *et al.,* 1994). Leptin was shown to have several functions that include, hepatic insulin sensitivity and glucose production (Cohen *et al.,* 1996), regulation of food intake (Stephens *et al.,* 1995), and inhibiting fatty acid synthesis and stimulating fatty acid oxidation resulting in triglyceride accumulation in peripheral tissues (Minokoshi *et al.,* 2002; Prieur *et al.,* 2008). Resistin, another adipokine produced by adipose tissues in animals, was shown to reduce glucose uptake by skeletal muscle cells (Moon *et al.,* 2003). It was suggested that insulin resistance and obesity in diabetic mouse can be attributed to the existence of resistin (Jamaluddin *et al.,* 2012). As will be described in detail in another section of this thesis, the role of resistin in obesity associated with T2DM in humans is unclear.

1.6. Resistin

1.6.1. Introduction

In humans, resistin is mainly secreted from immune cells (macrophages and monocytes), while in rodents (Mice), it is secreted by adipocytes (in adipose tissues). Adipose tissue, the main site for lipid storage, has been shown to function as an endocrine and secretory organ by secreting hormones e.g. adipokines (Kershaw and Flier, 2004). Adipokines compris a variety types of cytokines, chemokines, and hormone-like factors which participate in the regulation of physiological and pathological processes like catabolism and anabolism of metabolic fuel, immunity, and inflammation (Wellen *et al.,* 2005). In mice, resistin is derived of the adipose tissues (as a cytokine) and there is a lot of controversy about its physiologic role in obesity and T2DM (Lazar, 2007). Resistin was proposed as a potential link between obesity and diabetes (Janke *et al.,* 2002) and studies showed that resistin could interfere with insulin signalling and glucose tolerance (Steppan *et al.,* 2001). Obesity has been shown to contribute to insulin resistance which is considered a risk factor for development of (T2DM) (Kahn and Flier, 2000). Administration of a recombinant resistin to the obese Mice impaired glucose

tolerance, while suppressing resistin action by neutralizing resistin leads to improvement in insulin sensitivity and blood glucose in the obese Mice (Savage *et al.*, 2001).

In addition, resistin was shown to inhibit entry of glucose to muscle cells (Moon *et al*., 2003). Jamaluddin *et al*, observed that insulin resistance and obesity in diabetic mice was attributed to resistin (Jamaluddin *et al.*, 2012). In humans, the role of resistin in obesity associated with T2DM is unclear. Experiments in murine models showed marked increase in levels of resistin in both genetic and diet-induced obesity and these levels correlated with impaired glucose tolerance and insulin action (Nagaev and Smith, 2001). In addition, Thiazolidinedione treatment was shown to inhibit resistin gene expression and enhanced blood glucose uptake and insulin sensitivity in mice (Sentinelli *et al.,* 2002). It was suggested that resistin antagonizes insulin action and disrupts one or more component in insulin signalling pathways leading to insulin resistance. As stated previously, resistin plays an important role in insulin resistance and T2DM in the murine models, but the role of resistin in human obesity and diabetes is controversial. Resistin might have different roles in humans and mice. Resistin gene expression in adipocytes from overweight individuals has been reported to be absent (Nagaev and Smith, 2001) and there are controveries regarding the role of resistin in obesity and insulin sensitivity (Savage *et al.,* 2001; Rajala *et al*., 2003; Ribon *et al*., 1998 and Ribon and Saltiel, 1997). Studies showed that the expression of human resistin mRNA is very low in human adipocytes (Savage *et al.*, 2001).

The association between human resistin and T2DM has not been clarified as well (Engert *et al*., 2002). A recently study found a resistin gene promoter -420 G/G genotype to be associated with T2DM (Osawa *et al*., 2004). Higher serum levels of resistin were shown also to be associated with this genotype (homozygosity -420 G/G). Therefore, they suggested that this mutation -420 G/G might be associated with elevated resistin levels contributing to insulin resistance and T2DM in human (Osawa *et al*, 2005).

The association between elevated levels of resistin and T2DM in humans is very controversial (Cho *et al.,* 2004). Lee *et al* reported that there is no association between resistin levels and obesity or T2DM (Lee *et al.,* 2003). McTernan *et al* and Youn *et al* observed that serum resistin levels were higher in patients with T2DM, but are not associated with obesity (McTernan *et al.,* 2003 and Youn *et al.,* 2004). Fujinami *et al* and Azuma *et al* showed elevated levels of resistin in T2DM and in obese individuals (Fujinami *et al.*, 2004 and Azuma *et al.,* 2003).

In humans, resistin is mainly secreted by immune cells (monocytes/macrophages) and rarely by adipose tissue (Patel *et al.,* 2003). On the other hand, in mice resistin is expressed mainly

by adipose tissue (Steppan *et al.,* 2001). Macrophages infiltrating into adipose tissues could account for insulin resistance in obese mice. A study from Japan by Osawa *et al.* investigated the relation between human resistin and T2DM, and showed that the resistin promoter -420 genotype was associated with monocyte resistin mRNA expression and serum resistin levels. In addition, the study found that patients with T2DM had higher serum resistin levels as compared to their controls (Osawa *et al.,* 2005). Resistin expression was higher in patients with type 2 diabetes mellitus and dyslipidaemia and less in the control group. Resistin probably plays a role in the pathogenesis of hepatic insulin resistance and aggravates pathologic changes in the liver of patients with NAFLD (Gierej et al., 2017). Recently, a study has been reported on subjects with non-functional adrenal incidentaloma; they found not only significantly decreased serum adiponectin levels but also increased leptin, resistin levels as well as dislipidemia, hypertension and high insulin resistance index. All of which could affect insulin resistance and cardiovascular risk factors. The underlying mechanisms of these findings are unknown, hence further studies needed (Akkus *et al.,* 2018).

1.6.2. Structure and forms of Resistin

The resistin gene, referred to "Retn", encodes the resistin peptide (Steppan *et al.,* 2001). Resistin is found in two forms either high-molecular mass (HMM) which is the predominant form and contains a hexamer subunit, and low-molecular mass (LMM), which is monomeric and unable to form intertrimer disulphide bonds; this is considered the active form (Patel *et al.,* 2004). The resistin has a molecular weigh of approximately 12.5 kDa proteins that consist of 108 amino acids in human and 114 amino acids in Mouse and rat (Lazar, 2007). The gene that encodes human resistin is located on chromosome 19 whereas the Mouse resistin gene is located on chromosome 8 (Ghosh *et al.,* 2003). Mouse and human resistin share 46.7% similarity at the genomic DNA level, 64.4% sequence homology at the mRNA level, and 59% identity at the amino acid level. The mature segments are 55% amino acid (aa) identical between mice and human (Steppan *et al.,* 2001) and 72% aa identical between mouse and Rat (Del Arco *et al.,* 2003). Resistin also contains 11 cysteine (cys) amino acids residues 10 of which are involved in intramolecular disulfide bonding, the last one being (cys 26) which participates in dimer structure formation (Juan *et al.,* 2003). The resistin polypeptide consists of three domains, N- terminal which has 17 amino acids which represents, signal sequence, C-terminal is highly constant region, and variable region that lie in between N- terminal and C- terminal (Adeghat, 2004). There are three types of RELMs: Resistin-like molecule α-1 (RELM-α-1) (Found in Inflammatory Zone (FIZZ 1) is a secreted

protein that is present mainly in adipose tissue and found in the inflammatory zone in murine allergic pulmonary inflammation (Holcomb, 2000). RELM-β (FIZZ 2). It is secreted only in the gastrointestinal tract from goblet and epithelial cells, and it is markedly expressed in tumors, suggesting a possible role in proliferation. RELMs-γ the most recently discovered member of the RELM family is found in hematopoietic tissue, indicating cytokine- like functions (Gerstmayer *et al.,* 2003).

1.6.3. Resistin and Obesity

Obesity is a growing global health problem. It is associated with insulin metabolic syndrome, resistance, dyslipidaemia, and T2DM (Steppan and Lazar, 2002). Several factors were shown to be involved including hormones, cytokines, growth factors and adipokines. The later was shown to be involved in controlling insulin sensitivity linked to the pathophysiology of obesity (Steppan *et al.,* 2001; Kim *et al.,* 2001 and Holcomb et al., 2000). Resistin is proposed as a link between obesity and T2DM and may affect insulin signaling leading to insulin resistance (Steppan *et al.,* 2001; Sentinelli *et al.,* 2002 and Asano *et al.,* 2010). In vitro studies using adipocytes cell lines showed that neutralization of resistin with resistin antiserum was shown to lead to decreased insulin resistance and increase in glucose uptake by tissues (Steppan and Lazar, 2002). Increase in levels resistin were observed in high fed mice (Steppan and Lazar, 2002). Similar findings were observed in genetic models of obesity (ob/ob mouse) (Rajala *et al.,* 2004). The previous studies are in murine models, and the role of resistin in the pathophysiology of insulin resistance and T2DM is not clear. Several studies showed high levels of resistin mRNA expression in adipose tissue from obese diabetic subjects (McTernan *et al.,* 2002; McTernan *et al.,* 2002 and Heilbronn, *et al.,* 2004). Higher resistin levels were shown to be elevated in obese subjects as compared to non-obese subjects (Vendrell *et al.,* 2004; Schaffler *et al.,* 2004 and Degawa-Yamauchi *et al.,* 2003). A study involving adult, nondiabetic, obese and Caucasian first-degree subjects showed that resistin levels were highly heritable and suggested that resistin might play a pathogenic role in insulin resistance associated with obesity (Menzaghi *et al.,* 2006). However, in healthy young subjects, no correction was found between resistin levels and insulin resistance associated with obesity (Silha et al., 2003). Other studies showed correlation between resistin levels and waist circumference and body fat mass but not with the BMI (De Luis et al., 2003). The differences between studies raised question of ethnic influence on resistin levels. Ethnic variability might influence the association between resistin, obesity and insulin resistance.

Therefore, studying these associations in an ethnically uniform population is of great importance.

Moreover, positive correlation has been detected between serum resistin levels and body fat content (Rajala *et al.*, 2004). In obese Mice, it was observed that the levels of resistin drop during fasting and increase during re-feeding. It was suggested that resistin might be a hormone that works against the insulin and is considered as an independent factor for obesity. Some studies confirm that the resistin has been found at high concentrations in the high-fat-induced obese Mice and diabetics (Steppan *et al.*, 2001).

BMI = m/ h2 m (weight)

h (height)

Table 1.1 Body Mass Index

BMI (kg/m2)	**Classification**
<18.50	underweight
18.50-24.99	Normal weight
25-29.99	overweight
30-34.99	Class1 obesity
35-39.99	Class11 obesity
⩾40	Class111 obesity

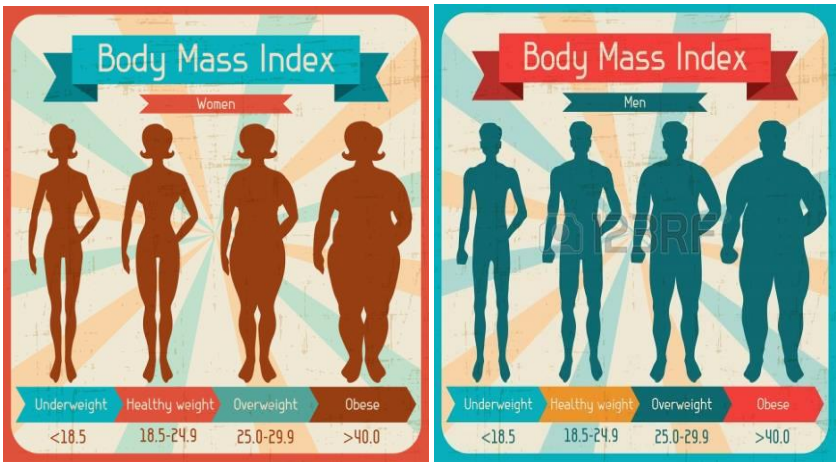


Fig 1.8 Body Mass Index of men and women

The diagram is provided from Zahn *et al.*, (2018)

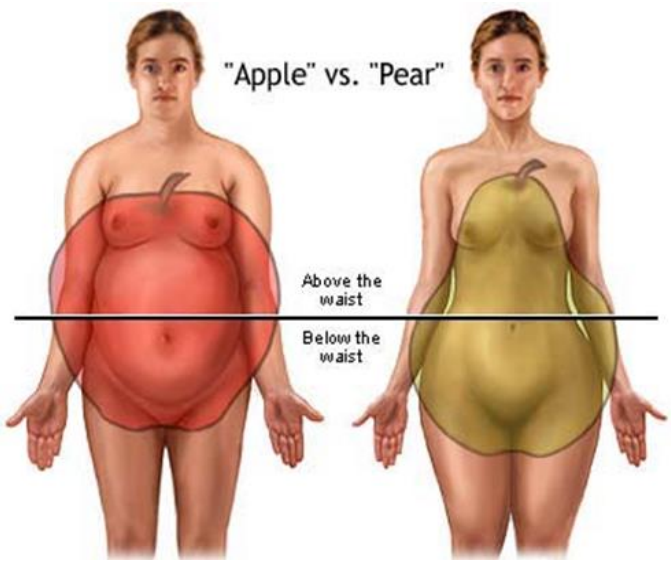


Fig 1.9 Body obesity: upper versus lower obesity.

The diagram is provided from Kirschner *et al.,* (1990)

Apple –shaped	Pear- shaped
Upper body obesity	Lower body obesity

1.6.4. Resistin, Insulin resistance and T2DM

Insulin action at target tissue starts when insulin binds with its receptor and subsequently sends a signal cascade to produce glucose transport for decreasing glucose levels in the plasma. Disruption of the insulin pathway leads to hyperglycaemia and then development of T2DM. It was suggested that the resistin had significant effects on insulin action, and link obesity with insulin resistance (IR) (Banerjee *et al.,* 2003). Injection of Mice with anti resistin antibody improved the action of insulin and glucose metabolism (Steppan, *et al.,* 2001). As well as treatment of normal Mice with recombinant resistin disrupt glucose tolerance and insulin action (Steppan *et al.,* 2001). Resistin gene knock-down led to a decrease in glucose by reducing gluconeogenesis, while giving resistin to these resistin-deficient Mice led to increases in sugar production in the liver (Banerjee *et al.,* 2004). Moreover, increased production of resistin disrupts glucose transport in skeletal muscle in Rats, while injection with recombinant resistin decreases insulin mediated glucose transport in muscle cells (Pravenec *et al.,* 2003). Furthermore, a study conducted by Rajala *et al* explored the physiological role of resistin in the development of insulin resistance in rodents (Rajala *et al.,* 2004). This study was explained that chronic hyperresistinemia impairs insulin signalling pathway in muscle, liver and adipose tissue, and indicated that resistin is considered one the factors which leads to insulin insensitivity and may participate to the development of insulin

resistance or diabetes in obese rodent. The role of resistin in the development of insulin resistance and T2DM was studied using Cell Cultures (Palanivel *et al.,* 2006) and animal's models but in human it remains controversial. Jenifer *et al* was found that there were association between resistin levels and obesity or insulin resistance in human and concluded that resistin hormone did not play a role in insulin resistance in human (Jenifer *et al.*, 2003). On the other hand, Silha *et al* reported that resistin was significantly associated with insulin resistance in obese individuals (Silha *et al.*, 2003). Hasegawa et al observed that resistin levels were higher with statically significant in patients with T2DM compared with healthy subjects, but this increase is not associated with obesity and insulin resistance (Hasegawa *et al.*, 2005).

1.6.5. Mechanism of action of Resistin

For biological action, insulin binds to its receptor to form a complex that in turn results in insulin receptor phosphorylation. Following receptor phosphorylation at tyrosine residue, the insulin receptor substrate (IRS) proteins -1 and -2 are attracted and are subsequently phosphorylated. This starts a cascade of reactions involving phosphoinositide 3-kinase (PI3K) and protein Kinase B (PKB), which it known as AKT. Adding resistin to cultures was shown to reduce phosphorylation of Insulin receptor substrate (IRS) induced by insulin (Karmiris *et al.*, 2005 & Kitagawa *et al.*, 2004). Karmiris *et al* shown decreased IRS-2 phosphorylation and protein level in adipose tissue, skeletal muscle and liver of mice after exposed to resistin (Karmiris *et al*., 2005). In contrast, Moon and Palanivel have observed that there is no effect of resistin on IRS-2 and IRS-1 proteins in cultured L6 myoblasts and muscle liver and therefore lack effect of resistin on the PI3K activity or the subsequent phosphorylation of AKT (Moon *et al*., 2003 & Palanivel *et al*., 2006). Following activation of AKT, glycogen synthase kinase (GSK)-3 phosphorylates glycogen synthase converting it to an inactive form. It was found that resistin increases the production of the inhibitor protein SOCS3 (a protein known to have ability to disrupt insulin signal transductions) (Muse *et al.,* 2007). SOCS3 interacts with the insulin receptor preventing its phosphorylation, and therefore inhibiting activation of IRS-1, PI3K, and AKT. The end result is reduction in glycogenesis, increase in gluconeogenesis and glycogenolysis leading to hyperglycemia and insulin resistance (Fig 1.10).

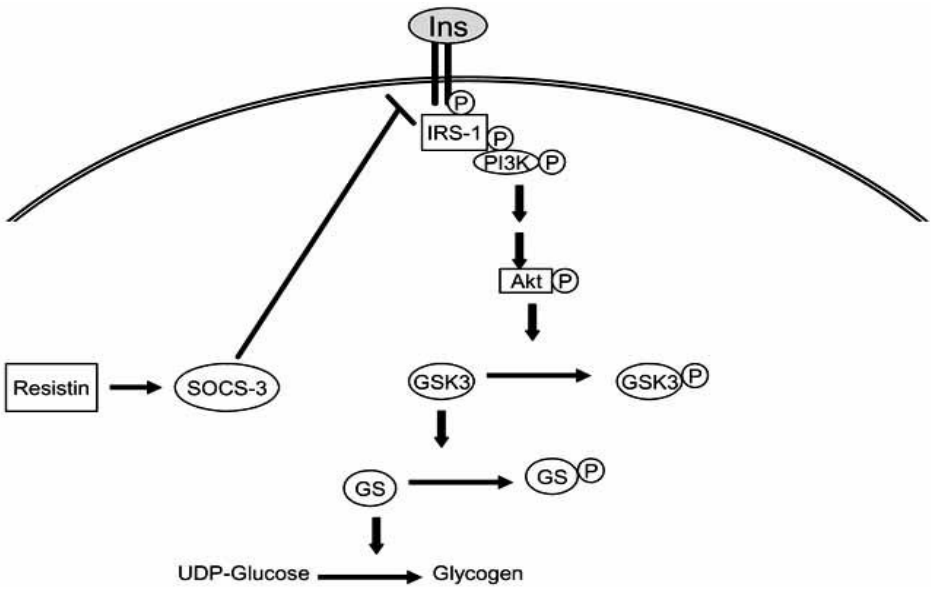


Fig 1.10 Action of resistin on the insulin signalling pathway:
resistin participates in the insulin resistance by activate SOCs-3 protein which in turn disrupt the insulin signaling by inhibiting Insulin receptor substrate 1 (IRS-1). The figure was reproduced from Song *et al.*, (2013).

1.7. Homocysteine

1.7.1. Structure and Function of Homocysteine

Homocysteine is an amino acid that is not present in the protein and contains sulfhydryl group (-SH) at the end (Hajjar, 2001). Homocysteine is a 2-amino-4 butyric acid, Fig1.8 (Finkelstein, 2003). Homocysteine was extracted for the first time from methionine after strongly heating with sulphuric acid (Butz and Vigneaud, 1932) Fig.1.11.

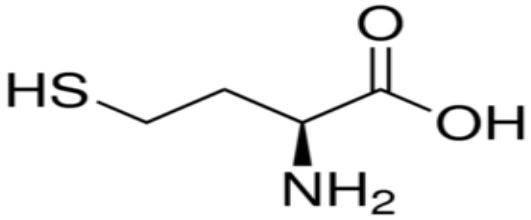


Fig 1.11 chemical formula of homocysteine

Normally homocysteine is not found in nature or in the diet, only as intermediate product in methionine metabolism, so it is considered as a main source of methionine production Fig1.12. Biologically, the homocysteine has three functions (i) a precursor of methionine formation Fig1.12. (ii) methyl group donor for the formation of choline (iii) metabolised to cystathionine and cysteine. (Finkelstein and Martin, 1986; Fig 1.12)

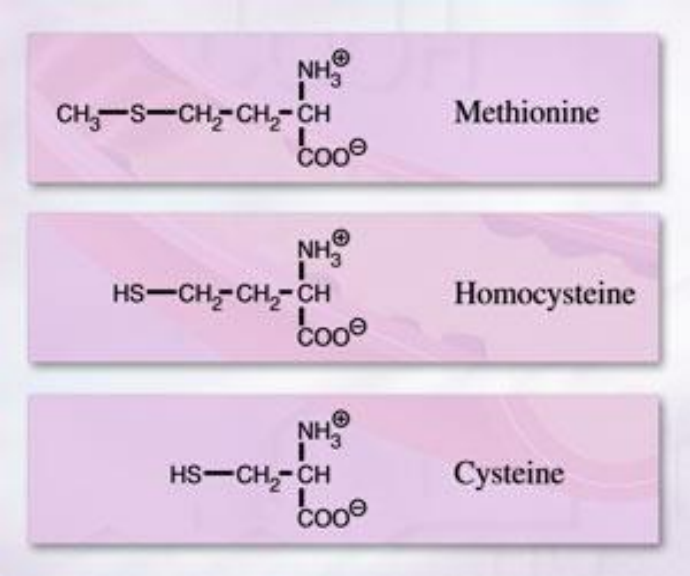


Fig 1.12 Methionine, Homocysteine and Cysteine Structure

1.7.2. Metabolism of the Homocysteine

Homocysteine is an intermediate product during metabolism of methionine (Met). The reaction starts by removing the methyl group from methionine, a reaction that requires ATP and S-adenosylmethionine synthetase to produce S-adenosylmethionine (SAM), (Fig1.13 reaction 1). SAM (methyl donor) is then converted to S-adenosylhomocysteine (SAH) after the methyl group has been removed and donated it to an acceptor, such as Creatine, DNA, RNA and Neurotransmitters (Fig1.13, reaction 2&3) , SAH is hydrolyzed to L-homocysteine and adenosine by a reaction catalysed by S-adenosylhomocysteine hydrolase (Fig1.13 reaction 4), (Fowler, 1997). Homocysteine can then be metabolized either to convert back to methionine via re-methylation pathway or by irreversible degradation to yield cystathionine and cysteine in the transsulfuration pathway (Mills *et al*., 1995; Selhub, 1999) (Fig1.13)

1.7.2.1. Metabolism of homocysteine via re-methylation pathway

The objective of the re-methylation pathway is the conversion of homocysteine to methionine. Homocysteine needs a methyl group that is obtained either from: 1) the conversion of 5-methyltetrahydrofolate to tetrahydrofolate (the active form of folate) catalysed by methionine synthase enzyme using vitamin B12 as a cofactor (Fig1.13 reaction 7). This reaction occurs in all tissues. To regenerate the production 5-methyltetrahydrofolate from tetrahydrofolate, methylenetetrahydrofolate reductase (MTHFR) and riboflavin (B2) are required. This reaction occurs in two steps, first tetrahydrofolate is converted to 5, 10 methylenetetrahydrofolate (Fig1.13 reaction 9), and then to 5-methyltetrahydrofolate

(Fig1.13 reaction 10). MTHFR is considered to be the key enzyme in the re-methylation pathway. The vitamins Folate, B2 (riboflavin), B6 (pyridoxal phosphate), and B12 (cobalamin) are required to complete the re-methylation pathway (Mattson and Shea, 2003). 2) The conversion of betaine to N, N-dimethylglycine catalysed by betaine-homocysteine methyltransferase enzyme (BHM) this pathway is considered a relatively minor pathway and vitamin B12 is not required as a cofactor. It found mainly in the liver. (Fig1.13 reaction 8; Perry, 1999).

1.7.2.2. Metabolism of homocysteine via Transsulfuration pathway

The main goal of the transsulfuration pathway is the production of cysteine from methionine via homocysteine as an intermediate (Medina *et al.*, 2001). The first step in this pathway is the formation of cystathionine from the condensation of homocysteine and serine catalysed by pyridoxal-5'-phosphate–dependent-cystathionine ß-synthase (CßS) using vitamin B6 as a coenzyme (Fig1.13 reaction 5). Cystathionine is cleaved into cysteine and ketobutrate a reaction catalysed by pyridoxal-5'-phosphate–dependent- cystathionine β-lyase with Pyridoxal phosphate (vitamin B6) as a co-factor (Fig1.13 reaction 6), (Mangoni and Jackson, 2002; Perry, 1999). Finally, cysteine is degraded into three compounds (sulfate, taurine and pyruvate) after removal CO_2 and NH_3. In addition to its main function, which is cysteine formation, this pathway also relieves the body from the excess homocysteine, mutations in transsulfuration pathway leads to homocystinuria (Medina *et al*., 2001). All these reactions take place in the liver and kidney (Blom *et al.*, 2006).

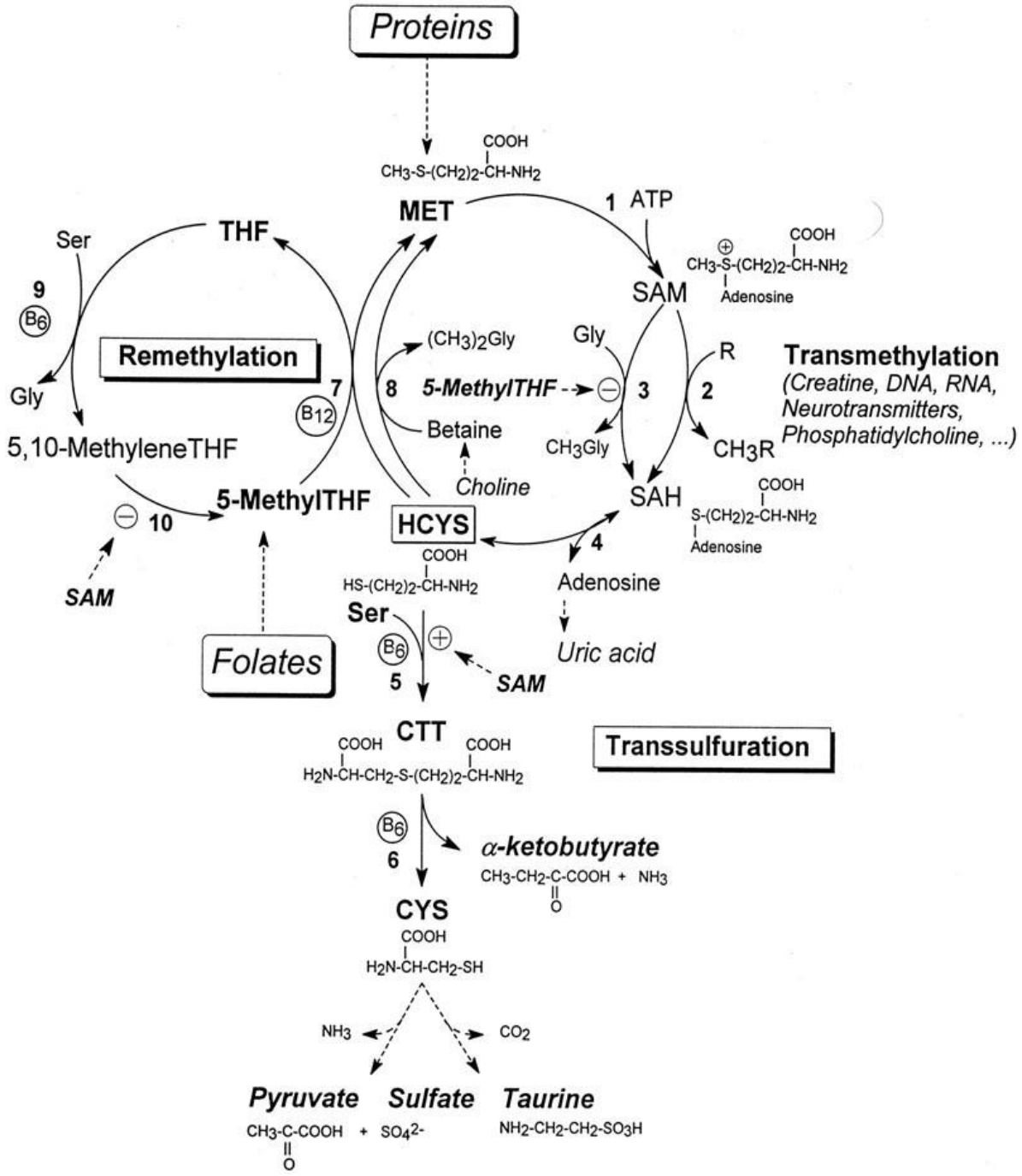


Fig 1.13 Homocysteine metabolism:

the toxicity of homocysteine can be removed either by re-methylation pathway where the homocysteine is converted to methionine or by transsu-lfuration pathway where the homocysteine is degraded to cysteine and α-ketobutyrate. The figure was taken from Finkelstein and Martin, (1986).

1.7.2.3. The roles vitamins in homocysteine metabolism

Many of the water-soluble vitamins have important roles, as coenzymes, in homocysteine metabolism:-

Vitamin B2

Flavin adenine dinucleotide (FAD) is considered the active form of vitamin B2 and it has an important role, as a coenzyme, in MTHFR. 0.6 mg intake daily from the diet was enough in modest reductions in homocysteine levels (Malinow *et al.*, 1999).

Vitamin B6

The active form of this vitamin B2 is Pyridoxal-5-phosphate (PLP) which plays a role as a coenzyme for many enzymes like cystathionine-β-synthase (CßS), β-lyase, γ-cystathionase and serine hydroxymethyltransferase (SHMT). The absorption of the vitamin B6 occurs in the upper small intestine afterward becomes transported to the liver where phosphorylation takes place (Merill and Henderson, 1990). Albumin is the main carrier of the PLP in the circulation and exported to extrahepatic tissues (Leklem, 1990).

Vitamin B12

The active form of vitamin B12 is methylcobalamin. It acts as a coenzyme for methionine synthase (MS) enzyme. In the stomach, methylcobalamin binds with haptocorrin forming a complex. In the duodenum, Vitamin B12 is released by pancreatic proteases and subsequently gets bound to intrinsic factor (IF) (Russell-Jones and Alpers, 1999). In the ileum, the complex (vitamin B12/IF) binds with its receptor- cubulin (Seetharam et al., 1999) and is absorbed by receptor-mediated endocytosis. It is transported in the circulation by transcobalamin (TC). The complex (vitamin B12/TC) enters cells by endocytosis via the transcobalamin receptor (TC-R) which is expressed on the cell surface in many tissues. After entering cells, B12 is released from its carrier, TC, and in metabolized in several steps before it becomes a methyl carrier in homocysteine metabolism (Seetharam and Li, 2000).

Folic acid

Folate is a biological carrier of the one-carbon unit; the active form is tetrahydrofolate when it binds with the methyl group becomes 5-methyltetrahydrofolate. It is a methyl donor in many reactions. It is formed upon reduction of 5, 10-methylenetetrahydrofolte by MTHFR. It is required by methionine synthase as a co-substrate (Castro *et al.*, 2006; Fig1.13 reaction 9 &10).

1.7.2.4. The role of enzymes in homocysteine metabolism

Three key enzymes play important roles in the metabolism of homocysteine:

Cystathionine β-Synthase (CβS)

Homocysteine can be converted to cysteine in the transsulfuration pathway, which requires the enzyme cystathionine- β-synthase (CβS). CβS requires vitamin B6 (pyridoxal 5-phosphate) as coenzymes (Finkelstein and Martin, 2000).

Methionine Synthase (MS)

Homocysteine binds with methyl group derived from 5- methyl-tetrahydrofolate (methyl donor) to become methionine. This reaction is catalysed by methionine synthase (MS), which requires vitamin B12 as coenzymes (Olteanu *et al*., 2001).

Methyltetrahydrofolate reductase (MTHFR).

The enzyme 5, 10 methylenetatrahdrofolate reductase (MTHFR) catalyses the conversion of 5, 10 methylenetatrahdrofolate into 5-methyl tetrahydrofolate. This is the major circulating form of folate. Folate in turn, is used in many biochemical pathways, including the methylation of homocysteine to methionine. Mutations in the MTHFR gene can be associated in reduction in the enzyme activity and can result hyperhomocysteinemia that can precipitate coronary artery disease CAD (Kluijmans *et al.*, 1997).

1.7.3. Forms of the homocysteine in plasma

In plasma, homocysteine was found in two forms (i) free homocysteine (it has a free sulfhydryl group) is present in trace amount (< 0.3 nmol/l, 1%). It is unstable and rapidly oxidized at neutral pH 7.3. (ii) The bound forms: are present in three forms: (a) Homocysteine (homocysteine-homocysteine). It is the second compound found in the plasma and is composed of two molecules linked together by a disulfide bridge. (b) Homocysteine binds with a cysteine molecule to form another large oxidized molecule (cysteine-homocysteine) which accounts for around 20-30% of total homocysteine in the plasma. (c) A large amount of the homocysteine in plasma binds covalently with a cysteine residue in protein especially those found in albumin which represents about 70%-80% of the total homocysteine (Ueland *et al.*, 1993).

The total homocysteine in the plasma is calculated of the sum of free and bound homocysteine and its abbreviated 'tHcy'(Hankey and Eikelboom, 1999; Perry, 1999).

1.7.4. Hyperhomocysteinemia

Elevated levels of homocysteine in the plasma above the normal level (5-15 µmol/l) is a considered clinically a pathological state known as hyperhomocysteinemia (Dudman *et al*., 1993). This elevation occurs due to defect in one of the enzymes in homocysteine metabolism or a deficiency in the vitamins which it contributes as cofactors to these enzymes in the homocysteine metabolism. According to this defects, hyperhomocysteinemia has been classified into three statuses depending on the level of homocysteine in plasma (i) mild (16-30 µmol) this occur due to deficiency of the vitamins (ii) moderate (31-100 µmol) due to genetic polymorphism of the methionine synthase enzyme (iii) severe (>100 µmol) condition of hyperhomocysteinemia. It happens in case of CβS deficiency or MTHFR deficiency (Mangoni and Jackson, 2002; Perry, 1999; Table 1.2).

Table 1.2 Classification of hyperhomocysteinemia.

Condition	Plasma total homocysteine	Aetiology
Severe	>100 mmol/l	Cystathionine-β- synthase deficiency Methylenetetrahydrofolate reductase deficiency Nutritional deficiencies ± minor genetic defect
Moderate	31 - 100 mmol/l	methionine synthase deficiency Compound heterozygosity of MTHFR Interallelic combination of genetic defects Nutritional deficiencies ± minor genetic defect
Mild	16 - 30 mmol/l	Interallelic combination of genetic defects Minor genetic defects Nutritional deficiencies ± minor genetic defect

Several factors participate in the elevation levels of homocysteine in plasma, which are summarized in either pathological or physiological condition.

1.7.5. Physiological Factors

1.7.5.1. Age and sex

Several authors have mentioned that homocysteine levels increases with age and found that men have a higher level of homocysteine compared with women (Dierkes *et al*, 2001; Hockly and Brown, 1999; Stein and McBride, 1998). Perry reported higher homocysteine in men by about 25% than premenopausal women (Perry, 1999). Previous studies showed that mean fasting homocysteine level in elderly patients (mean age 79±9 years) were 18.18 ±13.22

µmol/l) for men and 15.86 ± 12.14 µmol/l for women. The study concluded that the homocysteine levels in men were higher than in women (Ventura *et al.,* 2001). Other studies reported similar findings (Powers *et al.,* 2002).

1.7.5.2. Pregnancy

Normal levels of the homocysteine in non-pregnant women of childbearing age are 5.8-12.8 µmol/l (Vilaseca *et al.,* 1997). In pregnant women, lower levels of homocysteine (60% less than normal levels) were reported (Perry, (1999). Increased activity of the enzyme betaine homocysteine methyltransferase was shown to be associated with pregnancy, protecting pregnant women from developing hyperhomocysteinemia (Kang *et al.,* 1987). Several other factors might contribute to the reduced levels of homocysteine associated with pregnancy that includes among others: increase in estrogen levels, a physiological response to the pregnancy, increasing the need for methionine by the mother and the fetus and dilution of blood from increased plasma volume (de la Calle *et al.,* 2003). On the other hand, postmenopausal women have comparatively higher levels of homocysteine (11.5 µmol/l) than premenopausal women (10.7µmol/l), these findings support the hypothesis that estrogen reduces homocysteine levels (Hak *et al.,* 2000) by stimulating cystathionine β-synthase (CβS) (Dimitrova *et al.,* 2002).

1.7.5.3. Dietary factors

McCully recommended consumption of food that contains adequate amounts of vitamin B6, vitamin B12 and folic acid. It is recommended to consume sufficient quantities of fresh vegetables, fruits, whole grains and legumes, and small amount of the fresh meat and dairy products, as well as very little amount of fats and sugars. These vitamins lead to reduction the accumulation of homocysteine in the blood and tissues (McCully, 1998).

Table 1.3: allele frequencies of MTHFR C677T mutation and homocysteine and folate levels among different ethnic group and gender

Race and Gender	**Main finding**	**Reference**
12% of the Caucasian	Caring TT genotype of C667T MTHFR gene	Kauwell *et al.*, 2000
African-Americans	very low prevalence of this TT genotype	Kauwell *et al.*, 2000
black in South Africa	homocysteine was lower (11± 3.6 µmol/L)	Ubbink *et al.*, 1995)
white in South Africa	homocysteine was higher (18 ± 6.2 µmol/L)	Ubbink *et al.*, 1995)
premenopausal Black females	homocysteine was higher (8.32 µmol/L)	(Gerhard et al., 1999)
premenopausal whites females	homocysteine was lower (7.6 µmol/L)	(Gerhard et al., 1999)
premenopausal whites females	folate was higher (9.88 nmol/L)	(Gerhard et al., 1999)
premenopausal Black females	folate was lower (6.2 nmol/L)	(Gerhard et al., 1999)
Men in different population	Have high homocysteine levels 25% than women	(Cappuccio et al., 2002)
women in different population	Have low homocysteine levels 25% than men	(Cappuccio et al., 2002)
South Asian	Have high homocysteine levels	(Cappuccio et al., 2002)
whites	Have low homocysteine levels	(Cappuccio et al., 2002)
vegetarians and Hindus	Have high homocysteine levels	(Cappuccio et al., 2002)
non-vegetarians and other groups	Have low homocysteine levels	(Cappuccio et al., 2002)
Muslims and Whites	No variation of homocysteine levels	(Cappuccio et al., 2002)
In Whites	alleles frequencies of TT in MTHFR gene was 0.1	(Cappuccio et al., 2002)
In African origin	alleles frequencies of TT in MTHFR gene was 0.01	(Cappuccio et al., 2002)
in South Asians	alleles frequencies of TT in MTHFR gene was 0.02	(Cappuccio et al., 2002)
in White Italians in Burkina Faso	High prevalence of coronary heart disease / high homocysteine levels	(Simpore *et al.*, 2002)
Blacks population in Burkina Faso	low prevalence of coronary heart disease / low homocysteine levels	(Simpore *et al.*, 2002)

Table1.4: the role of pathological factors and vitamins B6 B12 and folate supplementation in homocysteine levels

Pathological factors	Main finding	Reference
Deficiency of vitamin B6, B12, folate	Hyperhomocysteinemia	(Samuels, 2003)
supplementation of vitamin B6, B12, folate	decrease homocysteine level in plasma	(Samuels, 2003)
daily requirement of vitamin B12 (0.5 mg)	optimize homocysteine level in plasma	(Clarke, 1998)
daily requirement of vitamin B6 (16.5 mg)	optimize homocysteine level in plasma	(Malinow *et al,* 1999).
daily requirement of folate (0.5 -5.0 mg)	optimize homocysteine level in plasma	(Malinow et al, 1999).
intake of vitamins B6, B12, folate	30% reduced in homocysteine levels in patients with recurrent venous thrombosis	(den Heijer *et al.*, 1998)
vitamins B6, B12 and Folate	Reduced homocysteine levels	(Marcucci *et al.,* 2005).
renal disease	Hyperhomocysteinemia	(Wilcken & Guptar., 1979)
85%-100% of end-stage renal disease(ESRD)	Hyperhomocysteinemia	(Robinson et al., 1996)
chronic renal failure	High concentration of homocysteine	(Kang et al., 2002).

Table1.5: association between life style, homocysteine and different vitamins levels

Life style	Main finding	Reference
coffee consumption	increased homocysteine levels in blood	(Hainaut *et al.*, 2002)
alcohol consumption	increased homocysteine levels in blood	(Carvo and Camilo, 2000)
smoking	increased homocysteine levels in blood	(O'Callaghan *et al.,* 2002)
alcohol abuse	severe hyperhomocysteinemia	(Carmel and James, 2002)
moderate consumption of red wine	Increase serum homocysteine levels	(Van der Gaag *et al.,* 2000)
moderate consumption of beer	No change in homocysteine levels	(Van der Gaag *et al.,* 2000)
alcohol administration in Rats	reduction in the methionine synthetase activity	(Barak *et al.,* 2002).
80% of alcoholic liver disease	Folic acid deficiency	(Halsted et al., 2002).
alcholics	High levels of homocysteine / brain shrinkage	(Bleich *et al.,* 2003).
caffeine (methyl xanthine)	act as vitamin B6 antagonist	(Urgert *et al.,* 2000)
daily coffee consumption	increases homocysteine concentration	Urgert et al., 2000
polyphenol in coffee and Black tea	raises total homocysteine concentrations	(Olthef *et al.,* 2001).
Smoking	hyperhomocysteinemia and increased risk of vascular disease and	(O'Callaghan *et al.,* 2002)
the smokers	cardiovascular disease increased 12-fold with plasma homocysteine levels above 12 µmol/l	(O'Callaghan et al., 2002)
current smokers	low levels of vitamins (B6, B12 and folic acid)	(O'Callaghan et al., 2002)
Smokers	positive association between elevated levels of plasma homocysteine and cardiovascular	(Wilcken *et al.,* 2002)
in smokers	low levels of folate/ higher levels of homocysteine	(Christensen et al., 1999).

1.7.6. Homocysteine and vasculers and heart diseases

1.7.6.1. Pathogenic effect of homocysteine

Although hyperhomocysteinemia was linked clinically to atherosclerosis, the mechanism of pathology induced by homocysteine on blood vessels is unclear. Several mechanisms were suggested to elucidate the pathogenic effects of homocysteine and these include;

(1) Homocysteine causes endothelial dysfunction and damage: the endothelium plays an active role in regulating blood flow, coagulation reactions, platelet activation, leukocyte adhesion and vascular muscle function. These effects are mediated by a variety of endothelial cell molecules, which include nitric oxide, prostacyclin, plasminogen activator and thrombomodulin. Impaired endothelial function was observed in experimental models of hyperhomocysteinemia and also in humans with acute hyperhomocysteinemia induced by oral methionine loading. The development of endothelial dysfunction in hyperhomocysteinemic animals was found to correlate with the degree of elevation of plasma homocysteine suggesting that the homocysteine is responsible for endothelial dysfunction (Lentz, 2005). (2) Acceleration of thrombin formation (Rodgers and Kane, 1986) by increasing the activity of the coagulation factors XII and V (Matthias *et al.*, 1996), this is in addition to the reducing activity of Protein C and thrombomodulin (Thambyrajah and Townend, 2000). Hyperhomocysteinemia may also predispose to thrombosis via upregulation of tissue factor on endothelium or monocytes or hyperactivation of platelets (Lentz, 2005), (3) Induction of vascular smooth muscle proliferation (Upchurch *et al.,* 1997), (4) Migration of monocytes (Upchurch *et al.,* 1997), (5) Increase of lipid peroxidation and oxidized low-density lipoprotein cholesterol (Thambyrajah and Townend, 2000) and (6) Inhibit thrombolysis by decreasing the effectiveness of tissue plasminogen activator (tPA) (Nappo *et al.,* 1999).

1.7.6.2. Coronary Heart Disease

Elevation of plasma homocysteine in cardiovascular disease in humans was reported from Australia in 1976 (Wilcken and Wilcken 1976). In that study, methionine metabolism was studied in 25 patients and 22 control subjects. Results suggested reduced ability to metabolize homocysteine in some patients with coronary artery disease (CAD). Nine years later, two studies investigated homocysteine metabolism in patients with CAD. The first study found that men with abnormal methionine intolerance had 7-fold increased risk for CAD (Murphy-Chutorian *et al.,* 1985). In the second study, the authors measured total plasma homocysteine levels in patients with CAD and in controls. The study showed a plasma homocysteine level of 5.41±1.62 nmol/ml in male patients, 4.37±1.09 nmol/ml in male healthy controls, 5.66±1.9 nmol/ml in female patients with CAD and 4.16 ± 1.62 nmol/ml in female healthy controls (Kang *et al.,* 1986). During the 1990's several studies were performed on the role of homocysteine in CAD. Clarke et al detected hyperhomocysteinemia in 30% of coronary vascular disease patients, and they showed that hyperhomocysteinemia is an independent risk

factor for CAD (Clarke *et al.,* 1991). Genest *et al.,* 1990 found higher homocysteine concentrations in patients with premature coronary artery disease than did control subjects, and they concluded that elevated homocysteine level is an independent risk factor for development of early CAD in men. Similar findings were reported by Finish (Sadeghian *et al.,* 2006) and Japanese research groups (Ogawa et al., 2003). Graham et al. showed that elevated homocysteine level is an independent risk of vascular disease similar to smoking or hyperlipidaemia (Graham *et al.,* 1997). In addition to the elevated homocysteine levels, low levels of folic acid were identified as risk factors for myocardial infarction (Christensen *et al.,* 1999). A limited number of studies on homocysteine came from the Arab world. A study from Syria showed that 61% of patients with CAD and 44% of controls were hyperhomocysteinemic (Hermann et al., 2003). Similar data was reported from an Egyptian population (El-Sammak et al., 2004). In addition to the above, large-sized studies from the US, Norway, France and UK investigated the association between elevated levels of plasma homocysteine and CAD. The US study concluded that moderately elevated levels of plasma homocysteine are associated with future risk of and myocardial infarction (MI), independent of other risk factors (Stampfer *et al.,* 1992), while the study from Norway concluded that serum homocysteine is an independent risk factor for CAD in the general population (Arnesen *et al.,* 1995). On the other hand, the British study showed that elevated serum homocysteine levels independently predict the risk for development of CAD in adults (Vasan et al., 2003), while the study from France concluded that elevated levels of homocysteine in healthy postmenipausal women increased the risk of future cardiac diseases (Ridker et al., 1999). Boushey et al (1995) performed a meta-analysis of 27 studies relating homocysteine to arteriosclerotic vascular disease. The conclusion of the meta-analysis was that elevated homocysteine levels were independent risk factors for arteriosclerotic vascular diseases. In addition the study also showed that high folic acid intake (200µg/d) was associated reduction in homocysteine levels and higher folic acid intake (that reduces homocysteine levels) can play a role in preventing development of arteriosclerotic vascular disease (Boushey et al., 1995). In Sudanese adults, homocysteine levels were higher with statistical signficance in patients with coronary heart disease (17.64; SD 11.68) and also, in children with protein-energy malnutrition (8.41; SD 1.61), as well as higher with statistical signficance in patients with recurrent venous thrombosis (5.06; SD 10.55) and recurrent malaria (13.61; SD 4.82) (Abdel, *et al.,* 2009).

1.7.7. Homocysteine and T2DM and its complication

Meigs *et al.* (2001) reported that there might be a link between increase levels of homocysteine and insulin resistance (Meigs *et al.,* 2001). Abu-Amero *et al.* (2003) found that homocysteine levels are increased in patients with the T2DM and metabolic syndrome (Abu-Amero *et al.,* 2003). In addition, in vitro studies observed that insulin receptor tyrosine kinase activity, phosphorylation of phosphatidylinositol 3-kinase (PI3K) and glycogen synthase kinase-3 (GSK-3) are inhibited by homocysteine thiolactone in rat HTC-IR hepatoma cells (Najib & Sanchez-Margalet, 2005), which in turn leads to inhibition of the glycogen synthase enzyme (Liu *et al.,* 2011). It has been shown that increase in the expression of phosphoenolpyruvate carboxykinase (PEPCK) gene and glucose output from the liver are associated with elevated levels of homocysteine (Yu *et al.,* 2009). Hoogeveen *et al.* (1998) found that hyperhomocysteinemia as a risk factor for cardiovascular disease in patients with T2DM but not in non-diabetic subjects (Hoogeveen *et al.,* 1998). A 5 years follow-up study reported that hyperhomocysteinemia is an independent major risk factor for mortality in patients with both cardiovascular and T2DM (Hoogeveen *et al.,* 1998). Hyperglycemia of diabetes was shown to be associated with long-term damage, dysfunction of the various organs includes eyes, kidneys, and artery, (Shera *et al.,* 2007). Diabetic retinopathy (DR) is one of the main complications of diabetes mellitus. Although several studies didn't report any association between hyperhomocysteinemia and diabetic retinopathy, recent studies showed a strong association between them (Satyanarayana *et al.,* 2011). A possible association between vitamin B12 deficiency and hyperhomocysteinemia in DR was suggested (Satyanarayana *et al.,* 2011). Homocysteine was significantly elevated in diabetic nephropathy more than normoalbuminuric patients or control (Mtiraoui *et al.,* 2007). Friedman *et al.* showed no association between the proteinuria and increased level of homocysteine in patients with type 2 diabetic nephropathy (Friedman *et al.,* 2002). In general, elevated homocysteine level can be contributed in the pathogenesis of several diseases; these include stroke, diabetes and its complications, cardiovascular disease CVD, coronary heart diseases CHD and ischemic heart disease (IHD) (Klerk *et al.,* 2002).

1.7.8. Genetic mutations in MTHFR gene and T2DM and its complication

MTHFR is an important enzyme in methyl cycle and it catalyzes the conversion of 5, 10 methyltetrahydrofolate into 5-methyltetrahydrofolate which participates in the vitamin-B12-

dependent-remethylation of homocysteine to methionine (Strain *et al.,* 2004). The MTHFR gene was found in the chromosome 1 (Goyette *et al.,* 1995). Suppression of the enzyme MTHFR activity was attributed to either presence of a genetic defect, in the gene encoding MTHFR enzyme, or deficiencies of the enzyme itself, which leads to increase level of homocysteine in the blood (Kluijtmans *et al.,* 1996). The activity of enzyme MTHFR was shown to be affected by presence of two genetic polymorphism, C677T and A1298 C, in MTHFR gene (Castro *et al.,* 2003). The most common MTHFR is a C to T transition at nucleotide 677 resulting in an alanine to valine substitution with 50% less activity (Engbersen *et al.,* 1995). The A 1298 C MTHFR variant is an A to C transversion at nucleotide 1298 resulting in a glutamate to alanine substitution, and the enzyme activity is decreased to 40% of the wild type enzyme (Weisberg *et al,* 1998). Increased plasma homocysteine levels in human have been associated with the presence of a common C677T mutation in the MTHFR gene, which reduced MTHFR activity (Frost et *al,* 1995).

There is a difference in frequency of the two mutations in the MTHFR gene (C677T and A1298C) among different geographical regions and ethnic populations (Esfahani *et al.,* 2003; Yang *et al.,* 2016). The prevalence of the C677T mutation polymorphism in the European populations from Norway, Ireland, Britain and Italy are was 22%, 32%, 37%, and 44%, respectively (Botto and Yang, 2000), while it ranged between 34% to 37% among whites from Australia, Brazil, Canada, and the United States (Botto & Yang., 2000). The prevalence of this polymorphism in other ethnic groups was 78%, 45.2%, 25.6%, and 44.4% in Mexicans, Hungarians, Thais, and Greek-Cypriots, respectively (Abu-Amero *et al.,* 2003). The prevalence of the C677T polymorphism is higher in Japan (34%) compared with other Asian countries (Botto & Yang., 2000). The frequency is 9% in two studies from Canada (Weisberg et al, 1998) and the Netherlands (Van der Put *et al.,* 1998). Falik-Zaccai et al mentioned that the prevalence of CVD among the Muslim Circassians, which they have been lived in Israel, were not related to the mutation C677T MTHFR gene or mutations in prothrombin gene and factor V gene (Falik-Zaccai *et al.,* 2003). Among healthy Blood donor group tested for C677T variant, the heterozygous C677T prevalence was 25.8%, comparable to the prevalence rates observed in other ethnic groups (Abu-Amero *et al,* 2003). The genetic polymorphisms of the MTHFR gene, C677T and A1298C, were associated with reduced enzyme activity, decrease production of active folate and consequently elevate the blood levels of homocysteine (Weisberg *et al,* 1998).

Increased levels of plasma homocysteine have been considered as an independent factor of CVD (Refsum *et al.,* 1998). In spite of the elevated level of homocysteine has been

associated with atherosclerosis, but there is no clear mechanism to explain this association. However, there are several suggestions to elucidate homocysteine causes pathology in blood vessels. Lentz reported that homocysteine is an independent factor that causes damage and dysfunction in endothelial cells of the blood vessels. The blood flow, platelet activation, coagulation reactions and vascular muscle function are regulated by the endothelium. So any defect in the endothelium can lead to atherosclerosis. Experimental models demonstrated how homocysteine causes impairment in the endothelial cells after oral methionine loading given to animals. It was found that there is a correlation between development of endothelial dysfunction and the degree of elevation of plasma total homocysteine in these animals (Lentz, 2005), Others reported that homocysteine decreases the concentration of HDL-cholesterol in plasma by suppressing the hepatic production of apoA1, the main apoprotein in HDL (Barter and Rye., 2006). Several studies have investigated the association between folic acid supplementation and cardiovascular disease. Li et al mentioned that folic acid supplementation may be effective for CVD prevention in patients with kidney disease, particularly in patients with end-stage renal disease (ESRD) or Advances in Chronic Kidney Disease (ACKD). In addition, they found that folic acid supplementation led to reduced (10%) risk of stroke, 4% lower risk of overall Cardiovascular disease (CVD) (Qin *et al.,* 2013; Li, *et al.,* 2016 & Zeng, *et al.,* 2014). In this meta-analysis study, MTHFR 1298C allele was considered a risk factor for coronary heart disease (CHD), but the authors suggested that further studies are needed to investigate the correlation of plasma homocysteine levels, enzyme activity, and folic acid supplementation with the risk of CHD (Yu, *et al.,* 2017).

To our knowledge, a few studies have been conducted in Africa and no reports are available on the prevalence of the C677T and A1298C MTHFR gene polymorphisms among Sudanese.

1.7.9. Homocysteine and resistin

Homocysteine is an amino acid (containing sulphur) which is formed during methionine metabolism. Elevated levels of homocysteine were proposed as a risk factor for coronary artery disease (Clarke *et al.,* 1991). It is a potent proinflammatory factor that was shown to promote inflammation in both *in vitro* and *in vivo* models (Wang *et al.,* 2000 and Wang *et al.,* 2002). Studies showed that elevated homocysteine can be associated with insulin resistance (Meigs et al., 2001 and Oron-Herman et al., 2003), this is in addition to an association between homocyteine and insulin levels in healthy subjects as well as in diabetic and obese subjects (Fonseca et al., 2003; Emoto et al., 2001 and Masaki et al., 2007). In *in vitro* models, homocysteine thiolactone was shown to induce oxidative stress that can lead to impaired

insulin signalling (Najib and Sanchez-Margalet, 2001; Najib and Sanchez-Margalet , 2005). However, whether Hcy can promote insulin resistance by directly regulating the expression and secretion of resistin— one of the adipokines that may link inflammation and obesity to insulin resistance. In a study using a murine model, homocysteine was shown to induce resistin expression in cultured adipocytes and to impair glucose transport (Steppan et al., 2001). In obese children, they found that Resistin might be an independent factor contributing to cardiovascular disease (Codoñer-Franch, et al., 2014). Esbah et al suggested that smoking might contribute to the elevated levels of resistin levels, and resistin led to insulin resistance in smokers. While, the BMI has no role in resistin levels in smokers or non-smokers and resistin. In addition, Resistin might not have a role in C-reactive protein, homocysteine and uric acid levels both in smokers and non-smokers (Esbah, et al., 2011).

1.8. Vitamin D

1.8.1. Vitamin D forms and sources

Vitamin D is a generic term that includes more than one type that includes vitamin D3 and vitamin D2. Vitamin D3 (cholecalciferol), the main type of Vitamin D, is derived from 7-dehydrocholesterol after exposure to ultraviolet light (Lips, 2006; Fig1.14).

The formation of vitamin D3 is affected by many factors among them is skin pigmentation (webb *et al.,* 1989), clothing, sunscreen and UV intensity (Holick, 1995). In addition to sunlight, Vitamin D3 can be obtained from food items which include: fish or their liver oils, milk, egg yolk and other sources (Mathieu *et al.,* 2005). On the other hand, Vitamin D2 (ergocalciferol) is obtained from plant sources (plants and plant materials) (Galea and Blundell, 2011; Fig1.14). Only 30% of vitamin D is obtained from the diet while the majority is obtained via the skin after exposed it to the U V- sun light. Chemically, Vitamin D2 differ from vitamin D3 in having a double bond between C22 and C23 in addition to a CH3 in C24 (Bikle, 2014). These differences are responsible for decreased affinity of Vitamin D2 for vitamin D binding protein **(**Galea and Blundell, 2011**)**, increased clearance from the blood, and lowered conversion to 25-vitamin D2 (25-(OH) D2) **(**Houghton and Vieth, 2006**)**.

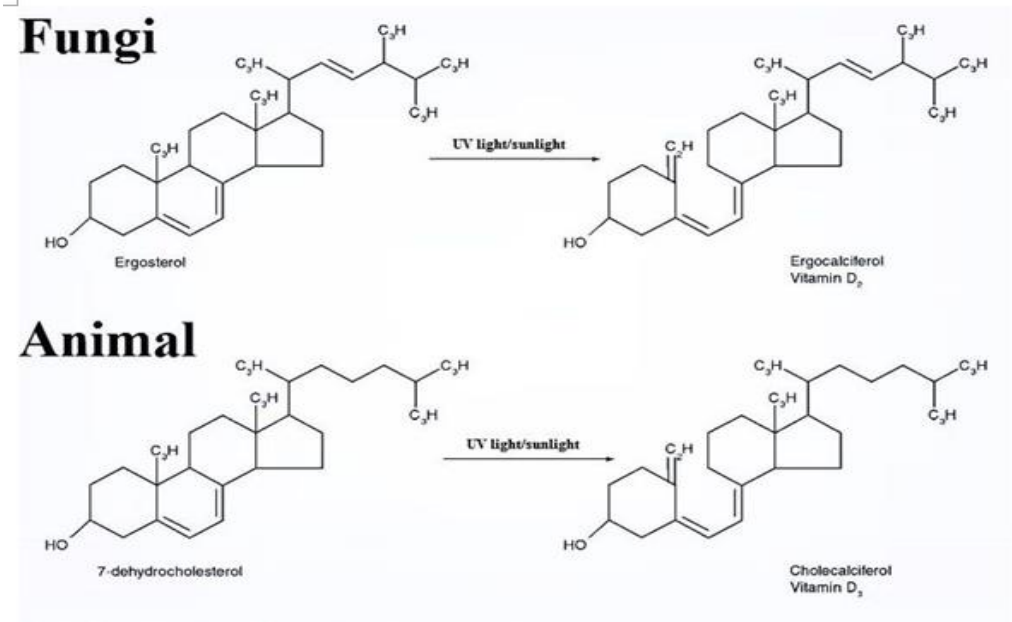


Fig 1.14 Forms of vitamin

1.8.2. Biological functions of Vitamin D and its Metabolism

The main role of vitamin D is to facilitate the absorption of calcium and phosphate from the gut and increases calcium reabsorption by the kidney (Deluca and Cantorna, 2001). Accordingly, the main function of vitamin D is to keep plasma calcium and phosphate within normal levels and bone mineralization (Zittermann, 2003).

For Vitamin D3 to get activated to 1, 25 (OH) 2 D3 (the active form) two hydroxylation steps are needed. In the liver, the initial hydroxylation takes place at carbon number 25 in vitamin D3 to produce 25-(OH) vitamin D3 (catalysed by 25-hydroxylase (CYP27A1) enzyme). Subsequently 25-(OH) D3 is transported to the proximal tubules in the kidney where a 2nd hydroxylation takes place at 1-carbon on 25-(OH) D3 to produce the active form (1, 25-(OH)2 vitamin D3) (1,25-(OH)2D3) (catalysed by 1-hydroxylase enzyme (CYP27B1) (Passeri *et al.,* 2008). Vitamin D2 is metabolized in the same manner. Several factors are involved in regulating the levels of the active vitamin D3 (1, 25 (OH) 2D3), including parathyroid hormone and directly via negative feedback inhibition. 1, 25(OH) 2D3 and 25-vitamin D3 are degraded by the mitochondrial enzyme, 24-hydroxylase or CYP24A1, which is also involved in regulation of the levels of 1, 25(OH) 2D3 and 25-vitamin D3 (Tanaka *et al.,* 1977). For the active form of vitamin D to do its biological function it has to bind to vitamin D receptor (VDR). Upon binding and signalling, this result in activation of more than 200 genes, among them are genes for proteins involved in facilitating absorption of calcium

and phosphate from the intestine to blood circulation (Bronner and Pansu, 1999; Deluca and Cantorna, 2001; Fig 1.15).

The extra-renal production of the active form of vitamin D (1, 25 dihydroxyvitamin D) seems important for paracrine regulation of cell differentiation and function (Peterlik and Cross, 2005). Vitamin D deficiency plays an important role in the pathogenesis of autoimmune disease and exposure of children aged 6-15 to sunlight prevents them from developing multiple sclerosis. Chiu et al, 2004 found that vitamin D affects the function of

β-cells of the pancrease and serum 25(OH) D were positively associated with insulin sensitivity to the target tissues (Chiu *et al.*, 2004; Fig 1.15).

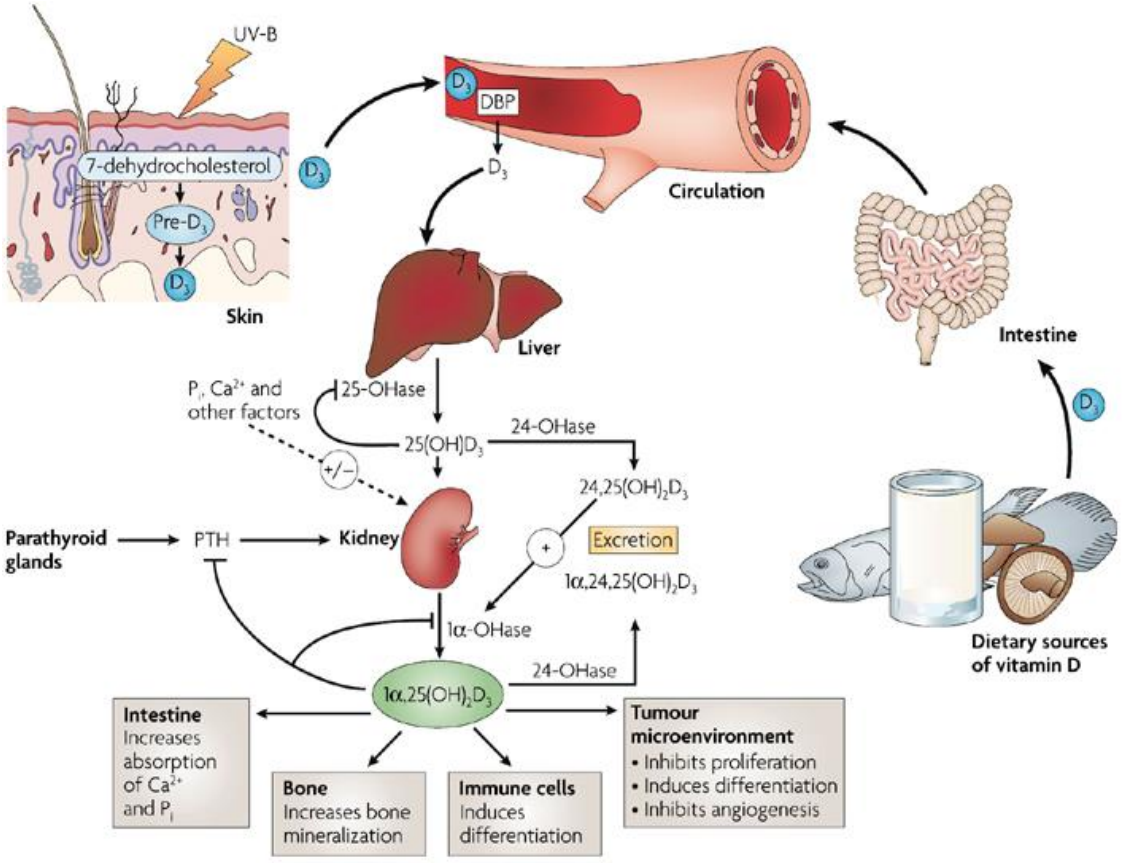


Fig 1.15 Sources and action of the active form of vitamin D

Sources of vitamin D3 (ultraviolet light UV and diet), transported in the circulation (by vitamin D binding protein DBP), formation of 25-hydroxyvitamin D3 in the liver (activated by 25-hydroxylase), formation of the active form of vitamin D (1,25 dihydroxyvitamin D3) in kidney (activated by 1-hydroxylase), metabolic activation form have , classical role (increasing calcium and phosphate level in plasma) and non-classical role of vitamin D3 (molecular action of the activeform). The diagram is reproduced from Hewison et al. (2004).

1.8.3. Vitamin D, β-cell function and T2DM

Vitamin D deficiency is a global problem, affecting more than one million people worldwide (Cavalier *et al.,* 2011). Vitamin D Receptor (VDR) was shown to be expressed on β-cells of the pancreas, and a role for vitamin D in insulin secretion was suggested (Johnson *et al.,* 1994). Several *in vivo* (in animal models) and *in vitro* studies showed that vitamin D is essential for normal insulin release and for glucose Homeostasis. Furthermore, Vitamin D deficiency was shown to contribute to impairment in glucose homeostasis and decreased insulin secretion (Nyomba *et al.,* 1984; Alvarez and Ashraf, 2010); Norman *et al.,* 1980).

A link between deficiency of vitamin D and type 1 Diabetes (T1D) was extensively studied (Mathieu *et al.,* 2005). Helper T (Th) 1 cell (Th1) secrete many cytokines includes, interferon-γ (IFN-γ), interleukin-2 (IL-2), and tumour necrosis factor-α. These cytokines participate in cell-mediated immune responses including host responses to tumors and intracellular pathogens (such as viruses). In autoimmune diseases, Th1 cells play an important role in pathogenesis of autoimmune diseases, like multiple sclerosis and T1DM (Cantorna and Mahon, 2004). It was found that vitamin D is involved in downregulating Th1 responses and suppressing the antigen-presenting capacity of macrophages (Lips, 2006). It was suggested that vitamin D supplementation can play an important role in preventing type 1 diabetes (Stene *et al.,* 2000; Gregori *et al.,* 2002; Mathieu *et al.,* 2005). On the other hand, subgroup analysis revealed that the effects of vitamin D supplementation on different glycemic measures were influenced by age, calcium coadministration, vitamin D deficiency, serum 25(OH)D level after supplementation, and duration of supplementation. Vitamin D supplementation and improved vitamin D status improved glycemic measures and insulin sensitivity and may be useful as part of a preventive strategy for T2DM. (Mirhosseini, *et al.,* 2018). Insulin secretion was shown to be decreased as a result of deficiency of vitamin D, and vitamin D supplementation could improve β-cell function and glucose tolerance (Boucher *et al.,* 1995). In addition, it was shown that vitamin D deficiency was associated with increased risk of T2DM and supplementation of vitamin D and calcium might have a beneficial effect in preventing T2DM (Pittas *et al.,* 2007A). Vitamin D might affect glucose metabolism in two ways: either directly by increasing insulin receptor expression therefore stimulating insulin response to glucose (Boucher et al., 1995), or indirectly by affecting calcium influx to ensure adequate calcium amount in the cell cytoplasm. In the tissues that expresses insulin receptors (skeletal muscles and adipose tissues) Ca^{+2} acts as a 2^{nd} messenger for the insulin signal inside the cell (Pittas *et al.,* 2007B).

It is generally believed that T2DM is associated with systemic inflammation which might play a role in insulin resistance (Hu *et al.,* 2004). It was suggested also that elevated levels of cytokines might have a role in the β-cell dysfunction by enhancing beta cell apoptosis. Vitamin D might have a fundamental role in improving insulin sensitivity to its receptor and enhance β-cell survival (Pittas, 2007A). Kedari and Hareesh (2014) have reported that there is substantial evidence to indicate that deficiency of vitamin D is associated with progression of T2DM and also, they suggested that there was an inverse association between blood glucose and insulin resistance and Vitamin D levels. Furthermore, it found that genetic polymorphism (allelic variations) in the vitamin D receptor (VDR) and Vitamin D-binding protein (DBP) might influence insulin secretion and glucose homeostasis (Ortlepp *et al.,* 2003), and therefore participating to genetic risk for T2DM. Accordingly, a possible role for vitamin D in regulation of insulin receptor gene expression and insulin secretion was suggested. In addition, a fundamental role for VDR in the pathogenesis and development of T2DM was also suggested. In beta cells, genetic polymorphisms in a VDR gene inhibit its association with an active form of vitamin D, leading to the decrease in insulin secretion and subsequent T2DM (Ortlepp *et al., 2*003).

1.8.4. Vitamin D receptor gene and type 2 diabetes Mellitus

Measurement of the vitamin D levels and its relation to T2DM development does not give a complete picture of vitamin D and its role in diseases. Sometimes individuals with normal or high levels of vitamin D may have suboptimal biological functions associated with vitamin D. Metabolising enzymes are needed for the formation of the biologically active form of vitamin D which binds with vitamin D receptor (VDR). Any defective of the metabolising enzymes might lead to failure of the body to produce the active form of the vitamin D. Furthermore, even when the active form of the vitamin D is produced normally, if there is a defect in the vitamin D receptor, the active form cannot bind with the receptor, thus leading to failure of function (DeLuca, 2004) as shown in Fig 1.16.

McCullough *et al* observed that the VDR gene have several allelic variations (McCullough *et al.,* 2007). Several polymorphisms in VDR gene were found to be associated with diabetes mellitus (Györffy *et al.,* 2002; Hitman *et al.,* 1998). Mayer et al (2018) found that decreased vitamin D levels were associated with reduction of insulin sensitivity, especially in those with GG genotype of rs2228570 VDR (Mayer *et al.,* 2018). Among VDR polymorphisms which are shown to be associated with diabetes are rs1544410 (BsmI), rs10735810 (Fok1).rs731236 (TaqI) and rs7975232 (ApaI). The single nucleotide polymorphisms (SNP) for Taq1 and

Bsml were T 65058 C and G 63980 A, respectively. Several studies showed associations between the two polymorphisms rs731236 and rs7975232 of VDR gene and elevated levels of fasting blood glucose (Li *et al.,* 2013), while others found no associations (Ye *et al.,* 2001; Malecki *et al.,* 2003; Cyganek *et al.,* 2006).

1.8.5. Vitamin D and resistin

Vitamin D deficiency has been shown to contribute to the development of T2DM (Bland *et al.,* 2004). Vitamin D may have similar fuction to resistin on insulin secretion, insulin action or both (van Etten, 2005), and also it was shown that viamin D inhibits production of several cytokines in in vitro studies (Khoo *et al.,* 2011). Furthermore, vitamin D was shown to be protective against several diseases like cancer and cardiovascular disease and autoimmune disease (Dusso *et al.,* 2005). The role of vitamin D deficiency in insulin resistance and the use of vitamin D in treatment of hyperglycaemia are still unclear. Resistin was shown to be a link between insulin resistances with obesity. Additionally, several studies reported a positive association between resisitin levels and pro-inflammation cytokines in diabetes (Osawa *et al.,* 2005). Vilarrasa *et al* reported no association between the levels of vitamin D and resistin. However, the findings showed a negative association between vitamin D and BMI waist and hip circumference (Vilarrasa *et al.,* 2010).

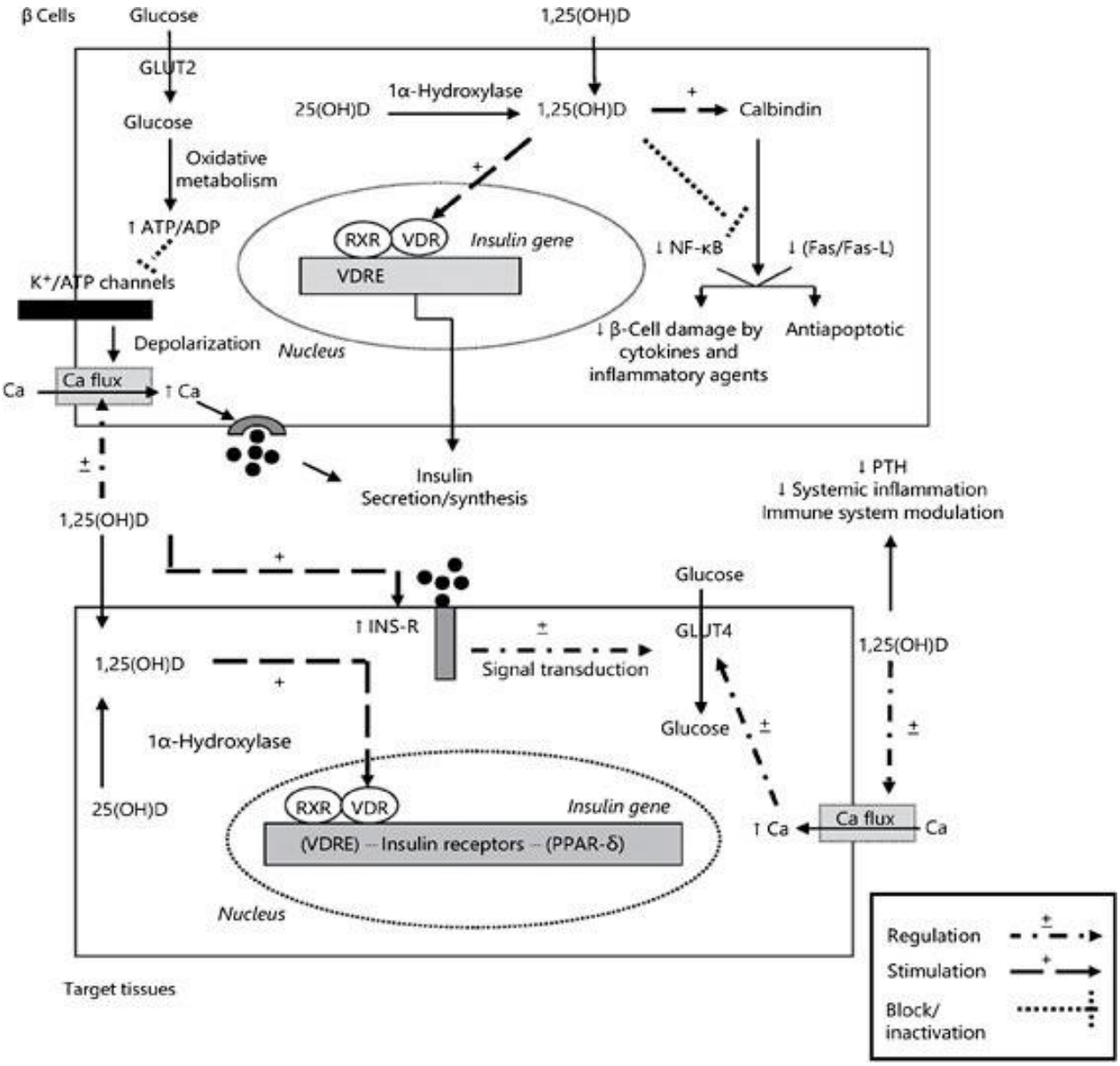


Fig 1.16 The role of vitamin D in the regulation of blood glucose and on β- Islet cells.
The active form of vitamin D (1, 25(OH) vitamin D) binds with VDR to activate several genes among them insulin gene which it led to increase insulin synthesis. The vitamin D regulates intracellular calcium levels by increasing ATP/ADP ratio, which in turn leads to insulin secretion and glucose tolerance. Insulin receptors and signalling transduction were produced after vitamin D activated to these genes, resulting translocation of GLUT4 to the membrane and glucose transport in peripheral tissues.

1.9. Rationale for the study

The role of homocysteine, resistin and vitamin D in type 2 diabetes mellitus (T2DM) has been studied in different populations and ethnic groups in Asia and Europe but limited numbers of studies have been conducted in African populations. To our knowledge, there is no reported study that has investigated the possible association of resistin, homocysteine and vitamin D with T2DM in Sudanese population. In addition, previous studies that have

investigated the role of vitamin D in T2DM were performed in populations from non-equatorial/tropical regions where sunlight exposure is seasonally variable and/or less intense. Thus, studying a Sudanese population, where sunlight intensity is less variable and more intense, provides the possibility of gaining a deeper insight into the role of vitamin D in the development of T2DM. The purpose of this study was to investigate the possible associations between levels of resistin and homocysteine, and levels of vitamin D with T2DM and in a representative population from Sudan.

1.10.General objectives

The primary aim of the current study is to investigate the association between levels homocysteine, resistin and vitamin D and T2DM in Sudanese study population. In addition, their associations with several demographic, clinical and metabolic parameters will be investigated.

1.11.Specific Objectives

a. To compare plasma levels of vitamin D (D2/D3), resistin and homocysteine levels between patients with T2DM and their healthy controls.
b. To investigate possible associations between resistin, Vitamin D and homocysteine levels with demographic, clinical and metabolic variables in female patients with T2DM compared to male patients with T2DM.
c. To compare plasma resistin and homocysteine levels between female and male patients with T2DM.
d. To compare plasma resistin levels between obese and non-obese subjects.
e. To investigate possible association between homocysteine, vitamin D levels and resistin in patients with T2DM.
f. To compare the prevalence of the MTHFR gene polymorphisms (C677T and A1298C) among patients with T2DM and their healthy controls.
g. To investigate possible associations between genetic polymorphisms of MTHFR gene (MTHFR C677T and A1298C) and plasma levels of homocysteine in patients with T2DM.
h. To investigate possible association between vitamin D receptor (VDR) gene variant T56058C and T2DM.

Chapter 2

2. Materials & Methods

2.1. Study design and population

A case-control hospital-based study was conducted with a study population of 395 Sudanese individuals: 200 patients with type 2 diabetes (T2DM) and 195 healthy controls. Ethical approval was obtained for the study from the Health Ministry in Khartoum (Sudan) and Middlesex University (UK). Written consent was obtained from each participant. The identities of the participants were anonymised. Confidentiality of each subject was assured by the research team members, and personal information of the participants was not shared with other parties.

2.1.1. Patient group

The patients had been previously diagnosed with type 2 diabetes (T2DM) and underwent regular follow-up at the diabetes clinic at the Military Hospital in Omdurman, Sudan. Although they all live in Omdurman, they belong to different tribes in Sudan. They were consecutively recruited from the diabetes clinic between September 2013 and December 2014. The patient group consisted of 200 participants (71 males and 129 females). Their age ranged between 37 and 70 years with a mean age of 52.3 ± 7.3 years and their mean of BMI was 27.2 ± 2.5 Kg/m^2. Patients with type 1 diabetes and those who did not give consent to participate in the study were excluded.

2.1.2. Control group

The participants in the control group were selected after confirmation they were not diabetic, are not known patients with chronic illness (especially heart and kidneys problems) with no family history of diabetes and all of them live in Omdurman, Sudan. The control group consisted of 195 individuals (130 males and 65 females). Their age ranged between 19 years and 82 years with a mean of 42.7 ± 14.4 years and their mean of BMI was 25.6 ± 3.9 Kg/m^2.

2.2. Blood sample collection, storage and plasma separation

Following 8-12 hours of fasting, blood samples of 3 ~ 4 ml were collected from the study population by venepuncture using disposable plastic syringes. Blood samples were dispensed

into tubes containing EDTA, heparin and fluoride oxalate, followed by separation of the plasma by centrifugation at 3000 x *g* for 10 minutes at room temperature. HbA1c was measured in whole blood, and part of the plasma was used to measure blood glucose, lipid profiles, urea and creatinine. For the analysis of resistin, homocysteine and vitamin D, part of the plasma was stored at -20 ^{O}C and later shipped from Sudan in a box containing dry ice to a laboratory at Middlesex University (UK).

2.3. Clinical history, base line parameters, anthropometric, and drug history

Baseline data recorded for the study participants included baseline parameters (Age, sex, Body Mass Index (BMI), Waist Circumference (W), Fasting Blood Glucose (FBG), 2-hour blood glucose (2hBG), Glycated Hemoglobin (HbA1c), cholesterol (CHOL), Triacylglycerol (TAG) , Low Density Lipoprotein (LDL), High Density Lipoprotein (HDL), Systolic Blood Pressure (SBP), Diastolic Blood Pressure(DBP) and anthropometric measurements (Body Weight and Height) and (BMI) (calculated as weight (kg) divided by height (m^2)). Subjects were regarded as normal, obese or overweight based on their BMI. Normal weight individuals have BMI between 18.5-24.9 (Kg/m^2), Obese have BMI > 30 (Kg/m^2) and overweight having a BMI ranging between 25-29.99 (Kg/m^2) (Flegal *et al.,* 2002: Read *et al.,* 2017). Data on medications were also collected including anti-diabetes drugs, Vitamin D, Folate, B12, Statins and Niacin. The data sheet used to collect the data is as in Appendix 1.

2.4. Study criteria and diabetes diagnosis

The diagnosis of diabetes was based on the definition of the American Diabetes Association and World Health Organization (WHO) (ADA, 2009; WHO, 1985). A doctor with special expertise in Diabetes was recruited during the sample collection period. An individual is considered to be diabetic when fasting blood sugar (FBG) level is ≥ 7.0 mmol/l (126 mg/dl) or had an oral glucose tolerance test (OGTT) samples taken within two hours of consuming 75g oral glucose dose) results of ≥ 11.1 mmol/l (200 mg/dl). In addition, HbA1c level ≥ 48 mmol/mol (≥ 6.5 %) is indicative of T2DM. Patients with type 1 diabetes and other types of diabetes were excluded from this study.

2.5. Quantification of resistin using sandwich ELISA assay

Manufacturer's instructions were followed to measure resistin in human plasma by using a Quantikine Human-Resistin Immunoassay (R&D system, Abingdon, Oxon, UK, Catalog Number SRSN00).

2.5.1. Reagents

The ELISA Kits were purchased from R&D Systems, Abingdon, UK. The assay kit contains: Resistin polystyrene microplate (96 wells coated with a mouse monoclonal antibody against human resistin); Resistin Conjugate (21 ml/vial of monoclonal antibody against Resistin Conjugated to horseradish peroxidase with preservatives), Assay diluent RD1-19 (11 ml/vial of a buffered protein base with preservatives), Resistin Standard (100 ng/vial of recombinant human resistin in a buffered protein base with preservatives; lyophilized), Calibrator Diluent RD5K (21 ml/vial of a buffered protein base with preservatives), Wash buffer concentrate (21 ml/vial of a 25-fold concentrated solution of buffered surfactant with preservatives), Colour Reagent A (12.5 ml/vial of stabilized hydrogen peroxide), Colour Reagent B (12.5 ml/vial of stabilized chromogen (tetramethylbenzidine) and Stop Solution (6 ml/vial of 2 N sulphuric acid). All reagents were stored at 2-8^{O}C. Before use, the reagents were left at room temperature for a period of half an hour for optimal conditions for the assay as recommended by the manufacturer.

2.5.2. Protocol for resistin analysis

Plasma samples were diluted 5-fold by adding 60 µl sample + 240 µl of the Diluent.

2.5.2.1. Reagent preparation

Concentrated wash buffer (20 ml) was diluted in 500 ml deionized water to prepare the working wash buffer solution. Substrate solution was prepared by mixing equal amounts of colour reagents A and B 15 minutes before use. 200 µl of the mixture was used per well. Resistin standard was reconstituted with1.0 ml of deionised water to prepare a 100 ng/ml stock solution. The Standard was incubated for 15 minutes and agitated prior to making serial dilutions. Seven tubes were prepared to be ready for dilutions. Calibrator Diluent RD5K (900 µl) was added into first tube containing resistin standard (100 ng/ml) to prepare a 10 ng/ml solution. Calibrator Diluent RD5K (500 µl) was added to the rest of the tubes. Taking 500µl from the first tube to prepare the next dilution, the following dilution series was

prepared: 10, 5, 2.5, 1.25, 0.62, 0.31, and 0.15 ng/ml, each tube was mixed thoroughly before the next transfer.

2.5.2.2. Procedure for the measurement of resistin

All reagents and samples were brought to room temperature before the start of the experiment. The 96 well microplates used were coated with monoclonal antibody specific for human resistin. All samples and standards were assayed in duplicates. A volume of 100 µl of assay Diluent RD 1-19 was added to each well before adding 100 µl of the standards and samples. The standards and samples of participants (patients and healthy individuals; controls) were added to separate wells and the 96-well microplates were covered with adhesive strips and incubated at room temperature for 2 h. After incubation, each well was washed four times with 400 µl wash buffer. At the final step, the plate was inverted and blotted against clean paper towels. Human Resistin conjugate (200 µl) was added to each well and covered by a new strip and incubated for 2 hours. After the incubation period, the same procedure in the previous step for washing was repeated. Substrate solution (200 µl; mixed in equal amounts of reagent A and reagent B) was added to each well and incubated for a further 30 minutes at room temperature. Finally, a stop solution (50 µl) was added to each well to stop the reaction and the colour changed from blue to yellow in all the wells.

2.5.2.3. Data analysis of the resistin results

Plasma samples for patients, controls and standards were tested in duplicate. The concentration of human resistin in each sample was calculated from the standard curve. additional informations are shown in appendix 2.

2.6. Measurement of Homocysteine using competitive EIA

Homocysteine concentrations in plasma were measured using Axis Homocysteine Enzyme immunoassay (EIA) (Axis-shield Diagnostics Ltd, Dundee, UK, Catalog Number FHC Y100). The experiment was carried out according to manufacturer's instructions.

2.6.1. Reagents

The homocysteine EIA Kits contained the following reagents:
Reagent A (assay buffer 45ml), Reagent B (adenosine/DTT 3.5ml), Reagent C (SAH hydrolase), Reagent D (Enzyme inhibitor 3.5ml), Reagent E (Adenosine deaminase 55ml), Reagent F (a-SAH antibody 25ml), Reagent G (enzyme conjugate 15ml), Reagent H

(substrate solution15ml), Reagent S (stop solution 20ml), washing buffer (phosphate buffer, merthiolate, Tween 20 , and BSA 60ml), CAL1-CAL (calibrators, S-adenosyl-L.homocysteine (2, 4, 8, 15, 30, 50 µmol/l) in buffer with preservative 6x1.5ml) and microtitre strips (coated with S-adenosyl-L.homocysteine 12x8 wells). Controls were provided in a separate kit containing control L, (7.0 µmol/l L.homocysteine in diluted serum samples of human origin and phosphate buffer); control M (12.5 µmol/l L.homocysteine in diluted serum samples of human origin and phosphate buffer); control H (25.0 µmol/l L.homocysteine in diluted serum samples of human origin and phosphate buffer).

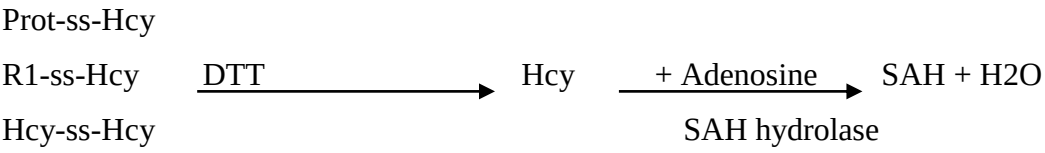
2.6.2. Principle of the homocysteine measurement

The level of total homocysteine in the plasma was determined by the EIA using the Axis-Shield Enzymatic Homocysteine assay. All Homocysteine forms such as that bound with protein or dimerised with another homocysteine, were reduced to free homocysteine and enzymatically converted to S-adenosyl-L-homocysteine (SAH) in a separate procedure (section 2.6.2.1) prior to the immunoassay.

2.6.2.1. Reduction process in samples and enzymatic conversion

Dithiothreitol (DTT) was added to the samples for reducing either mixed disulfide bonds (homocystine or other thiol compound homocysteine) or protein bound forms and

S-adenosyl- L-homocysteine was formed from homocysteine (Hcy) after addition SAH hydrolase and excess adenosine (Ad) in the test samples.



Prot: protein

R1 is any other thiol-residue

2.6.2.2. Competition assay for homocysteine

After reduction and enzymatic conversion, the samples were transferred to wells of an ELISA plate for the competition assay. The solid-phase enzyme immunoassay is based on competition between SAH in the samples and SAH bound to the walls of the ELISA plate for binding sites on a monoclonal anti-SAH antibody (capture). Unbound anti-SAH antibodies were washed and secondary rabbit anti-mouse antibody labelled with the enzyme horse

radishperoxidase (HRP) was added. The bound HRP activity was measured by the absorbance at 450 nm (FLUOstar Omega Microplate Reader (BMG Labtech, Aylesbury, UK) after the addition of enzyme substrate and is inversely related to the concentration of total homocysteine in the sample.

2.6.2.3. Preparation of the reagents and storage

All the kits were stored at 2-8^{O}C before use. All kit components were brought to room temperature and left over night before use. The samples, calibrators and controls were run in duplicate. Reagents A, B and C were mixed together to form sample pre-treatment solution, and this solution was valid for one hour after preparation. The wash buffer was diluted in ratio 1: 9 with deionized water before use. The diluted washing buffer was stable for 4 weeks when stored at room temperature (18-25^{O}C). Reagents D and H were stored in dark bottles to avoid exposure to light. The microtitre plate was kept dry in a sealed bag with drying capsules and stored in the refrigerator for at least 2 hours before use.

2.6.3. Protocol of homocysteine

2.6.3.1. Sample pre-treatment procedure

Reagents A, B and C were mixed together to form sample pre-treatment solution. This solution was valid for one hour after preparation. Volumes required per 10 samples (no dead volume calculated) are as follows: 4.5 ml from Reagent A, 0.25 ml from Reagent B and 0.25 ml from Reagent C (Mixing well using vortex).

Samples, calibrators and controls were diluted in glass tubes as follows:

Samples, calibrators and controls (25 µl) and sample pre-treatment solution (500 µl) were mixed well using vortex and covered by parafilm before incubating for 30 minutes at 37^{O}C. Reagent D (500 µl) was added to the tubes (containing samples, calibrators and controls and sample pre-treatment solution prepared in the previous steps). The solutions were mixed well and incubated for 15 minutes at room temperature, followed by the addition of reagent E (500 µl) and incubated for a further 5 minutes at room temperature.

2.6.3.2. Procedure for the measurement of homocysteine

After the sample pre-treatment was completed, diluted sample/control (25 µl) was added into the each well (coated with S-adenosyl-homocysteine), followed by an addition of reagent F (200 µl) to all the wells and incubated for 30 minutes at room temperature. The plate was

covered (with a lid provide with kits) for the incubation periods (30 minutes), then diluted washing buffer (350 µl) was added to each well for washing purposes. Subsequently, reagent G (100 µl) was added to each well and incubated for 20 minutes at room temperature. Each well was then washed with diluted washing buffer (350 µl) and after this, reagent H (100 µl) was added and the plate was incubated for a further 10 minutes at room temperature. Finally, reagent S (100 µl) was added and mixed into each well and the plate was read at 450 nm within 15 minutes using FLUOstar Omega Microplate Reader (BMG Labtech, Aylesbury, UK).

2.6.3.3. Data analysis of the homocysteine results

All samples, controls and calibrators were tested in duplicate. A calibration curve was drawn by plotting optical density against the concentration of the standards. The concentration of human homocysteine in each test sample was calculated by using the calibration curve. Two examples are shown in Appendix 3.

2.7. MTHFR C677T genotyping using real-time PCR

2.7.1. Focus 3M^{TM} integrated Cycler

Genotyping for MTHFR C677T polymorphism was performed using a real-time PCR with this instrument extraction of the genetic material (DNA or RNA) from the blood sample was not required, and the instrument has the ability to do two processes: real-time PCR amplification and fluorescence for detection of target. The instrument has the capability to use one of two types of disc for processing the samples and reagents: the Universal Disc and Direct Amplification Disc. A fluorescent signal is generated after separation of the fluorophore from the quencher as a result of the binding of a probe element to the extended DNA fragment synthesized during amplification. The instrument is controlled by an external computer running the Integrated Cycler Studio Software.

2.7.2. Amplification protocol of MTHFR C677T Direct

The PCR reactions contained master mix (4 µl), MTHFR C677T wild type primer (0.5µl), MTHFR C677T mutant type primer (0.5µl) (Oxford Biosystems Ltd, UK) and PCR grade water (3µl) (Life technologies, UK) and whole blood.

Blood (2 µl) diluted 1:4 in sterile PBS (30 µl PBS +10 µl whole blood). The 2.5 X Universal master Mix contains the enzyme DNA polymerase, buffers, deoxynucleoside triphosphates

(dNTP) and $Mgcl_2$. The bifunctional fluorescent probe-primers are used together with the reverse primers to amplify the MTHFR gene and the internal control. A fluorophore labels was incorporated for the gene targets: wild type (FAM 520) and mutant type (CFR 610).
The reagents and blood samples were pipetted into the appropriate test wells on the Universal Disc (96 wells). Briefly, 8 µl (master mix (4 µl), MTHFR 677 wild type primer (0.5), MTHFR 677 mutant type primer (0.5) and PCR grade water (3µl)) were pipetted into each test well in the universal disc, followed by added diluted blood sample (2µl) into appropriate test well. Finally, PCR grade water (2µl) was added into last test well instead of a blood sample to form the Negative control (NC).

The disc was covered with the adhesive disc cover and firmly smoothed with a blue plastic disc sealer to ensure the cover was secure. After placed the disc in the instrument and started running, the approximate run time was 55 minutes. PCR was performed under the following conditions: one cycle of 360 second at $97^{O}C$ with ramp rate $10^{O}C$/second followed by 40 cycles consisting of denaturation at $97^{O}C$ for 10 seconds at ramp rate $10^{O}C$/second, annealing at $60^{O}C$ for 30 seconds at ramp rate $10^{O}C$/second and no extend and final cycle were needed at all amplification steps. The emitted fluorescence was measured at the end of the annealing phase in each cycle using Focus 3M^{TM} integrated thermal cycler (3M-Focus Diagnostics). See Appendix 4 for raw data.

2.8. MTHFR (A1298C) and VDR (T65058C) genotyping

2.8.1. DNA Extraction

The DNA was extracted from the blood samples by using the PureLinkTM Genomic Mini Kit (Invitrogen, UK).
Reagents required for DNA extraction (Invitrogen, UK): Proteinase K (20 mg/ml), RNase A (20 mg/ml, in 50 mM Tris-HCI, pH 8.0,10 mM EDTA), lysis/binding buffer, wash buffers 1 & 2, elution buffer (10 mM Tris-HCI, pH 9.0, 0.1 mM EDTA), spin columns with collections tubes. Extra material needed included 96-100% ethanol.

2.8.2. Protocol for DNA Extraction

To extract genomic DNA from a blood sample, proteinase K (20 µl) was added to the tube followed by transfer blood (200 µl) and then RNase (20 µl). The mixture of the reaction tube was vortexed and incubated at room temperature for 2 minutes. This was followed by the

addition of the lysis/binding buffer (200 µl). After vortexing, the mixture was incubated in a water bath (55 ^{O}C) for 10 minutes. Before transferring the lysate mix to the spin column, absolute ethanol (200 µl) was added. The lysate mix was centrifuged at 10,000 x *g* for 1 minute; the spin column was removed from the collection tube and put into a clean collection tube. Wash buffer (500 µl) diluted with ethanol (96-100%) were added to the spin column and centrifuged at 10,000 x *g* for 1 minute (repeated twice using buffer 1 and 2). Finally, the elution buffer (50 µl) was added to the spin column and centrifuged at maximum speed (16,000 x *g*) for 1 minute at room temperature. Then the eluted purified DNA was quantitatively and qualitatively assessed.

2.8.3. DNA concentration and purity

To assess the quantity and quality of DNA in the eluted DNA from each blood sample, a Nanodrop spectrophotometry (Thermo scientific, UK) was used. The eluted DNA was mixed well first and 1µl was used. Purity (quality) of DNA was obtained by Nanodrop spectrometer by measuring the ratio of the absorbance at 260 nm and 280 nm (OD260 /OD280), with an ideal range between 1.6 to 2.0. All the DNA extraction gave DNA of acceptable quality and none of the samples gave abnormal purity result above range (contaminated with protein) or below it (contamination by RNA).

2.8.4. Genotyping for genetic variants using TaqMan SNP assays

The TaqMan Single nucleotide polymorphism (SNP) genotyping assays (Applied Systems, Foster City California) use TaqMan 5- nuclease chemistry for amplifying and detecting specific single nucleotide polymorphisms (SNP) (real-time) in the DNA extracted samples. The assays use TaqMan minor grove-binding (MGB) probes for allele discrimination. The two allele specific TaqMan MGB probes contain a pair of fluorescent dyes (FAM and VIC) and a pair of primers to detect the specific SNP targets. In addition, all MGB probes include a quencher (non-fluorescent) that attenuates the background fluorescence.

Each of the two MGB Probes with report dye at the 5'-end of each probe (FAM dye for the wild type allele and VIC dye for the mutant allele; with quenchers at the at the 3'-end) binds specifically with the DNA template if it is complementary, according to the base-pairing phenomena. During the extension phase of the amplification, the 5'-nuclease activity of the Taq polymerase degrades the hydrolysis probe leading to fluorescence. For illustration, please refer to Fig 2. 1.

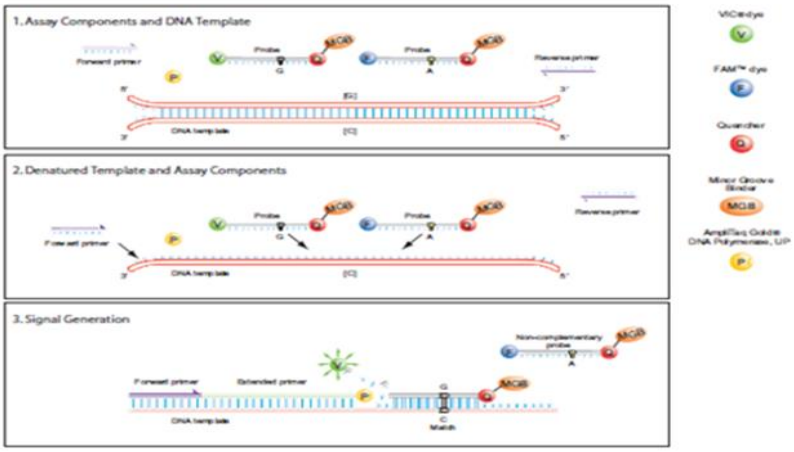


Fig 2.1 Method of genotyping by using TaqMan
the complementary TaqMan probe template and after cleavage
by AmpliTaq DNA polymerase, Ultra Pure (UP).

2.8.5. Genotyping for the MTHFR gene (rs1801131) and VDR gene (rs731236) variants

TaqMan predesigned SNP genotyping assays (Applied Biosystems, Foster City, California) were used to genotype two SNPs; T 65058 C (VDR gene) and A1298 C (MTHRF gene). The single nucleotide variation ATT ⇒ ATC is located at exon 9 of the VDR gene at position T65058C (rs731236) and is located at exon 9 in and it is a silent mutation. The alleles change (variation GAA ⇒ GCA), a result of a SNP in MTHFR gene, is located at exon 7 of the MTHRF gene at position A1298C (rs1801131).

All real-time PCR reactions were performed on 96- well plates on a real time Lightcycler 96 instrument (Roche, Munich, Germany) and the following reagents were provided by the manufacturer: Nuclease free water, 40X TaqMan primer/probe solution (Two primers (forward/reverse), two probes (VIC/FAM) and TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and the TaqMan master mix (AmpliTaq Gold ® DNA Polymerase, Deoxy ribonucleotide triphosphates (dNTPs), Passive Reference dye- Rhodamine X (ROX) and Buffer).

Nuclease free water was used to adjust the DNA quantity in the PCR reaction at a final concentration of 100-200 ng. The final PCR reaction volume in each well was 25µl diluted DNA (11.25µl) and primers/probes solution (1.25 µl) diluted in TE buffer (1:1) and TaqMan master-mix (12.5µl). Nuclease free water was put instead of diluted DNA in two wells to be Negative Template Control (NTC).

To prepare the PCR reaction on the 96-well plates, for each reaction TaqMan primers/probes (1.25 µl) and TaqMan master mix (12.5 µl) were added to each well. Then diluted DNA (11.25 µl) (or nuclease free water on the two negative control wells) were added.
Plate was covered with adhesive optical cover and centrifuged at 1500 rmp for 1 minute to ensure that reagent settled to the bottom of the wells without air bubbles before analysis.
Real-time PCR cycling on the LightCycler 96 (Roche, Germany) performed according the manufacturer's recommendations: 95^{O}C for 600s followed by 45 cycles at 92^{O}C for 15s, and then 60^{O}C for 60s). After the PCR run, the data transferred from the LightCycler 96 to the application software. The final results were reported as homozygous for VIC, heterozygous or homozygous for FAM (were translated to genotypes). See Appendix 5 and appendix 6 for some exmaples of raw data.

2.9. Quantification levels of vitamin D3/D2 in plasma by using Ultra High Performance Liquid Chromatography with ultraviolet detector (UHPLC -UV Detector)

2.9.1. General description of the analytical procedure

Plasma levels of total 25-OH -vitamin D3/D2 was measured using an UHPLC system with UV-detector. Briefly, plasma, calibrator and two-level controls were prepared separately before injection into the system. Before the analysis of vitamin D3/D2 in plasma, the calibrator and the two controls, the blank sample (no vitamin D3/D2 but spiked with the internal standard) was used. All samples (calibrator, controls and patients) were analysed. Vitamin D3/D2 concentration was calculated by using a well-established equation.

2.9.2. Reagents, sample preparation and instrument

2.9.2.1. Materials

Reagents required to measure total vitamin D3/D2 in 100 plasma samples were purchased from Waters Limited, UK. The kit components include: Mobile phase, Internal Standard (IS), serum Calibrator, serum control Level I, serum control Level II, test solution (blank), Precipitant P solution, sample preparation vials and the column (Waters ACQUITY UPLC BEH C18 1.7µm 2.1x50mm Col.).

2.9.2.2. Reagent preparation

Lyophilized calibrators and lyophilized controls Level I and II were reconstituted by adding 2 ml of the HPLC-grade water to each vial and mixed for 15 minutes. To prepare the Blank, 2 ml of the IS solution was added to the lyophilized blank and mixed well.

2.9.2.3. Sample, calibrator and control I/II pre-treatment

For sample pre-treatment, the following laboratory equipment was required: pipettes/pipette tips, table top centrifuge, HPLC-grade water and Vortex-mixer. Protein precipitation was used for pre-treatment of samples. Of the Precipitant P, 500 µl were added into sample preparation vials followed by addition of 400 µl of the plasma and equal volume (400 µl) of the Internal Standard. The contents were vortexed for 30 second and subsequently centrifuged for 5 minutes at 1000xg, 5 µl of the supernatant were injected into the UHPLC instrument. The same applies to the calibrator and the controls. Many exmaples are shown in Appendix 7.

Table 2.1 Chromatographic condition

UHPLC pump	Flow rate: 0.75 ml/minute
Mobile phase	1000 mL/L Methanol
Column	ACQUITY UPLC BEH C18 1.7 µm 2.1x50mm Col
Column temperature	40 ^{O}C
Detection wavelength	264 nm
Injection volume	5 µl
Injection interval	2 min
<u>Retention times:</u>	25-OH-vitamin D3 approx 1.3 min 25-OH-vitamin D2 approx 1.5 min Internal standard approx 1.3 min

2.9.2.4. Calculation method

The concentration of the unknown (vitamin D3/D2) was calculated by using the following: 1) Peak area patient. 2) Peak area internal standard (IS) patient. 3) Peak area

IS of the calibrator. 4) Peak area analyte of the calibrator. The calculation was done in two steps:

Calculation of the recovery rate (REC)

$$REC = \frac{\text{Area IS (sample)}}{\text{Area IS (calibrator)}}$$

Calculation of the analytes (vitamin D3/D2) concentration

$$C\,[(\text{Analytes, sample})\ (\mu g/l)] = \frac{\text{Area (Analyte, sample) X C (Analyte, calibrator) } [\mu g/l]}{\text{Area (Analyte, calibrator) X REC}}$$

2.9.2.5. Recovery (REC)

The recovery is 99-104% referred to a directly injected external standard solution

2.9.2.6. Precision (inter and intra assay Coefficient of variation)

Four samples with different concentration were used to evaluate intra - and inter assay

Table 2.2 Precision of vitamin D measurement (inter and intra assay Coefficient of variation)

	25-OH-Vitamin D2 (µg/l)	25-OH-Vitamin D3 (µg/l)
Sample 1	10.0	10.0
Sample 2	20.7	21.0
Sample 3	53.2	53.2
Sample 4	88.4	87.1

2.9.2.7. Detection limit and quantitation limit

Table 2.3 Detection limit and quantitation limit of vitamin D

	25-OH-Vitamin D2	25-OH-Vitamin D3
	µg/l	µg/l
LLOD	2.8	2.2
LLOQ	4.6	3.7
LLOD: Lower limit of detection , LLOQ: Lower limit of quantitation		

2.10. Statistical Analysis

Data are presented as median and interquartile range (IQR) or mean±standard error (SE). *P*-Value <0.05 was considered as statistically significant. Normality tests were performed before the analysis of difference (between resistin and homocysteine in patients with type 2 diabetes and healthy control, as well as between resistin and homocysteine in the males and females; also between resistin and homocysteine in difference BMI), normality tests were performed for each group, The results inferred that the data do not follow a normal distribution pattern, so non-parametric, Mann Whitney test (MW) was used to determine the association between them. A correlation test was performed to study possible association between the resistin or homocysteine and the demographic, clinical and metabolic variables (Age, Duration of DM, BMI, waist, FBG 2hBG, HbA1c%, CHOL, TAG, LDL, HDL, SBP and DBP) in all participants. A chi-square test was used to analyse the association between the MTHFR genotypes and allelic frequencies.

Chapter 3

3. Results

3.1. Resistin study

3.1.1. General feature of the participants

In this study, 200 patients with type 2 diabetes (T2DM) and 195 healthy controls were recruited. The characteristics of the study population are summarised in the Table 3.1. Demographic and clinical variables (Age, sex, 2 hour Blood Glucose (2hBG), Fasting Blood Glucose (FBG), Glycosylated Hamoglobin (HbA1c), Body Mass Index (BMI), Cholesterol (CHOL), Triglycerides (TAG), Low-Density Lipoprotein (LDL) and High-Density Lipoprotein (HDL) Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), creatinine, Waist circumference (waist) and plasma levels of resistin, vitamin D and Homocysteine (Hcy) are also presented in Table 3.1.

In patients with T2DM, the median (range) age is 50.0 (47.0-58.0) years, while it is 42.5 (31.0-50.0) years in the control group. The average BMI is higher, with statistically significant difference in patients with T2DM compared to the control group {26.8 (25.6-28.4) (kg/m^2) vs 24.9 (23.0 - 28.0) (kg/m^2); $p<0.001$}. There was no statistically significant difference in levels of CHOL, LDL and HDL in patients as compared to controls. TAG levels are higher with statistical significance in controls compared with patients {153.5 (96.0 - 224.0) (mg/dl) vs 131.0 (84.3-164.0) (mg/dl); $p< 0.0001$)}. Also the waist circumference was lower with statistical significance in T2DM patients compared to the healthy control group {78.0 (74.0, 86.0) (cm) vs 86.0 (73.0, 94.0) (cm); $p= 0.0079$}.

All variables (clinical and metabolic) remaining (median values and ranges) are summarised in Table 3.1.

Table 3.1 General characteristic of the study participants

Variable	Patient (T2DM) Median (IQR)	Control Median (IQR)	(95% CI) Point Estimates	Mann-Whitney (*p*-value)
Number (n)	200	195	N/A	N/A
Age (y)	50.0 (47.0-58.0)	42.5 (31.0-50.0)	10.0 (8.0,12.9)	< 0.0001
Males [n (%)]	71 (35.5)	130 (66.7)	N/A	N/A
Females [n (%)]	129 (64.5)	65 (33,3)	N/A	N/A
2hBG (mg/dl)	215.0 (156.5 -284.0)	100.0 (88.0-116.8)	108.0 (91.0, 126.0)	< 0.0001
FBG (mg/dl	132.0 (98.5, 196.5)	N/A	N/A	N/A
HbA1c (%)	8.0 (7.0-9.0)	5.0 (5.0-6.0)	3.0 (2.4, 3.0)	< 0.0001
BMI (kg/m^2)	26.8 (25.6-28.4)	24.9 (23.0-28.0)	1.8 (1.2, 2.4)	< 0.0001
CHOL (mg/dl)	172.0 (144.0 -201.0)	176.0 (150.0,203.3)	-5.0 (-14.0, 4.0)	0.2405
TAG (mg/dl)	131.0 (84.3-164.0)	153.5 (96.0-224.0)	-13.0 (-48.0, -16.0)	< 0.0001
LDL (mg/dl)	102.5 (86.0-123.0)	103.0 (79.1-121.0)	1.0 (-6.0, 8.0)	0.7481
HDL (mg/dl)	42.0 (36.0-48.0)	44.1 (37.8-49.2)	-1.7 (-4.0, 0.40)	0.1256
SBP (mmHg)	130.0 (120.0-140.0)	120.0 (110.0-120.0)	10.0 (10.0, 10.0)	< 0.0001
DBP (mmHg)	90.0 (85.0-100.0)	80.0 (80.0- 85.0)	10.0 (10.0, 10.0)	< 0.0001
Creatinine (mg/l)	0.70 (0.50, 0.90)	0.70 (0.80, 0.90)	-0.10 (-0.20,0.00)	0.002
Waist (cm)	78.0 (74.0, 86.0)	86.0 (73.0, 94.0)	-4.0 (-7.0, -1.0)	0.0079

SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure, BMI: Body Mass Index, BG: Blood Glucose, CHOL: Cholesterol, TAG: Triglycerides, LDL: Low Density Lipoprotein and HDL: High Density Lipoprotein N/A: not applicable Hypertension known as SBP Systolic Blood Pressure/ DBP Diastolic Blood Pressure , Point Estimates (95% confidence Interval) and *p*-value < 0.05 for patients with T2DM vs control group.

3.1.2. Plasma resistin levels between patients with T2DM and healthy controls

Comparing resistin levels between patients with T2DM and healthy controls in representative group of Sudanese population. Plasma resistin levels were investigated in patients with T2DM (n=200) and healthy controls (n=195). The results showed that plasma resistin levels were higher with statistical significance in control group compared with patients with T2DM, 3.80 (1.80, 7.40) ng/ml vs 2.70 (2.00 4.28) ng/ml, $p< 0.000$.

Table 3.2 Difference of the plasma resistin concentration in patients with T2DM and control

Variable	Patient (T2DM) Median (IQR)	Control Median (IQR)	(95% CI) Point Estimates	Mann-Whitney (*p*-value)
Number (n)	200	195	N/A	N/A
Resistin ng/mL	2.70 (2.00 4.28)	3.80 (1.80, 7.40)	-0.90 (-1.50, -0.30)	*p*< 0.000.

Data were presented as mean ± standard deviation (SD) 95 % CI.

Mann Whitney test. *p*-value is signification at < 0.05.

Point Estimates (95% confidence Interval)

3.1.3. Correlation between plasma resistin levels in patients with T2DM and Healthy control and the different variables.

One of the objectives of this study was to evaluate the correlation between the plasma resistin levels in patients with T2DM and clinical and metabolic variables, such as age, waist, FBG, 2hBG, HbA1c %, CHOL, TAG, LDL, HDL, SBP, DBP. The correlation between plasma resistin levels in all participants, patients, and controls were summarized in Tables 3.3. There is a positive but a weak correlation between resistin levels and 2hBG and HbA1c % in all participants (r = 0.121; *p*= 0.035 and r = 0.237; *p*<0.001), respectively), as well as weak positive correlation between resistin levels and FBG, log BMI in patients with T2DM (r = 0.188; *p*= 0.008 and r = 0.140; *p*=0.048, respectively). In addition, the results also showed a negative correlation between resistin levels and vitamin D (r = 0.261; *p*<0.0001) and positive correlation between resistin and homocysteine (r = 0.335; *p*<0.0001) in patients with T2DM. There was no correlation between resistin levels and the remaining variables (demographic, clinical and metabolic) in all participants and in patients. Results also showed that there is no correlation between resistin and all variable in the control group. It worth mentioning that, the number of females exceeded the number of male in individuals with diabetes, while in the control group the number of males was more than females.

Table 3.3 Correlation between plasma resistin values and subject characteristic variables listed for combined, patients with T2DM and healthy control groups.

Variable	**All**		**Patient (T2DM)**		**Control**	
Number (n)	395		200		195	
	r	*p*	r	*p*	r	*p*
Age (y)	0.005	0.921	0.098	0.172	0.112	0.120
2hBG (mg/dl)	0.121	0.035	0.069	0.368	0.065	0.456
FBG (mg/dl	0.188	0.008	0.188	0.008	N/A	N/A
HbA1c (%)	0.237	0.000	-0.018	0.806	0.111	0.271
BMI (kg/m^2)	-0.047	0.351	0.133	0.060	-0.040	0.585
CHOL (mg/dl)	-0.018	0.751	0.081	0.261	-0.170	0.051
TAG (mg/dl)	0.008	0.887	-0.018	0.798	-0.098	0.266
LDL (mg/dl)	-0.076	0.170	-0.200	0.005	0.063	0.471
HDL (mg/dl)	-0.006	0.919	-0.006	0.933	-0.069	0.436
SBP (mmHg)	-0.031	0.570	0.091	0.199	0.036	0.681
DBP (mmHg)	-0.060	0.281	0.033	0.646	0.082	0.355
Creatinine (mg/l)	0.036	0.556	0.095	0.269	-0.012	0.890
Waist (cm)	0.055	0.318	-0.031	0.664	0.058	0.507
Vitamin D (µg/l)	-0.119	0.018	-0.261	0.000	0.108	0.134
Hcy (µmol/l)	-0.025	0.615	0.335	0.000	0.011	0.879
Log BMI (kg/m2)	-0.059	0.243	0.140	0.048	-0.045	0.536

(r) Person correlation coefficient

3.1.4. Difference of resistin levels in patients with T2DM and healthy controls according to the gender:

Resistin levels in T2DM patients (n=200) were comparable to levels in healthy controls (n=195), (Fig. 3.1). However, there was a statistically significant increase in plasma resistin levels in males compared to females in both the All and control groups; (4.4±0.2 ng/ml vs 3.8±0.2 ng/ml; *p*= 0.002) and (5.1±0.3ng/ml vs 4.2±0.49 ng/ml; *p*= 0.007), respectively. The same applies to male patients with T2DM versus female patients with T2DM, where there was no statistical significantly difference in plasma resistin levels between them (3.0±0.21ng/ml vs 3.6±0.20 ng/ml; *p*=0.09).

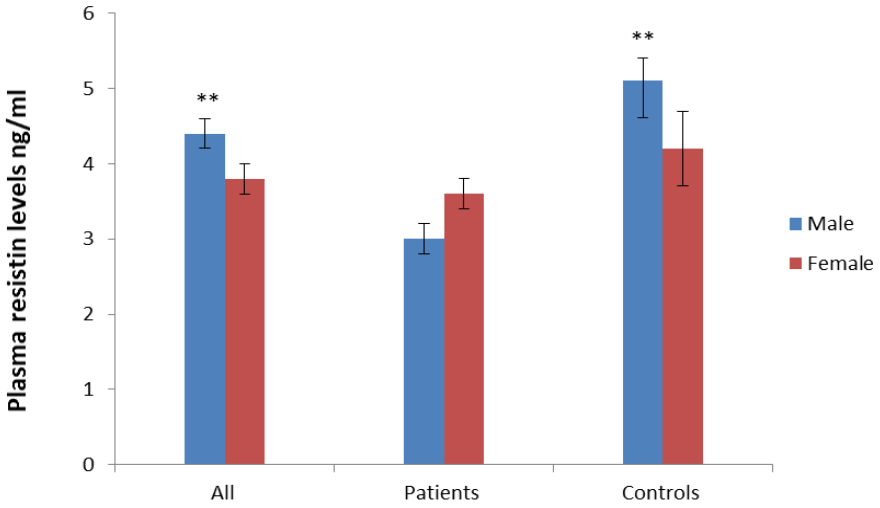


Fig 3.1 Comparison of human plasma resistin levels between males and females in all participants, patients with T2DM and healthy controls group Data were expressed as mean ± standard error and, *p < 0.05, **p <0.01 and ***p <0.001).

3.1.5. Difference between resistin levels in patients with type 2 diabetes and healthy controls according to the BMI

To investigate the levels of resistin according to BMI, participants were classified into two groups: those with BMI >25 (kg/m^2) and those with BMI <25(kg/m^2) and the data are summarised in Fig 3.2. Among each group, levels of resistin were compared between patients with T2DM and healthy controls, between male patients and male healthy controls and between female patients and female healthy controls. The results showed that levels of resistin were statistically significantly higher in healthy controls compared to patients in both BMI groups (4.7±0.33 ng/ml vs 3.5±0.16 ng/ml, p=0.03 for those with BMI >25(kg/m^2); 4.9±0.37ng/ml vs 2.9±0.27ng/ml, p=0.03 for those with BMI <25) (kg/m^2). The results showed that the resistin levels were statistically significantly higher in male healthy controls as compared to male patients with T2DM in both BMI groups (4.9±0.36 ng/ml vs 3.1±0.21 ng/ml, p<0.001, for males with BMI >25(kg/m^2); 5.4±0.44 ng/ml vs 2.5±0.50 ng/ml; p=0.007 for males with BMI <25) (kg/m^2). There was no statistical difference between the levels of resistin between female healthy controls and their female patients with T2DM in both BMI groups ((4.2±0.76 ng/ml vs 3.7±0.21 ng/ml; p=0.16 for females with BMI <25(kg/m^2); 4.3±0.64 ng/ml vs 3.1±0.32 ng/ml; p=0.45 for females with BMI >25) (kg/m^2).

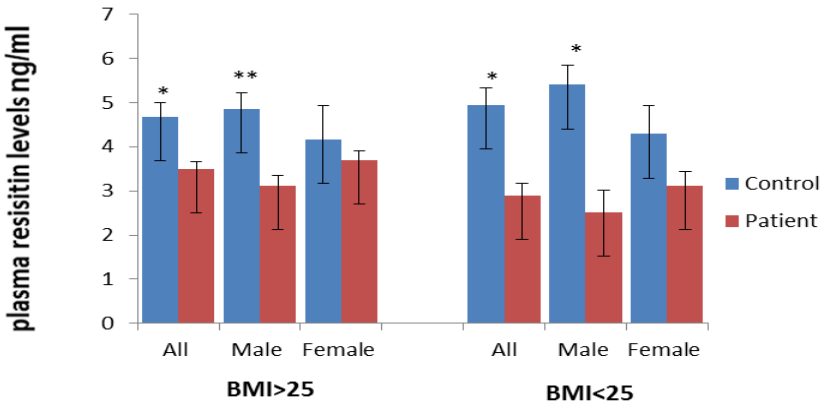


Fig 3.2 Comparison of plasma resistin levels between patients with T2DM and healthy control according to BMI group. Data were expressed according to the mean ± SEM. (*$p < 0.05$, **$p < 0.001$ and ***$p < 0.0001$)

3.1.6. Difference between resistin levels in patients with type 2 diabetes and healthy controls according to the waist circumference

To verify the levels of Resistin according to waist circumference, participants were classified into two groups according to the gender (female group and male group) and the data were summarised in fig 3.4. Among each group, levels of resistin were compared between patients with T2DM and healthy controls, between female patients and female control have WC >80, also between female patients and female control have WC <80 cm, as well as, between male patients and male control have WC >94 cm, also between male patients and male control have WC <94 cm. The result showed that there were no statistically significant of resistin levels between female patients and female control which have WC > 80 cm (4.0±0.35 ng/ml vs 2.7±1.7 ng/ml, p=0.1024); , but the results showed that the resistin levels were higher with statistically significant in diabetic female compared with female control which have WC<80 cm (3.5±0.22 ng/ml vs 2.5±0.47 ng/ml, p=0.0070). while as in two male groups, the results showed that levels of resistin were statistically significant higher in patients with T2DM compared to health controls in both WC groups (2.5±0.22 ng/ml vs 4.9±0.49 ng/ml, p=0.0018 for those with WC>94 cm;(3.2±0.24 ng/ml vs 2.5±0.47 ng/ml, p=0.0292 for those with WC<94 cm).

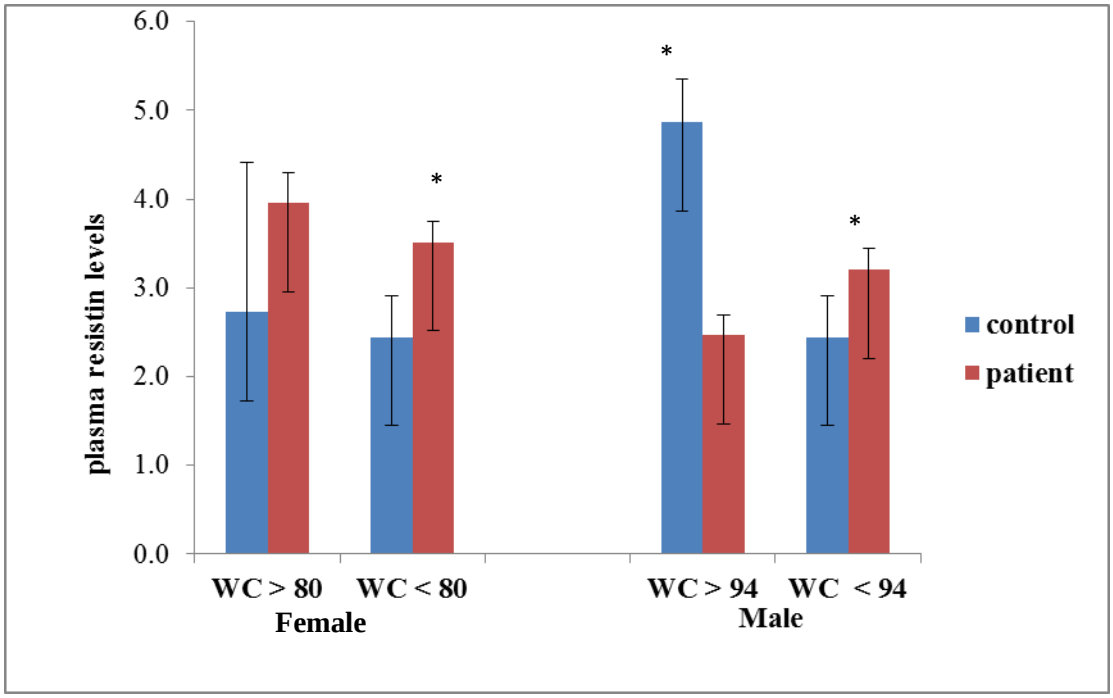


Fig 3.3 comparison of human plasma resistin levels between female and males in all patients with T2DM and all control group according to waist circumference (WC) group. Data were expressed according to mean± SEM (*p < 0.05, **p <0.001 and ***p <0.0001)

3.1.7. Resistin levels in patients with T2DM and their healthy controls according to the HDL

Levels of Resistin were compared between patients and healthy control according to HDL. Participants (patients with T2DM and control) were divided in two groups, those with HDL > 50 (mg/dl) and those with HDL > 50 (mg/dl) and the data are summarised in fig 3.5. Among each group, levels of resistin were compared between patients with T2DM and healthy controls. The results showed that there was no statistically significant between patients with T2DM and healthy controls in those with HDL>50 mg/dl (3.4 ±0.34 ng/ml vs 5.1 ±0.62 ng/ml, p=0.08). Those with HDL<50 mg/dl, levels of resistin were statistically significant higher in healthy controls compared to patients in HDL<50 mg/dl (3.1±0.14 ng/ml vs 4.7±0.29 ng/ml, p<0.0000).

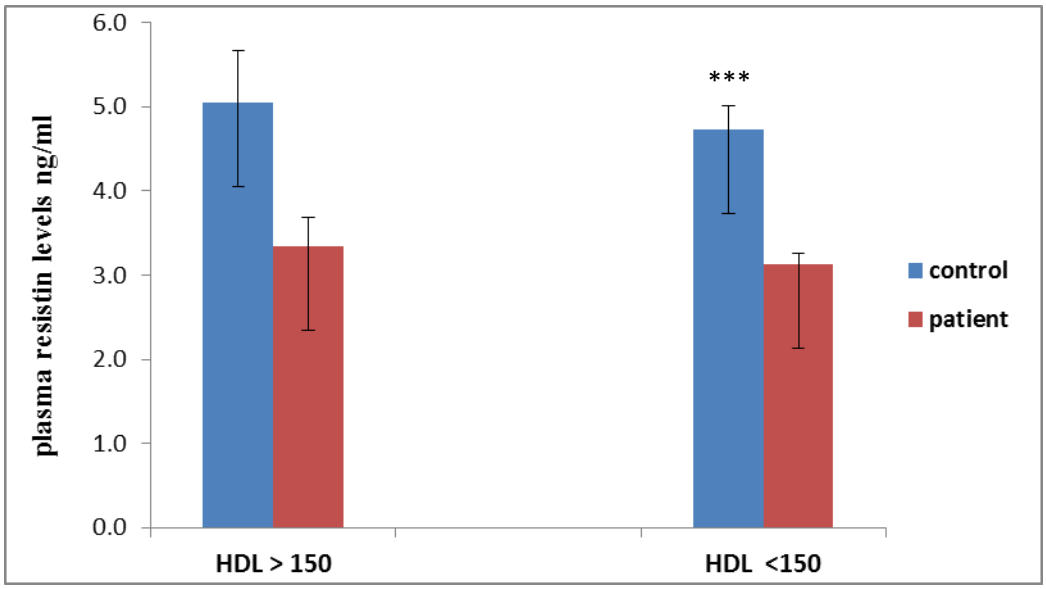


Fig 3.4 Comparison of human plasma resistin levels between patients with T2DM and healthy controls group according to HDL. Data were expressed as mean ± standard error and, *p < 0.05, **p <0.01 and ***p <0.001).

3.1.8. Resistin levels in the study population (patients with T2DM and Healthy controls) according to triglycerides

The levels of resistin were compared between patients with T2DM and healthy controls according to triglyceride (TAG). the participants were classified into two groups: those with TAG>150 mg/dl and those with TAG<150. the results showed that the plasma resistin levels

were higher with statistically significant difference in healthy control compared with diabetic patients in both TAG groups (4.6±0.37ng/ml vs 3.4±0.22 ng/m; p=01 for those with TAG >150 mg/dl; 4.8±0.37ng/ml vs 3.5±0.19 ng/m; p=002 for those with TAG <150 mg/dl).

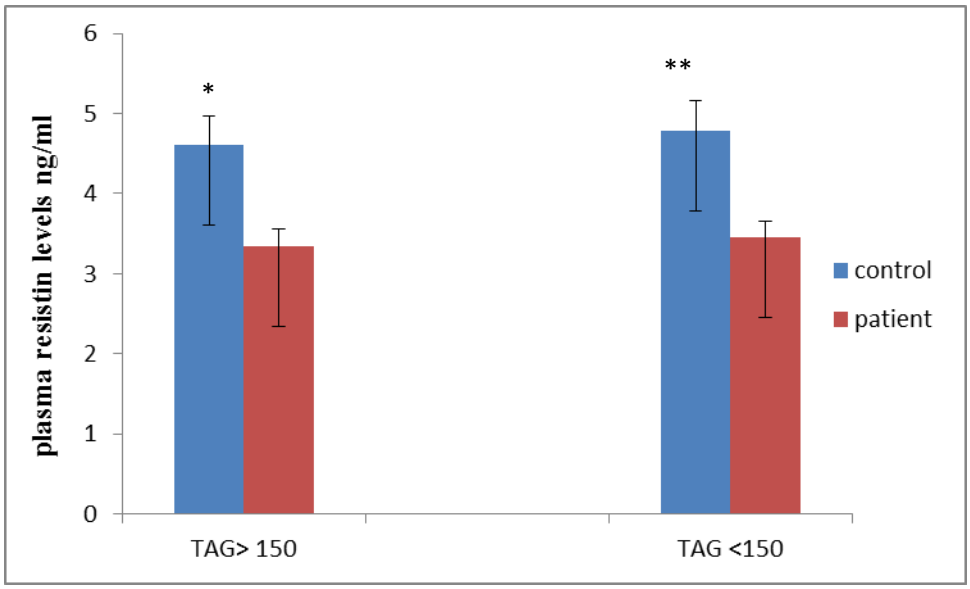


Fig 3.5 Comparison of human plasma resistin levels between patients with T2DM and healthy controls group according to TAG. Data were expressed as mean ± standard error and, *p < 0.05, **p <0.01 and ***p <0.001).

3.2. Homocysteine study

3.2.1. Plasma homocysteine levels between patients with T2DM and healthy controls

The hypothesis: to compare between the levels of homocysteine in patients with T2DM and healthy controls. Homocysteine levels were tested in 200 patients and 195 healthy controls. Our finding showed that homocysteine were significantly higher in patients with T2DM compared to controls (17.3 (13.2, 22.1) µmol/l vs 5.2 (2.2, 13.3) µmol/l, p< 0.0001).

Table 3.4 Difference of the plasma homocysteine concentration in patients with T2DM and control

Variable	**Patient (T2DM) Median (IQR)**	**Control Median (IQR)**	**(95% CI) Point Estimates**	**Mann-Whitney (*p*-value)**
Number (n)	200	195	N/A	N/A
Hcy (µmol/l)	17.3 (13.2, 22.1)	5.2 (2.2, 13.3)	10.3 (9.00, 11.6)	p< 0.0001

Data were presented as mean ± standard deviation (SD) 95 % CI.

Mann Whitney test. *p*-value is signification at < 0.05.

Point Estimates (95% confidence Interval)

3.2.2. Correlation between plasma homocysteine levels in patients with T2DM and Healthy control and the different variables

The association between homocysteine (Hcy) and demographic and metabolic variables (Age, 2hBG, FBG, HbA1c %, BMI, CHOL, TAG, LDL, HDL, SBP, DBP, creatinine and Waist) were investigated in the studied population and the results are summarised in Table 3.5. Homocysteine levels positively correlated with Age, 2hBG, HbA1c, BMI, SBP and DBP, (r = 0.231; p<0.001, r = 0.361; p< 0.001, r = 0.298; p< 0.001, r = 0.126; p= 0.013, r = 0.213; p<0.001 and r = 0.269; p< 0.001, respectively), however, the correlations were weak. Homocysteine also had a negative correlation with TAG, r= -0.234; p< 0.001, waist, r= -0.165; p<0.001in all participants. In patients with T2DM, homocysteine levels had a weak positive correlation with Age (r = 0.212; p= 0.003), BMI (r=0.186; p= 0.008) and SBP (r= 0.181; p= 0.011). The results showed that there was a weak negative correlation between homocysteine levels and Log-vitamin D (µg/l) in patients with T2DM and all participants, r= -0.138; p= 0.052 and r = -0.100; p= 0.050, respectively. In addition, in the healthy control group, there was only a negative correlation between homocysteine levels and LDL levels (mg/dl) and Waist (cm), r= -0.189; p= 0.030 and r= -0.321; p= 0.000, respectively.

Table 3.5 Correlation between plasma homocysteine values and subject characteristic variables listed for combined, patients with T2DM and healthy control groups.

Variable	**All**		**Patient (T2DM)**		**Control**	
Number (n)	395		200		195	
	r	*p*	r	*p*	r	*p*
Age (y)	0.231	< 0.001	0.212	0.003	-0.047	0.516
2hBG (mg/dl)	0.361	< 0.001	0.087	0.253	-0.211	0.015
FBG (mg/dl	0.083	0.247	0.083	0.247	N/A	N/A
HbA1c (%)	0.298	0.000	0.022	0.762	-0.151	0.133
BMI (kg/m^2)	0.126	0.013	0.186	0.008	-0.107	0.139
CHOL (mg/dl)	-0.094	0.088	0.002	0.973	-0.126	0.151
TAG (mg/dl)	-0.234	0.000	-0.022	0.764	-0.150	0.086
LDL (mg/dl)	-0.048	0.390	0.011	0.876	-0.189	0.030
HDL (mg/dl)	-0.010	0.861	0.051	0.492	0.066	0.459
SBP (mmHg)	0.213	0.000	0.181	0.011	-0.134	0.127
DBP (mmHg)	0.269	0.000	0.092	0.195	-0.015	0.862
Creatinine (mg/l)	-0.030	0.626	0.010	0.903	-0.072	0.414
Waist (cm)	-0.165	0.002	0.094	0.184	-0.321	0.000
Vitamin D (µg/l)	0.078	0.122	-0.064	0.365	-0.002	0.980
Resistin ng/l	-0.025	0.615	0.335	0.000	0.011	0.879
Log-vitamin D (µg/l)	-0.100	0.050	-0.138	0.052	-0.061	0.409

(r) Person correlation coefficient

3.2.3. Distribution of MTHFR 677 C> T gene polymorphism in patients with T2DM and their healthy controls

The allele frequency and genotype distribution of the MTHFR gene (677 C>T) were investigated in 200 Sudanese patients with T2DM and 195 healthy controls. As shown in Table 3.6, the percentage of patients carrying the CC (wild type homozygotes), TT (mutant type homozygotes) and CT (Heterozygotes) genotypes is 85.5%, 0.5% and 14.0%, respectively; while among healthy controls it is 88.7%, 11.3 % and 0.0%. There is no statistically significant difference in the genotype CT distribution between patients and healthy control (χ^2=0.660, p= 0.417) and the same applies to the "C" and "T" allele frequencies (χ^2=1.11, 1, p= 0.292).

Table 3.6 Distribution of MTHFR C677T genotypes and allelic frequencies among patients with T2DM and their healthy controls

	Patient (T2DM) (n =200) (400 alleles)	**Control (n = 195) (390 alleles)**	χ^2 / ***p*. value**
CC n (%)	171 (85.5)	173 (88.7)	0.909 / 0.340
CT n (%)	28 (14.0)	22 (11.3)	0.660 / 0.417
TT n (%)	1 (0.5)	0 (0.0)	N/A
Allele C (%)	370 (92.5)	368 (94.4)	1.110 / 0.292
Allele T (%)	30 (7.5)	22 (5.6)	

P-values obtained for the χ^2-test for the CT genotypes distribution of allele T frequencies l between patients and healthy control. N/A = χ^2 Not calculated due to zero TT genotype. CC (wild type homozygotes), TT (mutant type homozygotes) and CT (Heterozygotes)

3.2.4. Distribution of MTHFR A1298 C gene polymorphism in patients with T2DM and their healthy controls

The genotype distribution of the MTHFR gene (1298 A>C) and allele frequency were investigated in 200 Sudanese patients with T2DM and 195 healthy controls. As summarized in Table 3.7. The distribution of genotypes of the MTHFR gene (1298 A> C) in patients was as follows: AA (65.0%), AC (29.0%) and CC (6.0%): while among the control group was as follows: AA (65.1%), AC (29.8%) and CC (5.1%). There is no statistically significant difference in frequencies of AA (χ^2= 0.001 / p = 0.098), AC (χ^2= 0.026 / p = 0.87) and CC (χ^2= 0.143 / p = 0.71) genotypes of the MTHFR gene between patients with T2DM and healthy controls. The same applied for alleles "A" and "C" frequencies of the MTHFR gene, where results showed that there is no statistically significant difference between, alleles "A" and "C" frequencies, patients with T2DM and healthy controls (χ^2= 0.031 / p = 0.86).

Table 3.7 Distribution of MTHFR A1298 C genotypes and allelic frequencies among patients with T2DM and their healthy controls

	Patients (T2DM) (n =200, 400 alleles)	**Control (n = 195, 395 alleles)**	χ^2 **/** ***p*****. values**
AA n (%)	130 (65.0)	127 (65.1)	0.001 / 0.098
AC n (%)	58 (29.0)	58 (29.8)	0.026 / 0.87
CC n (%)	12 (6.0)	10 (5.1)	0.143 / 0.71
Allele A (%)	318	312	0.031 / 0.86
Allele C (%)	82	78	

P-values obtained for the χ^2-test for the AA/AC /CC genotypes distribution of allele A and C frequencies between patients and healthy control. AA (wild type homozygotes), CC (mutant type homozygotes) and AC (Heterozygotes)

3.2.5. Association between plasma homocysteine levels and MTHFR gene polymorphisms, C677T and A1298C, in patients with T2DM and control group.

Here we investigated possible association between the genetic polymorphisms in MTHFR gene (C677T and A1298C), and levels of Hcy in plasma. Results showed that the levels of Hcy were significantly higher in patients with T2DM carrying genotypes C677T (18.6 (14.4,21.9) µmol/l) , A1298C (16. 3 (12.3, 21.3) µmol/l) and C1298C (20.9 (15.3, 25.9) µmol/L) compared with the control group carrying the same genotype polymorphisms C677T (3.6 (1.0, 16.3) µmol/l , A1298C (4.7 (1.6, 13.3) µmol/l) and C1298 C (4.7 (2.9, 13.3) µmol/l; $p<0.001$ (Table 3.8).

As shown in table 3.6, it was observed that the heterozygosity at both alleles (C677T/ A1298C) are statistically significantly associated with elevated level of homocysteine in patients (14.1 (11.1, 25.0) µmol/l) than in healthy controls (1.6 (0.9, 3.9) µmol/l); $p=0.03$.

Table 3.8 Homocysteine levels in MTHFR 677C>T and 1298 A>C carries

Genotype	Patients (T2DM) Median (IQR)	Control Median (IQR)	(95% CI) Point Estimates	Mann-Whitney (P-value)
C 677 C	17.1 (12.9,22.1)	5.6 (2.5, 13.30)	10.0 (8.5, 11.4)	p<0.001
C 677 T	18.6 (14.4,21.9)	3.6 (1.0, 16.3)	12.5 (7.7, 15.9)	p<0.001
A 1298 A	17.4 (13.8, 21.9)	5.9 (2.1, 14.1)	10.2 (8.5, 11.8)	p<0.001
A 1298 C	16. 3 (12.3, 21.3)	4.7 (1.6, 13.3)	9.8 (7.1, 12.3)	p<0.001
C 1298 C	20.9 (15.3, 25.9)	4.7 (2.9, 13.3)	13.5 (7.4, 20.8)	p=0.001
C 677 T/ A 1298 C	14.1 (11.1, 25.0)	1.6 (0.9, 3.9)	12.1 (6.3, 26.8)	p=0.03

P-values obtained for the Mann-Whitney test for the AA/AC /CC and CC/CT/genotypes distribution between patients and healthy control.

3.2.6. Homocysteine levels in the study population (patients and healthy Controls) according to Gender:

The levels of Hcy were compared between males and females. In all participants, the results showed that the plasma Hcy levels were higher with statistically significant difference in females compared with males (15.56±0.56 µmol/l vs 11.09±0.71 µmol/l; p<0001). In addition, in the healthy controls, plasma Hcy levels were higher with statistically significant differences in females compared with the males (11.47±1.16 µmol/l vs 6.75±0.63 µmol/l; p<0001). However, in the patients group, the plasma Hcy levels were not statistically significant different between males and females (19.03±1.15 µmol/l vs 17.63±0.53 µmol/l; p= 4498). All data are summarised in Fig 3.3.

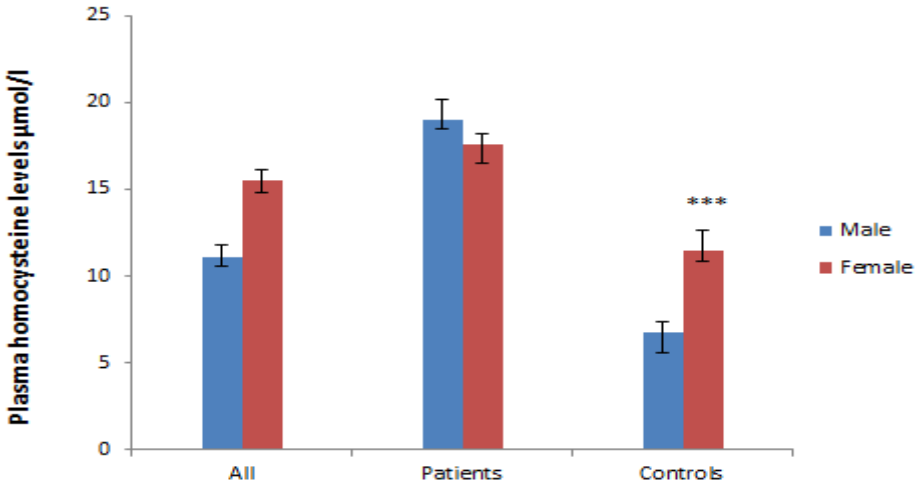


Fig 3.6 Comparison of plasma homocysteine (Hcy) levels between females with males in all participants, patients with type 2 diabetes and healthy controls. Data is expressed as mean ± SEM

3.2.7. Investigation of the relationship between homocysteine and resistin hormone levels in patients with T2DM.

The possible association between levels of Hcy and resistin in patients with T2DM was investigated. Table 3.2 showed that resistin levels were significantly higher in control group than in patients with T2DM (3.8 (1.80, 7.40) ng/ml vs 2.7 (2.00 4.28) ng/ml; *p*= 0.002). In addition, Table.3.4 showed that Homocysteine levels were a significantly higher in patients with T2DM than in control group (17.3 (13.2 - 22.1 µmol/L vs 5.2 (2.2 - 13.3µmol/L; *p*< 0.0001). As shown in Fig 3.4 and 3.5 there is a positive Pearson correlation between Hcy levels and resistin levels in patients with T2DM (r = 0.335; *p*<0.001) and no correlation between Hcy levels and resistin levels in the controls (r = -0.011; *p*= 0.879).

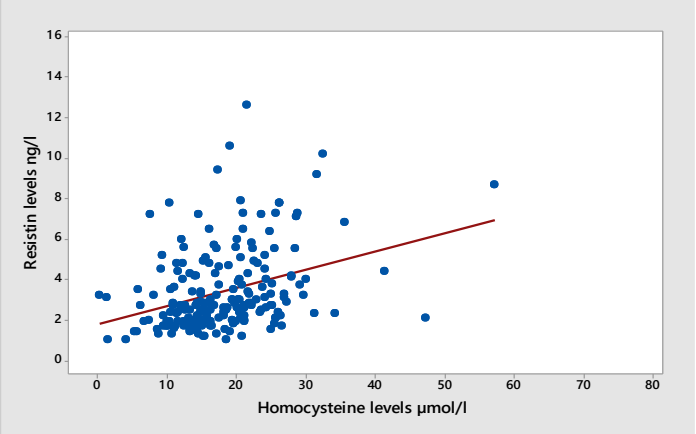


Fig 3.7 Correlation between Hcy and resistin **in** patients

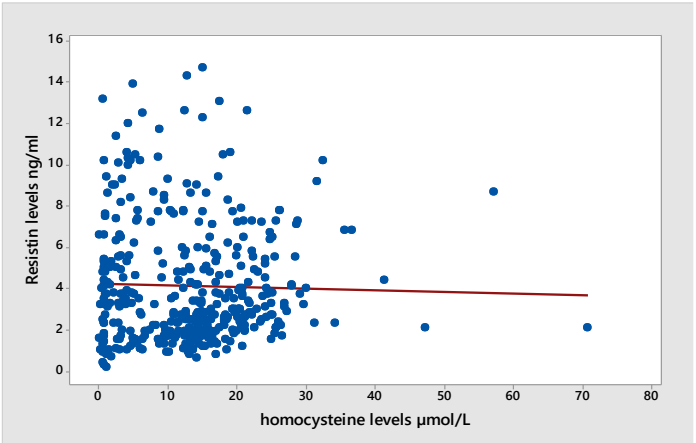


Fig 3.8 Correlation between Hcy and resistin in control

3.3. Vitamin D study

3.3.1. Plasma vitamin D levels patients with T2DM and healthy controls

One of the objectives of this study was investigate any association between vitamin D deficiency in individuals with T2DM. Total vitamin D levels were measured on 200 patients with T2DM and 195 healthy controls. The results showed vitamin D levels were higher with statistical significance in patients with T2DM compared with control groups. The median (range) for total vitamin D is 51.0 (35.3 - 116.3) µg/l in T2DM patients and 21.8 (6.9 - 79.9) µg/l in control groups (p< 0.0001; Table 3.9).

Table 3.9 Difference of the plasma vitamin D concentration in patients with T2DM and control

Variable	**Patient (T2DM) Median (IQR)**	**Control Median (IQR)**	**(95% CI) Point Estimates**	**Mann-Whitney (p-value)**
Number (n)	200	195	N/A	N/A
Vitamin D (µg/l)	51.0 (35.3 -116.3)	21.8 (6.9 - 79.9)	27.7 (20.6, 34.1)	< 0.0001

Data were presented as mean ± standard deviation (SD) 95 % CI.

Mann Whitney test. p-value is signification at < 0.05.

Point Estimates (95% confidence Interval)

3.3.2. Vitamin D supplementation based analysis of clinical and laboratory characteristics of patients with type 2 diabetes (T2DM)

Clinical and metabolic features of patients with T2DM are summarized in Table 3.10. The characteristics of the patients were presented according to vitamin D supplementation. Among patients with T2DM, 58 were using vitamin D supplementation, while 142 were not using a vitamin D supplementation. The median age of those not taking vitamin D was higher with statistically significant difference than those taking vitamin D supplementation (52.0 (48.0, 59.8) years vs 49.0 (46.0, 51.0) years; p= 0.0008). The BMI is significantly higher in patients not using vitamin D supplementation as compared to patients using vitamin D supplementation (27.0 (25.9, 28.8) Kg/m^2 vs 26.1 (25.4, 28.2) Kg/m^2; p< 0.05). The fasting Blood glucose (FBG) was higher with statistical significant in the patients not taking vitamin D compared with patients taking vitamin D (143.5 (102.3, 207.5) mg/dl vs 114.0 (91.0, 171.0) mg/dl; p = 0.04). No significant difference in HA1c % between the patients not taking vitamin D compared with patients taking vitamin D (8.0 (7.0, 9.0) vs 8.0 (7.0, 9.0); p= 0.870).

Plasma vitamin D levels are higher with a statistically significant difference in patients taking vitamin D compared with patients not taking vitamin D (106.8 (50.7, 327.2) vs 43.2 (32.2, 73.6); $p< 0.0001$. In addition, the results showed that resistin levels were higher with statistical significantly difference in patients not taking vitamin D compared with patients taking vitamin D (2.85 (2.18, 4.80) vs 2.45 (1.70, 3.43); $P= 0.0039$.

Table 3.10 General characteristics of patients with and without vitamin D supplementation

Variable	Patient (T2DM) with Vitamin D Median (IQR)	Patients (T2DM) without vitamin D Median (IQR)	(95% CI) Point Estimates	Mann-Whitney (*p*-value)
Number (n)	58	142	N/A	N/A
Age (y)	49.0 (46.0, 51.0)	52.0 (48.0, 59.8)	4.0 (1.0, 6.0)	0.0008
Males [n (%)]	16 (27.6)	55 (38.7)	N/A	N/A
Females [n (%)]	42 (72.4)	87 (61.3)	N/A	N/A
2hBG (mg/dl)	177.0 (143.0, 274.0)	228.5 (162.3, 291.3)	28.0 (-2.0, 56.0)	0.0697
FBG (mg/dl	114.0 (91.0, 171.0)	143.5 (102.3, 207.5)	-18.0 (-37.0, -1.0)	0.0391
HbA1c (%)	8.0 (7.0, 9.0)	8.0 (7.0, 9.0)	0.0 (-0.8, 1.0)	0.8695
BMI (kg/m^2)	26.1 (25.4, 28.2)	27.0 (25.9, 28.8)	0.70 (-0.0, 1.3)	0.0465
CHOL (mg/dl)	169.0 (143.8, 205.0)	175.0 (143.5, 200.0)	-0.0 (-14.0, 13.0)	0.9635
TAG (mg/dl)	140.00 (87.5, 168.8)	125.0 (84.0, 164.0)	-7.0 (-23.0, 8.0)	0.3525
LDL (mg/dl)	111.50 (88.50, 129.7)	99.8 (84.5, 120.00	-10.0 (-19.0, -0.2)	0.0432
HDL (mg/dl)	43.0 (37.0, 49.5)	42.0 (36.0, 48.0)	-2.0 (-4.0, 1.0)	0.2516
SBP (mmHg)	130.0 (120.0, 140.0)	130.0 (120.0, 140.0)	-0.0 (-0.0,-0.0)	0.4421
DBP (mmHg)	90.0 (83.8, 100.0)	90.0 (85.0, 100.0)	-0.0 (0.0, 5.0)	0.2871
Creatinine (mg/l)	0.70 (0.54, 0.81)	0.60 (0.50, 0.90)	0.0 (-0.1, 0.1)	0.9443
Waist (cm)	76.0 (72.0, 83.0)	80.0 (76.0, 86.0)	3.0 (1.0, 6.0)	0.0114
Resistin ng/ml	2.45 (1.70, 3.43)	2.85 (2.18, 4.80)	0.6 (0.2, 1.0)	0.0039
Vitamin D (µg/l)	106.8 (50.7, 327.2)	43.2 (32.2, 73.6)	-55.2 (-94.2,-30.2)	< 0.0001
Hcy (µmol/l)	16.2 (11.8, 20.0)	18. 5 (14.1, 23.1)	2.4 (0.50, 4.60)	0.0174

3.3.3. Correlation test was done between vitamin D levels and different variables in all participants, patients and control groups

Correlation between Vitamin D levels and clinical metabolic and analytical variables were shown in table 3.11. The results showed that there is a strong negative correlation between vitamin D and resistin in all participants (r = -0.172; p<0.001), and in patients with T2DM (r = -0.261; p<0.001), but not in control group (r = 0.017; p< 0.827). In addition, no correlation between vitamin D and homocysteine levels in all groups (all participants, patients and control subjects, (- r =0.054; p=0.331), (r = -0.064; p=0.366) and (r= -0.001; p= 0.994, respectively). There is a negative correlation between the vitamin D and both (FBG & SBP) in patients (r = -0.177; p=0.013) & (r= -0.242; p= 0.001), respectively. As shown in table 3.9, there is no correlation between vitamin D levels and the other clinical and metabolic parameters.

Table 3.11 Pearson correlation (r) between vitamin D and all other variables

Variable	**All**		**Patient (T2DM)**		**Control**	
Number (n)	395		200		195	
	r	p	r	p	r	p
Age (y)	0.056	0.266	-0.036	0.615	-0.034	0.636
2hBG (mg/dl)	0.058	0.316	-0.023	0.762	-0.186	0.032
FBG (mg/dl	-0.177	0.013	-0.177	0.013	N/A	N/A
HbA1c (%)	0.058	0.317	-0.050	0.486	0.163	0.104
BMI (kg/m^2)	-0.004	0.938	-0.082	0.250	-0.041	0.570
CHOL (mg/dl)	-0.044	0.424	-0.042	0.562	-0.016	0.855
TAG (mg/dl)	-0.019	0.727	0.086	0.232	-0.058	0.512
LDL (mg/dl)	0.062	0.262	0.127	0.076	-0.121	0.167
HDL (mg/dl)	0.030	0.597	0.130	0.079	-0.120	0.178
SBP (mmHg)	-0.067	0.224	-0.242	0.001	0.103	0.241
DBP (mmHg)	0.025	0.645	-0.107	0.131	0.152	0.082
Creatinine (mg/l)	-0.029	0.638	-0.062	0.471	0.020	0.824
Waist (cm)	-0.054	0.331	-0.112	0.116	0.076	0.389
Resistin ng/ml	-0.119	0.018	-0.261	0.000	0.108	0.134
Hcy (µmol/l)	0.078	0.122	-0.064	0.365	-0.002	0.980

(r) Person correlation coefficient

3.3.4. Distribution of VDR (T 56058 C) gene polymorphism (TaqI) in patients with T2DM and their healthy controls

The distribution of VDR gene polymorphisms (65058 TC) in patients with T2DM is CC (36.5%), TC (46%), TT (17.5%), while it is CC (28.7%), TC (52.8%) and TT (18.5 %) for the healthy controls; while the allele frequency is C (238) and allele T (162) for patients with T2DM and C (215) and allele T (175) in the healthy controls. The results showed that there are no statistically significant differences in genotype frequencies, CC (χ^2 = 2.719, p = 0.099) TC (χ^2 = 1.838, p= 0.175) and TT (χ^2 = 0.062, p = 0.803), between patients with T2DM and their healthy controls and also that there is no statistically significant difference in alleles frequencies "T" and "C" (χ^2 = 2.084, p = 0.149) between patients and control group. (Table 3.12).

Table 3.12 Distribution of VDR genotypes and allelic frequencies among patients with T2DM and their healthy controls

	Patient (T2DM) (n =200, 400 alleles)	**Control (n = 195, 395 alleles)**	χ^2 / ***p*. values**
CC n (%)	73 (36.5)	56 (28.7)	2.719 / 0.099
TC n (%)	92 (46)	103 (52.8)	1.838 / 0.175
TT n (%)	35 (17.5)	36 (18.5)	0.062 / 0.803
Allele C (%)	238	215	2.084 / 0.149
Allele T (%)	162	175	

p-values obtained for the χ^2-test for the CC, TC AND TT genotypes distribution of allele C and T frequencies l between patients and healthy control.

3.3.5. Association between vitamin D levels and elevated levels of resistin in patients with T2DM in representative group of the Sudanese population.

The hypothesis: There is an association between vitamin D deficiency and elevated levels of resistin in patients with T2DM. Results showed that vitamin D levels were statistically significantly higher in patients with T2DM compared with control group (51.0 (35.3 - 116.3) µg/L vs 21.8 (6.9 - 79.9) µg/L; $p< 0.0001$) Table 3.9. In contrast, resistin levels were statistically significant higher in healthy controls than in patients with T2DM (3.8 (1.80, 7.40) ng/ml vs 2.7 (2.00 4.28) ng/ml; $p= 0.002$). Table 3. 2. Pearson correlation test is presented in Fig 3.6 and Fig 3.7.

Fig 3.6 shows that there is a negative correlation between vitamin D levels and resistin levels in patients with T2DM. Whereas Fig 3.7 revealed that there is no correlation between vitamin D levels and resistin levels in the control group.

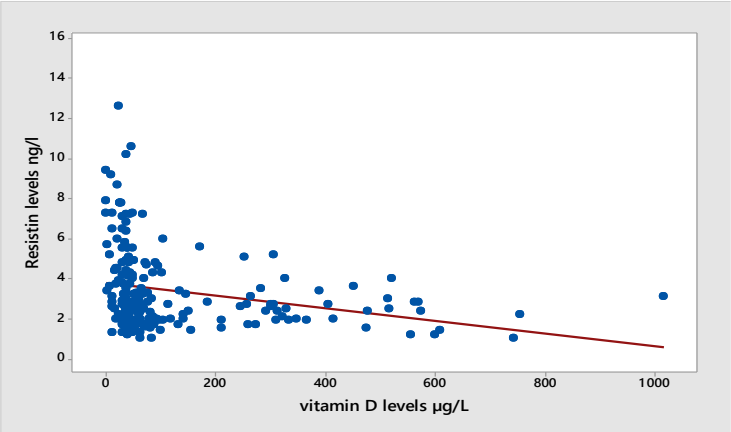


Fig 3.9 Correlation between vitamin D resistin in patients

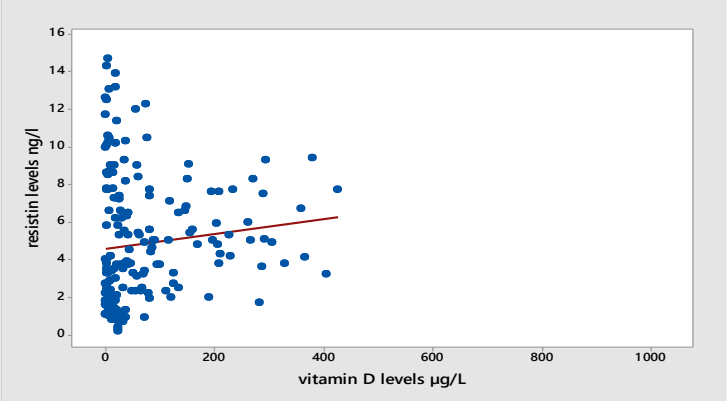


Fig 3.10 Correlation between vitamin D in control subject

Chapter 4

4. Discussion

In this case-control study I investigated the association between resistin, homocysteine and vitamin D, and T2DM in a Sudanese population, this is in addition to investigating possible associations between them and T2DM. Firstly, the levels of resistin, homocysteine and vitamin D were measured in patients with T2DM and healthy controls. Secondly, a correlation test was done to show the association between resistin, homocysteine and vitamin D and demographic, metabolic and clinical variables. Thirdly, we have tested the genetic polymorphisms in the MTHFR gene (C677T and A1298C) and the VDR gene (T 56058 C) and association of the former with homocysteine levels and the latter with vitamin D levels in patients with T2DM.

Our findings suggested possible associations between resistin, homocysteine and vitamin D, individually and together in the development of the T2DM. Our study was not limited to investigating the role of resistin in T2DM but it was extended to investigate possible association (in patients with T2DM) between resistin and vitamin D on one hand, and between resistin and homocysteine on the other hand.

Resistin is a hormone which is secreted from adipocytes and inflammatory cells, especially macrophages (Osawa *et al.*, 2005). Obesity was shown to be associated with decreased levels of vitamin D due to trapping of vitamin D in the adipose tissue and further increased risk of vitamin D deficiency (El-Tahir *et al.*, 2016). Subsequently, it is possible that vitamin D deficiency may contribute to insulin resistance and the development of metabolic syndrome secondary to inflammation aggravated by the increased resistin levels in diabetics. (El-Tahir *et al.*, 2016).

Several studies suggested an association between homocysteine and insulin resistance (Meigs *et al.*, 2001; and Oron-Herman *et al.*, 2003). An *in vitro* study using a hepatoma cell line suggested that the oxidative stress resulting from homocysteine thiolactone led to disruption of the insulin signalling pathway (Najib *et al.*, 2001). In addition, homocysteine may promote insulin resistance by inducing resistin secretion, which links inflammation and obesity with insulin resistance. Homocysteine was shown to induce resistin expression in cultured hepatocytes in mice but not in humans (Li *et al.*, 2004).

Previous studies showed that resistin is synthesized during adipogenesis and is secreted by fat cells and it contributes to insulin resistance in both humans and animals. There is a

controversy about the association between obesity in T2DM and levels of human resistin (Steppan *et al.,* 2001). In this study, levels of resistin in Sudanese patients with T2DM and their possible association with demographic, clinical and metabolic parameters were investigated. Our findings showed that the plasma resistin levels were higher in healthy controls in comparison with the patients with T2DM. Similarly, others have reported no association between resistin levels and T2DM (Stejskal *et al.,* 2003 and Lee *et al.,* 2003). However, in contrast, findings are not consistent with previous studies that demonstrated that resistin levels were elevated in patients with T2DM (Hasegawa *et al.,* 2005 and McTernan *et al.,* 2003). In the current study, we found that BMI was lower in the healthy controls compared with patients with T2DM, while there were higher levels of blood glucose (2hBG) and HbA1c in the diabetic patients. In addition, results from this study showed a positive correlation between resistin levels and FBG, 2hBG, HbA1c, log BMI and homocysteine levels.

Plasma resistin levels were higher in males compared with females in all the study population and also among the healthy controls. Findings from Fujinami *et al.,* (2003) reported that resistin concentration in diabetic patients was higher in females than in males (Fujinami *et al.,* 2003). Differences in resistin levels according to gender were also observed by Silha *et al.,* (2003), where plasma resistin levels were higher in females compared to males. Chen *et al.,* (2005) reported no significant difference in levels of resistin between males and females, which is in contrast with our study findings.

In overweight and obese individuals, our findings showed that plasma resistin levels were higher in healthy controls and in male controls compared with patients and males patients. Steppan *et al.,* (2002) demonstrated that plasma resistin levels were significantly associated with obesity and insulin resistance. I showed that resistin was lower in patients with T2DM and obese individuals and in contrast to our findings, El-Shal *et al.,* (2013) reported that resistin levels are increased in obese patients with T2DM in an Egyptian population. Most of studies on role of resistin in T2DM were from Europe and Japan. Limited numbers of studies were conducted in Africa. In addition to the study by El-Shal *et al.,* Azab *et al.,* demonstrated that serum resistin was higher in patients with T2DM compared with healthy controls *(*Azab *et al.,* 2012). This study is the first to be done in the Sudanese population.

In this study, a negative correlation was found between vitamin D levels and resistin levels in patients with T2DM. This finding is consistent with the El-Tahir *et al.,* who reported a negative correlation with resistin levels and vitamin D in a Saudi population (El-Tahir *et al.,* 2016). In addition, our studies showed increased levels of resistin in patients not taking

vitamin D supplementation compared with those who are not indicating that lower plasma resistin levels in patients with T2DM can be due to vitamin D supplementation.

The association between the increased levels of homocysteine and T2DM was reported by Hoogeveen *et al.,* (2000) and Shaikh *et al.,* (2008). Most of the studies were conducted in Europe and Asia but very few studies were conducted in Africa, whereas, one study in Sudan showed high level of homocysteine in patients with ischemic hemi disease but not in individuals with diabetes (Adbel *et al.,* 2009). Our results showed that plasma homocysteine levels were higher in patients with T2DM compared with the healthy controls. The findings are consistent with previous studies that that showed that homocysteine was elevated in patients with T2DM compared with healthy controls (Huang *et al.,* 2006 and Shaikh *et al.,* 2012). Our results showed a positive correlation between the 2hBG and HbA1c% and the levels of homocysteine in plasma. This was considered as evidence for the association between the homocysteine and T2DM. *In vitro* studies observed that insulin receptor tyrosine kinase activity, phosphorylation of phosphatidylinositol 3-kinase (PI3K) and glycogen synthase kinase-3 (GSK-3) are inhibited by homocysteine thiolactone in rat HTC-IR hepatoma cells (Najib and Sanchez-Margalet, 2005), which in turn leads to inhibition of the glycogen synthase enzyme (Liu *et al.,* 2011). It was also shown that increased expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene and glucose output from the liver are associated with elevated levels of homocysteine (Yu *et al.,* 2009).

A study by Dominguez *et al.,* (2010) observed that homocysteine levels increase with age. Our study found a positive correlation between levels of homocysteine and age in patients with T2DM but not in healthy controls. This is in addition to the positive correlation between homocysteine and Blood pressure, DBP and SBP, in all participants and in patients with T2DM but not in control subjects. Our findings are in agreement with a previous study that reported elevated levels of homocysteine in hypertensive compared with normotensive subjects (Sutton-Tyrrell *et al.,* 1997 and van Guldener *et al.,* 2003).

Several studies investigated the possible association between MTHFR gene polymorphism, levels of homocysteine and T2DM (Kluijtmans *et al.,* 1996; Abu-Amero *et al.,* 2003 and Mtiraoui, *et al.,* 2007). There is no report on the prevalence of the MTHFR genotype and T2DM was documented for the Sudanese population. Differences in the ethnicities and population genetics might contribute to differences in allele frequencies and hence disease susceptibility. The association between MTHFR gene polymorphism and complications associated with T2DM was suggested by several studies: cardiovascular disease (Clarke *et al.,* 1991), occlusive arterial disease and thrombosis (Kang *et al.,* 1992; Malinow 1994; Den

Heijer *et al.,* 1995) and diabetic nephropathy (Miraoui, *et al.,* 2007). Here we investigated the association of the MTHFR gene polymorphism and T2DM among Sudanese population in a case-control hospital based study. Our findings showed that there was no association between the MTHFR C677T polymorphisms and T2DM among Sudanese population, which is consistent with the study by Schneider *et al.,* 1998 and Sarah *et al.,* 2016. The later study showed that the frequency of the TT genotype in sub-Saharan population was zero percentage. Previous studies indicated that the prevalence of the MTHFR C677T depend on the geographical location and ethnicity (Liew *et al.,* 2012). Others reported that T allele frequency to be higher in the Italian and the Hispanics population (Botto *et al.,* 2000), and lowest in the Germans (Girelli *et al.,* 1998 and Abbate *et al.,* 1998) and American Blacks and the frequency of T homozygosity allele was shown to be zero percentage in the sub-Saharan African (Pepe *et al.,* 1998). Our results showed that the percentage of the TT genotype among the Sudanese population to be 6%, which is comparable with the prevalence observed in other ethnic group (Abu-Amero *et al.,* 2003). The MTHFR gene polymorphism was shown to be associated with increased level of the homocysteine in the plasma which has negative effects on the diabetes complication (Santos *et al.,* 2010). Hence, T2DM patients with MTHFR polymorphism may be at greater risk of Diabetic nephropathy, and atherosclerosis. Therefore, we investigated the relationship between the MTHFR C677T polymorphism and homocysteine levels in 200 patients with type 2 diabetes and 195 healthy controls. The results show that homocysteine levels were higher with statistical significance in patients with T2DM that carrying MTHFR C677T compared with the healthy controls carrying the same alleles.

With regard to the polymorphism, A1298C, in the MTHFR gene, Yan *et al.,* observed a significant association between T2DM and MTHFR A1298C gene polymorphism in an Asian population (Yan *et al.,* 2014). Conversely, our finding showed no significant association between MTHFR A1298C gene polymorphism and T2DM. Our result is consistent with a study conducted on the Caucasian population which found no association between T2DM and MTHFR A1298C gene polymorphism (Yan *et al.,* 2014). The frequencies of the 1298 CC genotype were 6% among patients with T2DM and it was 5.1% among the healthy controls. Differences in results can be related to differences in ethnicities. The frequencies of the MTHFR gene A1298C alleles and genotype was lower compared to other ethnic groups: Canada (9%), Netherlands (9%), Germany (13.8), China (17%) and Brazil (41.1%) (Abu-Amero *et al.,* 2003).

The findings also showed that the levels of homocysteine were higher in patients with T2DM carrying C1298C and A1298C alleles compared with control group carrying C1298C and A1298C and also homocysteine levels were higher in patients with T2DM who are heterozygosity for both alleles (C677T/ A1298C) than in healthy control. Van der Put *et al.*, 1998 and Moczulski *et al.*, 2003) reported that heterozygosity at both alleles decreases the MTHFR enzyme activity and elevated plasma homocysteine levels. A study from Tunisia showed that double heterozygosity (C677T/ A1298C) in patients with T2DM had not effect on the plasma homocysteine levels (Mtiraoui et al, 2007).

In addition, we compared the levels of homocysteine between the females and males. In all participants and control group, the results were higher in females compared with the males. Other studies were not in agreement with our findings, where they reported that males in general have higher homocysteine levels compared with females (Powers *et al.*, 2002). However, a study suggested that homocysteine levels increase in the females after menopause due to deficiency or completely lost of the estrogen hormone (Powers et al, 2002). Estrogen has been shown to affect plasma homocysteine levels (Kim et al, 1997 & Giri et al, 1998). In our study the age of female patients and healthy controls was (51.2±7.2) and (39.9±17.7), respectively.

In this study, has been explained for the first time the relation between levels of the homocysteine and the resistin in human plasma of the patients with T2DM. These findings revealed that the resistin levels were lower in patients compared with control, and homocysteine levels were higher in patients than in control. However, our results showed that there is a positive correlation between homocysteine and resistin levels in patients with T2DM.

Several *in vitro* and experimental studies have demonstrated that hyperhomocysteinemia can induce the ROS-PKC-NF-kB pathway to produce resistin from adipocytes (Li *et al.*, 2008).

Homocysteine was considered a strong proinflammatory factor which boosts inflammation both *in vitro* (Wang *et al.*, 2000) and in rat (Wang *et al.*, 2002). Zeng et al., mentioned that homocysteine induces synthesis and secretion of proinflammatory cytokines from macrophage, by the mediation of oxidative stress in human monocytes (Zeng *et al.*, 2003). Previous studies investigated the associate between insulin resistance and increase levels of homocysteine (hyperhomocysteinemia) (Golbahar *et al.*, 2007). In contrast, others found no association between insulin resistance and hyperhomocysteinemia (Goldsland *et al.*, 2001).

Recently, Li *et al.*, suggested that homocysteine can contribute to insulin resistance by inducing the production of adipokines (Li *et al.*, 2008).

The discovery of the association between resistin and insulin resistance has drawn the attention of many researchers and scientists to pursue further studies in this field. In mice, the association between obesity, resistin and insulin resistance appears strong, but in humans, still unclear (de Luis, 2011). In humans, Patel *et al.*, reported that resistin is mainly secreted from the macrophages (Patel *et al.*, 2003), although, fat cells secrete a huge amount of resistin (Pagano *et al.*, 2005). Recently, a link between insulin resistance and inflammatory condition was suggested (Vgontzas *et al.*, 2004). The association between diabetes and inflammation was supported by studies that used bacterial endotoxin (inflammation inducing substance) which led to insulin resistance (Cani *et al.*, 2007). The effects of inflammation on the insulin signalling attenuated by the use of aspirin as an anti-inflammatory medicine and subsequently, improve insulin resistance (Yuan *et al.*, 2001). *In vitro* study showed increased expression of resistin following exposure to pro-inflammatory factors. This study supported that resistin is the link between inflammation and obesity associated with insulin resistance (Kaser *et al.*, 2003). Najib et al observed that thiolactone (part of homocysteine) has the ability to induce oxidative stress which in-turn lead to disruption of insulin signalling pathway in rat hepatoma cell line (Najib *et al.*, 2005). *In vitro* and in experimental studies by Li *et al.*, was showed that homocysteine (thiolactone) enhances insulin resistance via up-regulation of resistin expression and secretion from the adipocytes (Li *et al.*, 2008).

Although insulin resistance and change in insulin secretion are considered as main factors contributing to T2DM, the precise pathogenesis of T2DM remains unclear. Recently, it has been suggested that environmental factors and genetic factors might play important roles (accelerating or protective effect) in the development or protection from T2DM by affecting insulin secretion and its action or both. Among the environmental factors is “hypovitaminosis D” that is due to deficiency which has been suspected to be a risk factor for glucose intolerance.

In this study I investigated possible association between vitamin D levels and T2DM in a study population from Sudan, where there is no seasonal variation in sunlight. Our hypothesis is that our results might be different from that from other populations due to differences in lifestyle, ethnicity and genetic factors. My first findings note that vitamin D levels were higher remarkably in patients with T2DM compared with healthy controls group. Among patients with T2DM, some used vitamin D supplementation while others not. This can be attributed to the fact that some of the individuals with T2DM are already on vitamin D

supplement. Also, these results showed a marked decrease of FBG and 2hBG in patients who took vitamin D compared to those who didn't. Furthermore, in patients with T2DM, a negative correlation was found between vitamin D and several variables including fasting blood glucose (FBG), Systolic blood pressure (SBP) and resistin levels. In addition, I found that resistin levels were higher in healthy controls compared to their patients with T2DM as well as lower in patients who took vitamin D compared with patients who did not. these results indicate that vitamin D supplementation improves glucose levels and reduces resistin levels. It is worth mentioning that resistin was considered as one of the main factors that contribute to insulin resistance and subsequently T2DM and its complication (El-Tahir *et al.,* 2016).

Scragg *et al* (2004) observed that there is an inverse association between vitamin D status and diabetes in Mexican American and non-Hispanic white but not in non-Hispanic Black (Sragg *et al.,* 2004). Authors questioned the lack of association between vitamin D status and diabetes in non-Hispanic Black, particularly taking into account that their low serum vitamin D levels. An explanation for this lack of association could be the existence of variable threshold effect among different ethnic groups (Scragg *et al.,* 2004). A study from New Zealand found that vitamin D levels were lower in newly diagnosed patients with T2DM and impaired glucose compared with matched healthy control (Scragg *et al.,* 1995). Kedari and Hareesh (2014) have reported that deficiency of vitamin D is associated with the progression of T2DM. On the other hand, a Bangladeshi group at high risk for T2DM was shown to have low vitamin D levels compared with those with no and low risk of T2DM (Boucher *et al.,* 1995). This study indicated also that T2DM was more prevalent among Bangladeshis than British Caucasians, concluding that vitamin D plays an important role in the pathogenesis of the T2DM. Overall, long term studies revealed that there is a negative relation between the vitamin D and development of T2DM (Mitri *et al.,* 2011).

Boucher et al showed that Vitamin D deficiency led to reduction in insulin secretion and Vitamin D supplementation led to improvement in the function of β- cell and glucose tolerance (Boucher et al., 1995). These results showed reduced glucose levels in Sudanese patients with T2DM who took Vitamin D supplementation. Several studies showed that treatment of osteomalacia for long time led to improve glucose tolerance (Norman *et al.,* 1980 and Boucher *et al.,* 1995).

On the other hand, Boucher et al (1998) reported that vitamin D supplementation does not improve glucose tolerance in peoples without hypovitaminosis D (Boucher *et al.,* 1998), indicating that the effect depends on the vitamin D status in the diabetic patients.

Cavalier *et al.* (2011) observed an increase in the levels of fasting blood glucose and HbA1c in patients with T2DM in the winter and a subsequent decrease in the summer. The seasonal variation in glycaemic parameters in Europe indicates the importance of exposure to sunlight. In Sudan, there is no seasonal variation in sunlight.

The association between Obesity and metabolic syndrome or T2DM is very well established. Obesity was shown to be associated with a depletion of vitamin D (Palomer *et al.*, 2008). Our results showed higher waist circumference (W) and lower vitamin D levels in the healthy controls compared with patients with T2DM. In addition, these results showed lower waist circumference and BMI in patients who took vitamin D supplementation compared with those who did not. Accordingly, there is an inverse correlation between obesity and vitamin D levels. Holick reported that vitamin D is stored in adipocytes, and obesity individuals are chronically vitamin D deficient (Holick, 2004) i.e. fat cells trap vitamin D leading to reduction in its bioavailability and therefore increase risk of vitamin D deficiency. Zittermann found that the vitamin D deficiency associated with obesity is associated with increased levels of parathyroid hormone (PTH) (Zittermann, 2003), which in turn lead to overproduction of glucose (glucose intolerance) (Ivarsson, 2014). It was shown also that vitamin D can suppress PTH and stimulates insulin secretion by β- cells (Bikle, 1992). It was suggested that PTH and insulin stimulate synthesis of vitamin D, and deficiency of insulin (in case of diabetes) may reduce vitamin D production (Bikle, 1992). In addition, it was shown that patients with hyperparathyroidism are at high risk of developing T2DM and insulin resistance (Ivarsson, 2014).

Here I also studied the association between vitamin D and resistin levels in Sudanese patients with T2DM. I showed that vitamin D levels was lower in the healthy controls compared with patients, and lower in patients with T2DM who took Vitamin D supplementation compared with those who did not. In both controls and patients, taking vitamin D supplementation was associated with lower resistin levels. In addition, there was a negative correlation between vitamin D and resistin levels in patients with T2DM but not in the healthy controls. These results are not in agreement with the study by Vilarrasa et. al. that reported no significant association between plasma vitamin D and resistin levels (Vilarrasa *et al.*, 2010), but these results were in agreement with the study by EL-Tahir et al that showed a significant negative correlation between vitamin D and resistin levels in patients with T2DM (El-Tahir *et al.*, 2016). Therefore, the negative correlation between vitamin D and resistin levels observed in our study can be attributed to vitamin D supplementation.

VDR gene polymorphism was studied among different ethnic populations. It was reported that VDR is expressed in different tissues in the body among them pancreatic β- cells and fat cells (Reis *et al.,* 2005 and Howard *et al.,* 1995), and it was suggested that VDR polymorphisms might influence glucose metabolism and insulin secretion (Oh and Barrett, 2002). Several studies investigated the possible association of VDR polymorphisms and T2DM, and it is generally believed that there is an association between VDR polymorphisms and clinical variables (glucose levels, obesity and insulin levels) of T2DM, but the other studies reported no association between VDR polymorphism and T2DM (osman et al,2016). Polymorphism in the Taq-I (rs731236) was found to be lower in Africans compared with Caucasians (Smolders *et al.,* 2009). In the present study, we investigated the possible association of the VDR polymorphism Taq-I (rs731236) with T2DM in a Sudanese study population. Our results showed that there is no association between TT/TC/CC genotype nor T/C allele frequencies. Studies by Malecki et al and Cyganek et al were in agreement with our findings, where they found no association between Taq (rs731236) and Bsm (rs1544410) VDR gene polymorphisms and T2DM (Malecki *et al.,* 2003; Cyganek *et al.,* 2006). In addition, our results showed that allele and genotype distribution of the Taq (rs731236) in the Sudanese population is similar to that in the Emirati population (Osman *et al.,* 2015). In contrast, several studies showed an association between the two SNPs Taq (rs731236) & Bsm (rs1544410) and T2DM (Ortlepp et al., 2003). A study from Greece by Panierakis et al found a strong association between the genetic polymorphisms in VDR gene, rs 731236 (Taq-I) and T2DM) (Panierakis *et al.,* 2009). Taneja et al reported that the Taq-I polymorphism in VDR gene is prevalence in Indians and is associated with T2DM (Taneja *et al.,* 2016).

4.1. Conclusion

Previous studies suggested a role for Vitamin D deficiency and elevated levels of plasma resistin and homocysteine levels in the pathogenesis of insulin resistance and T2DM. Our results are in agreement with previously reported high levels of homocysteine in patients with T2DM. In addition, a positive correlation was observed between levels of homocysteine, resistin, FBG, HbA1c, as well as a positive correlation between resistin and FBG and HbA1c, and a negative correlation between vitamin D and resistin, homocysteine and FBG levels in patients with T2DM. The negative association between vitamin D and resistin/blood glucose levels suggest that vitamin D might have an impact on resistin levels in patients with T2DM and improved insulin action/blood glucose levels and T2DM. Taken together, our results

suggest a possible role for vitamin D, resistin and homocysteine in the development of insulin resistance and T2DM.

4.2. Recommendation for further study

Further Sudanese population studies are needed to investigate the following:

1. The role of resistin in Obesity
2. The mechanism of expression of resistin gene by vitamin D deficiency or hyperhomocysteinemia or both.
3. *In vivo* studies (animal experiments) to explore how Vitamin D deficiency induces/modifies expression of resistin gene.
4. *In vitro* studies to explore the effect of hyperhomocysteinemia on the expression of resistin gene.
5. The role of Vitamin D in glycaemic status in patients with T2DM and its role in keeping normal blood glucose levels.
6. Develop sensitive techniques to assess insulin sensitivity and develop markers associated with obesity.
7. Large samples size studies are needed to investigate the allele and genotype frequencies of two MTHFR gene variants (C677T and A1298) in the Sudanese population. It is worth mentioning that limited studies were conducted in African populations investigating these gene variants in the MTHFR gene.
8. Large samples size studies are needed to investigate the allele and genotype frequencies of VDR T56058C/rs731236 (*Taq*) mutation and other gene variant in VDR: rs 544410 (*Bsml*), rs7975232 (*Apal*) and rs 10735810 (*Fok1*)) among different Sudanese tribes and ethnic groups living in Sudan.

4.3. Study limitations

Although the sample size was adequate for most of the association studies, a larger sample size (based on the VDR and MTHFR allele frequencies) would be required to improve the power of the statistical analysis. This is in addition to the financial constraints that aborted our plans to investigate the following:

1. Associations between genetic variants of vitamin D metabolism associated with vitamin D deficiency and T2DM e.g. vitamin D 25-hydroxylase (CYP2R1), sterol 27-hydroxylase (CYP27A1), 25-Hydroxyvitamin D3 1-alpha-hydroxylase (CYP27B1), 1,25-dihydroxyvitamin D3 24-hydroxylase (CYP24) and GC gene (encoding the Vitamin D binding protein).

2. The effect of resistin on insulin resistance and glucose output, via the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glycogen synthase genes (GS) using a hepatocyte cell line for *in vitro* culture mod

3. The role of ethnicity as one of the limitation and that this can be done in further studies

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Appendix:1 Questionnaire

Homocysteine, resistin and vitamin D level in type 2 diabetic patients

Code :		Name :	
Age:	Sex : M [ ] F [ ]	Chart No. :	

- **Type of DM** **Type 1** ☐ **Type 2** ☐

- **Drug history** **B_{12}** ☐ **Folate** ☐
  Statins ☐ **Vitamin D** ☐
  Niacin ☐ **anti-diabetes drugs** ☐

Peripheral neuropathy ☐

- **SBP :** ☐ **DBP :** ☐ **Height :** ☐ **Weight :** ☐ **BMI :** ☐
- **Fasting blood sugar :** ☐ **Cholesterol :** ☐ **Triglyceride :** ☐
- **LDL :** ☐ **HDL :** ☐ **HbA1C :** ☐
- **Albumin :** ☐ **Serum creatinine :** ☐
- **Homocysteine level :** ☐
- **Vit. B_{12} level :** ☐
- **Serum folate level :** ☐

Appendix 2 Measurement of human resistin

The blank, standards and samples (patients and healthy controls) in the Layout of ELISA plate were duplicated

Table 1 A2 Layout for ELISA (Assay -1/resistin)

B	B	C1	C1	C9	C9	C17	C17	P5	P5	P13	P13
S1	S1	C2	C2	C10	C10	C18	C18	P6	P6	P14	P14
S2	S2	C3	C3	C11	C11	C19	C19	P7	P7	P15	P15
S3	S3	C4	C4	C12	12	C20	C20	P8	P8	P16	P16
S4	S4	C5	C5	C13	113	P1	P1	P9	P9	P17	P17
S5	S5	C6	C6	C14	C14	P2	P2	P10	P10	P18	P18
S6	S6	C7	C7	C15	C15	P3	P3	P11	P11	P19	P19
S7	S7	C8	C8	C16	C16	P4	P4	P12	P12	P20	P20

Table 2.A2 Raw data of resistin absorbance (Assay -1/resistin)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.047	0.046	1.848	2.009	0.803	0.831	1.196	1.357	0.633	0.62	1.979	1.941
B	2.525	2.522	2.356	2.405	1.369	1.334	1.006	1.056	0.851	0.837	0.743	0.762
C	1.278	1.29	1.237	1.295	1.282	1.32	0.422	0.434	1.216	1.212	1.373	1.398
D	0.609	0.62	1.876	1.914	1.202	1.24	0.928	0.942	0.603	0.565	1.829	2.118
E	0.318	0.285	1.677	1.654	0.641	0.619	0.999	1.006	0.564	0.553	0.921	0.955
F	0.161	0.155	1.394	1.335	1.133	1.107	1.211	1.266	1.119	1.072	0.557	0.538
G	0.092	0.088	0.929	0.97	1.116	1.05	0.453	0.426	0.647	0.673	0.618	0.66
H	0.063	0.058	0.601	0.557	0.688	0.704	0.641	0.671	0.634	0.654	1.405	1.373

Table 3. A2 Raw data of resistin concentration (Assay -1/resistin)

	1	2	3	4	5	6	7	8	9	10	11	12
A			7.312	7.948	3.169	3.279	4.728	5.365	2.495	2.444	7.831	7.681
B	9.995	9.984	9.327	9.518	5.414	5.275	3.972	4.17	3.358	3.304	2.93	3.004
C	5.051	5.101	4.89	5.118	5.066	5.22	1.656	1.704	4.807	4.792	5.427	5.528
D	2.398	2.441	7.424	7.572	4.749	4.903	3.663	3.72	2.374	2.225	7.236	8.384
E	1.244	1.115	6.632	6.544	2.526	2.438	3.946	3.973	2.221	2.179	3.638	3.772
F	0.622	0.6	5.513	5.278	4.475	4.374	4.787	5.003	4.419	4.233	2.194	2.117
G	0.348	0.335	3.669	3.83	4.408	4.148	1.781	1.674	2.551	2.651	2.437	2.602
H	0.235	0.216	2.369	2.192	2.711	2.776	2.524	2.643	2.5	2.579	5.555	5.427

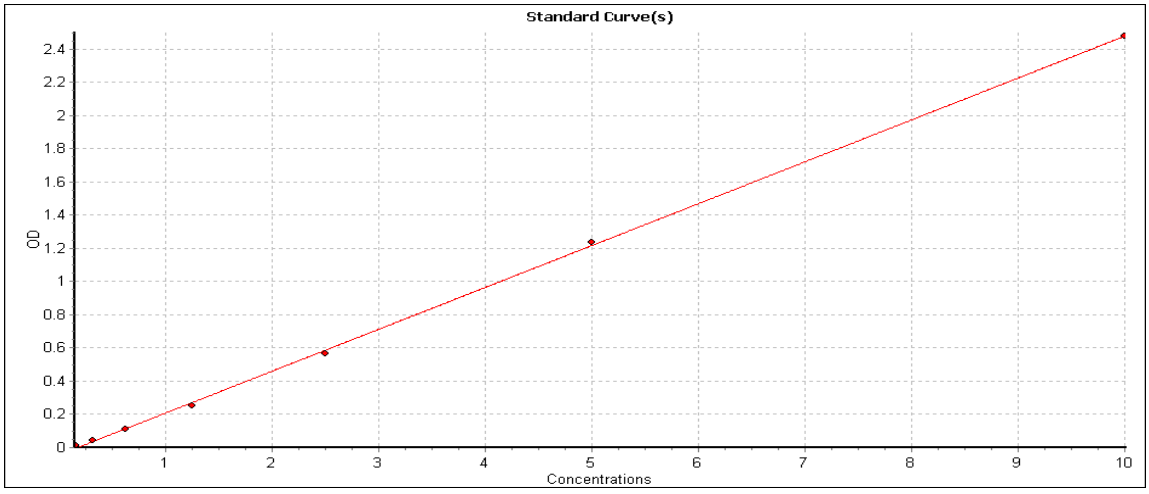


Fig 1. A2 Standard curve for ELISA (Assay -1/resistin)

Table 4. A.2 Layout for ELISA (Assay -6/ resistin)

B	B	P132	P132	P142	P142	P150	P150	C45	C45	C53	C53
S1	S1	P134	P134	P143	P143	P153	P153	C46	C46	C54	C54
S2	S2	P135	P135	P144	P144	P154	P154	C47	C47	C55	C55
S3	S3	P136	P136	P145	P145	P155	P155	C48	C48	C56	C56
S4	S4	P137	P137	P146	P146	C41	C41	C49	C49	C57	C57
S5	S5	P139	P139	P147	P147	C42	C42	C50	C50	C58	C58
S6	S6	P140	P140	P148	P148	C43	C43	C51	C51	C59	C59
S7	S7	P141	P141	P149	P149	C44	C44	C52	C52	C60	C560

Table 5.A2 Raw data of resistin absorbance (Assay -6/resistin)

	1	2	3	4	5	6	7	8	9	10	11	12
A			5.113	5.357	2.502	3.41	7.874	7.784	12.518	13.754	4.163	4.288
B	9.621	10.306	2.501	2.626	6.31	6.583	7.308	7.312	14.254	14.352	7.603	7.813
C	4.901	5.263	2.581	3.062	10.162	11.03	7.912	7.892	8.502	8.713	9.081	9.137
D	2.388	2.67	2.56	2.73	5.43	5.6	7.174	7.165	8.897	8.245	8.158	8.516
E	1.287	1.072	3.708	4.797	7.101	7.45	7.893	7.472	7.913	7.635	5.062	4.943
F	0.527	0.598	2.805	3.26	8.823	8.636	14.653	14.763	6.905	6.615	7.351	7.751
G	0.258	0.388	5.685	5.368	2.681	2.792	7.626	7.697	8.142	8.466	5.085	4.766
H	0.209	0.199	5.199	4.291	4.904	5.358	7.941	9.061	9.186	9.498	7.687	7.806

Table 6. A2 Raw data of resistin concentration (Assay -6/resistin)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.059	0.052	0.986	1.031	0.503	0.671	1.497	1.48	2.356	2.585	0.81	0.833
B	1.82	1.947	0.503	0.526	1.207	1.258	1.392	1.393	2.677	2.695	1.447	1.485
C	0.947	1.014	0.517	0.607	1.92	2.081	1.504	1.5	1.613	1.652	1.72	1.731
D	0.482	0.534	0.514	0.545	1.045	1.076	1.367	1.366	1.686	1.565	1.549	1.616
E	0.278	0.238	0.726	0.928	1.354	1.418	1.5	1.422	1.504	1.453	0.976	0.955
F	0.137	0.151	0.559	0.643	1.672	1.638	2.751	2.771	1.317	1.264	1.4	1.474
G	0.088	0.112	1.092	1.033	0.536	0.557	1.451	1.464	1.546	1.606	0.981	0.922
H	0.079	0.077	1.002	0.834	0.947	1.031	1.509	1.716	1.74	1.797	1.462	1.484

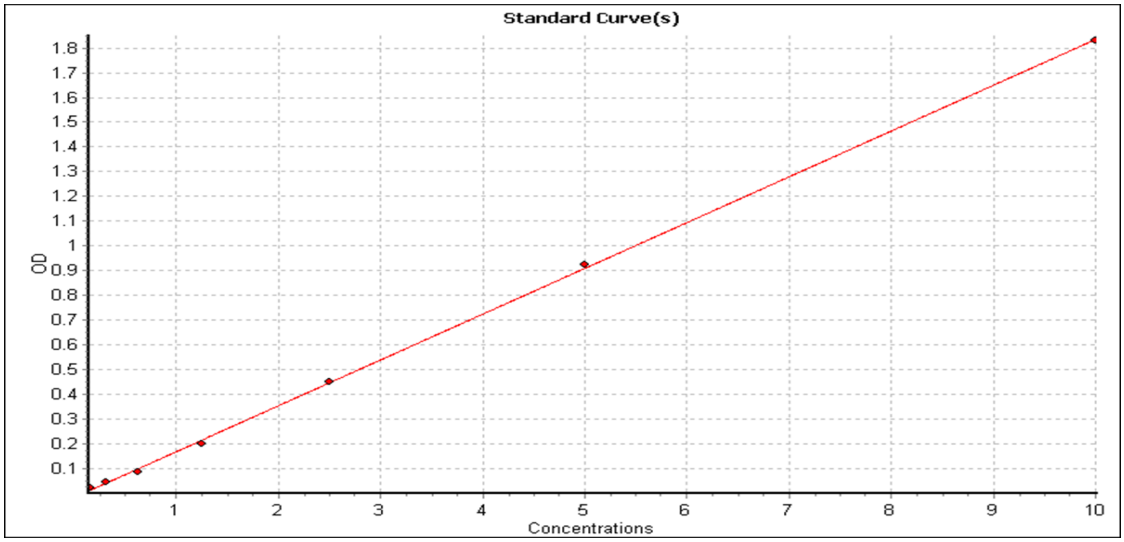


Fig 2. A2 Standard curve for ELISA (Assay -6/resistin)

Appendix 3 Measurement of the homocysteine

The standards, controls and samples (patients and healthy controls) in the Layout of ELISA plate were duplicated

Table 1A3 Layout for ELISA (Assay -5/homocysteine)

S1	S1	C_H	C_H	P108	P108	P116	P116	C64	C64	C72	C73
S2	S2	P101	P101	P109	P109	P117	P117	C65	C65	C73	C73
S3	S3	P102	P102	P110	P110	P118	P118	C66	C66	C74	C74
S4	S4	P103	P103	P111	P111	P119	P119	C67	C67	C75	C75
S5	S5	P104	P104	P112	P112	P120	P120	C68	C68	C76	C76
S6	S6	P105	P105	P113	P113	C61	C61	C69	C69	C77	C77
C_L	C_L	P106	P106	P114	P114	C62	C62	C70	C70	C78	C78
C_M	C_M	P107	P107	P115	P115	C63	C63	C71	C71	C79	C79

Table 2.A3Raw data of homocysteine absorbance (Assay -5/homocysteine)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.803	2.835	2.203	2.271	2.764	2.739	2.545	2.603	2.602	2.708	2.471	2.546
B	2.877	2.877	2.674	2.652	2.646	2.634	2.108	2.143	2.499	2.451	2.255	2.283
C	2.748	2.744	2.335	2.285	2.56	2.637	2.382	2.494	2.577	2.567	2.428	2.492
D	2.658	2.651	2.584	2.679	2.647	2.616	2.692	2.696	2.329	2.186	2.163	2.32
E	2.107	1.984	2.611	2.567	2.69	2.625	2.74	2.722	2.603	2.684	2.57	2.662
F	1.649	1.524	2.405	2.529	2.689	2.542	2.361	2.478	2.329	2.393	2.514	2.475
G	2.796	2.788	2.556	2.476	2.568	2.547	2.623	2.516	2.433	2.469	2.318	2.252
H	2.753	2.755	2.716	2.662	2.739	2.75	2.683	2.647	2.67	2.604	2.586	2.594

Table 3.A3 Raw data of homocysteine concentration (Assay -5/homocysteine)

	1	2	3	4	5	6	7	8	9	10	11	12
A	4.231	3.886	20.706	17.269	4.693	5.005	8.362	7.17	7.189	5.435	10.189	8.346
B	3.475	3.475	5.944	6.31	6.404	6.611	26.566	24.268	9.451	10.731	18.011	16.751
C	4.896	4.947	14.575	16.654	8.043	6.559	12.867	9.575	7.685	7.895	11.408	9.626
D	6.212	6.317	7.54	5.87	6.389	6.926	5.669	5.608	14.836	21.652	23.011	15.177
E	26.644	36.873	7.031	7.901	5.703	6.776	4.995	5.242	7.17	5.789	7.829	6.145
F	89.653	124.796	12.127	8.737	5.713	8.444	13.617	9.989	14.812	12.525	9.081	10.079
G	4.31	4.402	8.122	10.044	7.874	8.322	6.817	9.026	11.246	10.23	15.257	18.164
H	4.833	4.804	5.329	6.149	5.01	4.86	5.805	6.382	6.01	7.157	7.512	7.359

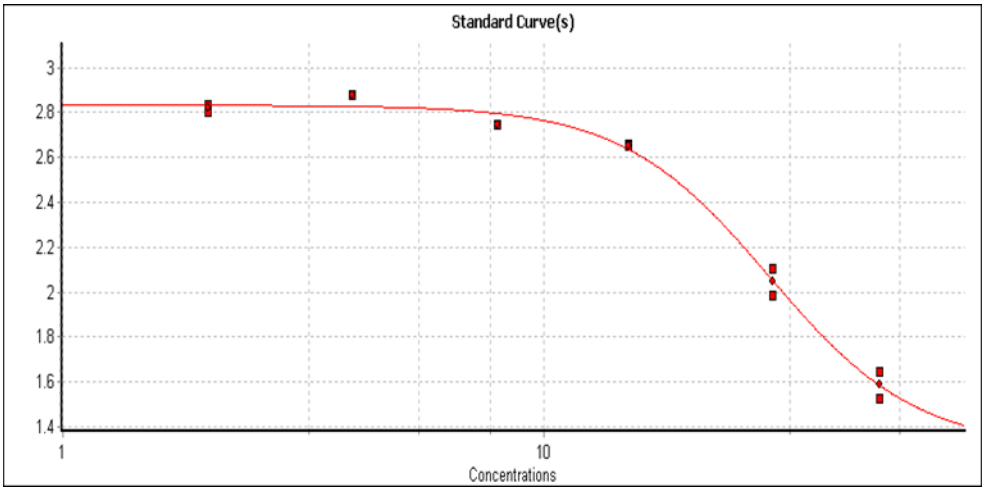


Fig 1. A3Standard curve for ELISA (Assay -6/homocysteine)

Table 4A3 Layout for ELISA (Assay -5/homocysteine)

S1	S1	C_H	C_H	P88	P88	P96	P96	C45	C45	C53	C53
S2	S2	P81	P81	P89	P89	P97	P97	C46	C46	C54	C54
S3	S3	P82	P82	P90	P90	P98	P98	C47	C47	C55	C55
S4	S4	P83	P83	P91	P91	P99	P99	C48	C48	C56	C56
S5	S5	P84	P84	P92	P92	P100	P100	C49	C49	C57	C57
S6	S6	P85	P85	P93	P93	C42	C42	C50	C50	C58	C58
C_L	C_L	P86	P86	P94	P94	C43	C43	C51	C51	C59	C59
C_M	C_M	P87	P87	P95	P95	C44	C44	C52	C52	C60	C60

Table 5.A3 Raw data of homocysteine absorbance (Assay -5/homocysteine)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.68	2.71	1.647	1.716	2.333	2.363	1.9	1.967	1.813	1.762	1.912	2.014
B	2.563	2.539	2.26	2.33	1.969	1.889	2.437	2.49	2.144	1.976	2.339	2.345
C	2.455	2.395	2.201	2.293	1.891	1.809	2.024	2.124	2.171	1.901	2.045	2.073
D	1.919	1.889	2.126	2.105	1.813	1.846	2.154	2.27	1.88	1.912	2.284	2.288
E	1.399	1.33	2.355	2.402	1.983	1.939	1.992	2.191	2.105	2.078	1.965	2.124
F	1.103	1.136	2.26	2.308	2.54	2.502	2.047	2.157	1.241	1.246	2.18	2.206
G	2.457	2.456	2.194	2.328	2.258	2.219	1.871	1.965	1.86	1.617	2.046	2.026
H	2.207	2.203	1.623	1.677	2.073	2.168	2.217	2.342	2.302	2.198	2.172	2.251

Table 6.A3 Raw data of homocysteine concentration (Assay -5/homocysteine)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.455	-0.141	25.896	23.751	7.689	7.033	18.429	16.62	20.882	22.377	18.1	15.39
B	2.779	3.275	9.37	7.759	16.577	18.724	5.412	4.287	12.117	16.392	7.555	7.421
C	5.025	6.323	10.75	8.591	18.666	20.992	15.113	12.607	11.461	18.415	14.579	13.86
D	17.922	18.735	12.561	13.075	20.885	19.936	11.869	9.134	18.988	18.094	8.801	8.71
E	34.416	37.091	7.214	6.174	16.204	17.364	15.965	10.984	13.075	13.757	16.673	12.593
F	46.882	45.299	9.349	8.257	3.252	4.036	14.535	11.789	40.708	40.498	11.249	10.629
G	4.997	5.004	10.91	7.815	9.407	10.322	19.23	16.681	19.555	26.876	14.553	15.08
H	10.603	10.701	26.663	24.949	13.868	11.523	10.365	7.493	8.398	10.815	11.439	9.557

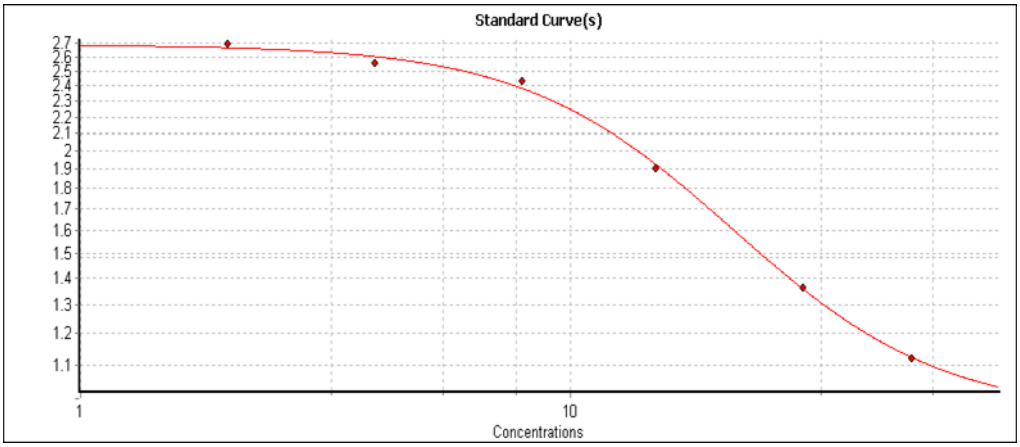


Fig 2. A3 Standard curve for ELISA (Assay -5/homocysteine)

Appendix 4 Method of genotyping (C677T) using 3M™ integrated Cycler

All samples were placed in the disc below, which it contains 12 column (numbered 1, 2, 3, 4 etc. …12) and each column have 8 wells (numbered and then covered and placed into the machine (3M™ integrated Cycler's). all samples and NTC were duplicated

ɔort

Assay Name	MTHFR 677 Direct	**Run Name**	abdelrouf4 9-2-2015 C677T 02-09-2015 At 1207
Test By	ICS Administrator	**Report By**	ICS Administrator
Lot Number	26075	**Lot Expiration**	08/2015
Instrument	200057	**Software**	5.0.0.17
Disc Id	A10167272	**Test Date**	2/9/2015 12:32:45 PM

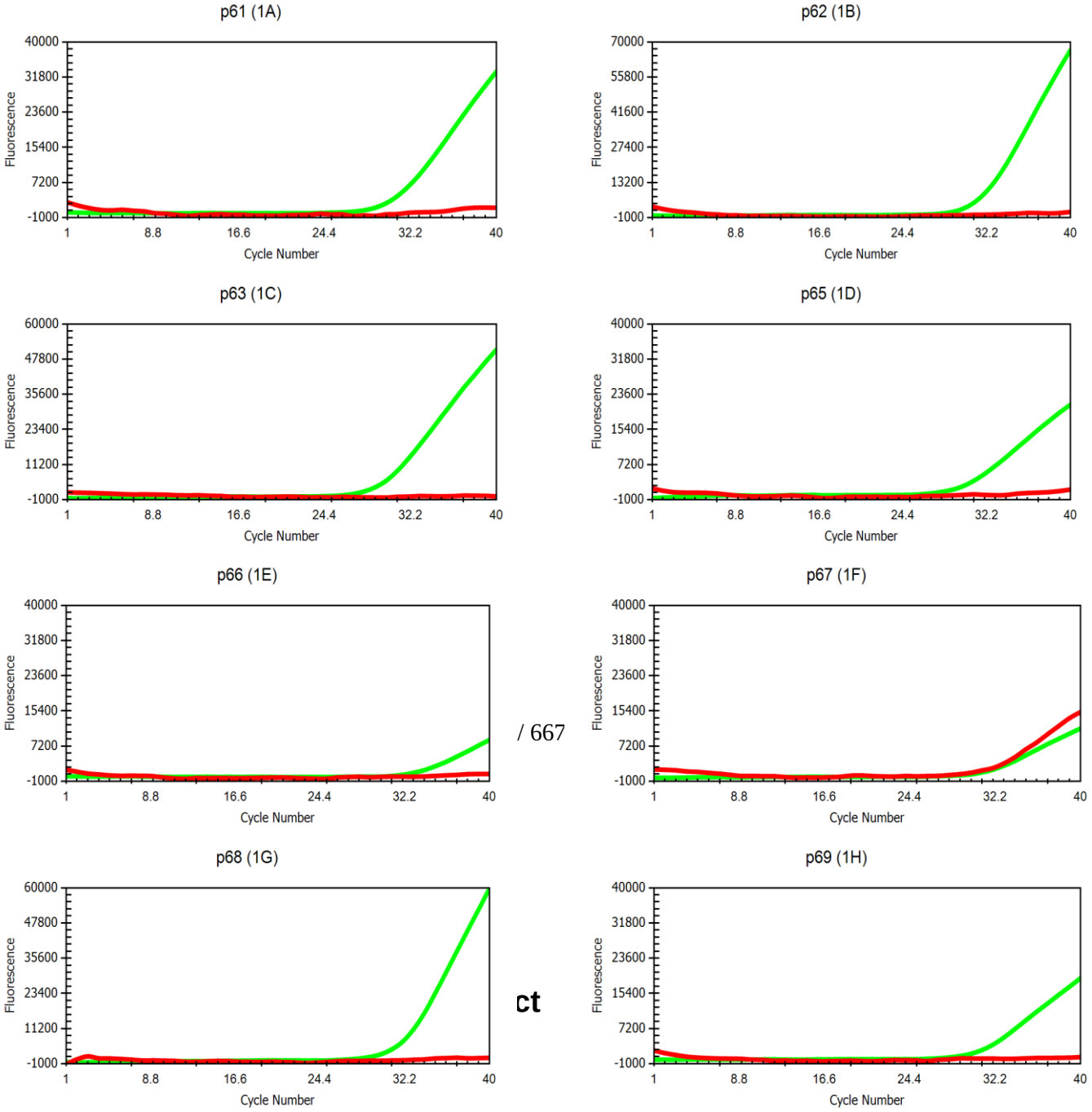
Well	S	Sample Type	677 WT (FAM)	677 M (CFR610)	QC Statement/Notes
1A		pUnknown 6	32.2	0	
1B		pUnknown 6	31.5	0	
1C		pUnknown 6	30.4	0	
1D		pUnknown 6	33	0	
1E		pUnknown 6	38.6	0	
1F		pUnknown 6	36.4	37	
1G		pUnknown 6	32	0	
1H		pUnknown 6	33.9	0	
2A		pUnknown 7	30.9	0	

2B	pUnknown	33.9	0
2C	NUnknown	0	0

MTHFR 677 Direct_1 Segment Report

Assay Name	MTHFR 677 Direct	**Run Name**	abdelrouf4 9-2-2015 C677T 02-09-2015 At 1207
Test By	ICS Administrator	**Report By**	ICS Administrator
Lot Number	26075	**Lot Expiration**	08/2015
Instrument	200057	**Software**	5.0.0.17
Disc Id	A10167272	**Test Date**	2/9/2015 12:32:45 PM

-677 WT -677 M



/ 667

ct

Actual Run Time: 50 Minutes

Hold 1 Cycle	360.0	97.0	10.0	
Hold 2	Not Used			
Denaturation	10.0	97.0	10.0	
Anneal	30.0	60.0	10.0	Yes
Extend 1	Not Used			
Extend 2	Not Used			

Final Cycles	Time(s)	Temperature(C)	Ramp Rate(C/s)	Capture
Final 1 No	Not Used			
Final 2 No	Not Used			

Initial Cycles	Time(s)	Temperature(C)	Ramp Rate(C/s)	Capture

Main Cycles	Time(s)	Temperature(C)	Ramp Rate(C/s)	Capture

Appendix 5 Method of genotyping (A1298C) using TaqMan

The reagents (master mix and TaqMan), DNA samples (patients and controls) and Negative template control all were duplicate

Table 1A5 Layout of real time PCR plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	**P46**	**P47**	**P48**	**P49**	**P50**	**P51**	**P52**	**P53**	**P54**	**P55**		NTC
B	**P46**	**P47**	**P48**	**P49**	**P50**	**P51**	**P52**	**P53**	**P54**	**P55**		**NTC**
C	**P56**	**P57**	**P58**	**P59**	**P60**	**P61**	**P62**	**P63**	**P64**	**P65**		**NTC**
D	**P56**	**P57**	**P58**	**P59**	**P60**	**P61**	**P62**	**P63**	**P64**	**P65**		**NTC**
E	**C137**	**C141**	**C145**	**C150**	**C151**	**C152**	**C153**	**C155**	**C157**	**C159**		
F	**C137**	**C141**	**C145**	**C150**	**C151**	**C152**	**C153**	**C155**	**C157**	**C159**		
G	**C160**	**C163**	**C168**	**C171**	**C181**	**C182**	**C183**	**C185**	**C1**	**C2**		
H	**C160**	**C163**	**C168**	**C171**	**C181**	**C182**	**C183**	**C185**	**C1**	**C2**		

MTHFR gene polymorphism (A1298C) according to the Layout of real time Lightcycler 96 plate

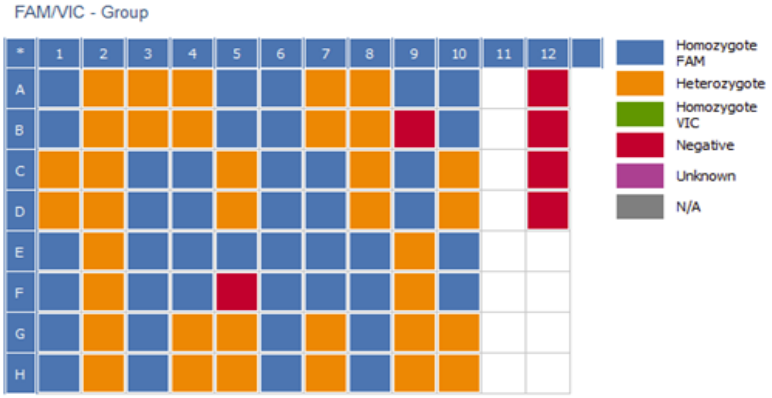


Fig 1A5 MTHFR gene polymorphism (A1298C) were obtained by real time Lightcycler 96 instrument (amplification) and LightCycler 96 application software,

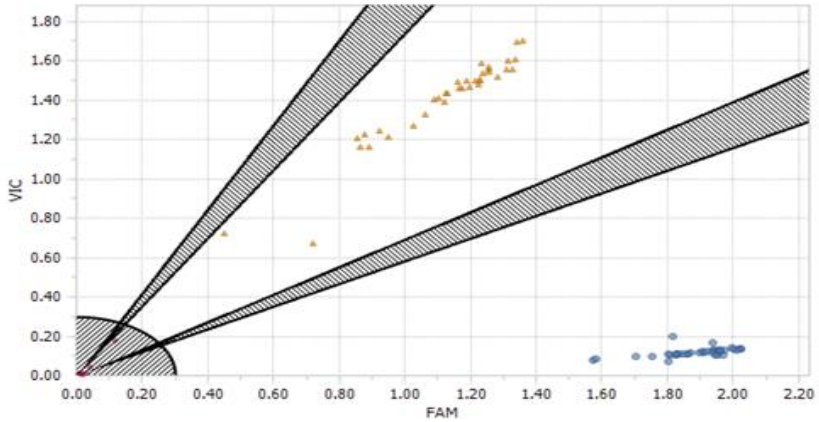


Fig 2A5 scatter plot for genetic polymorphisms separation

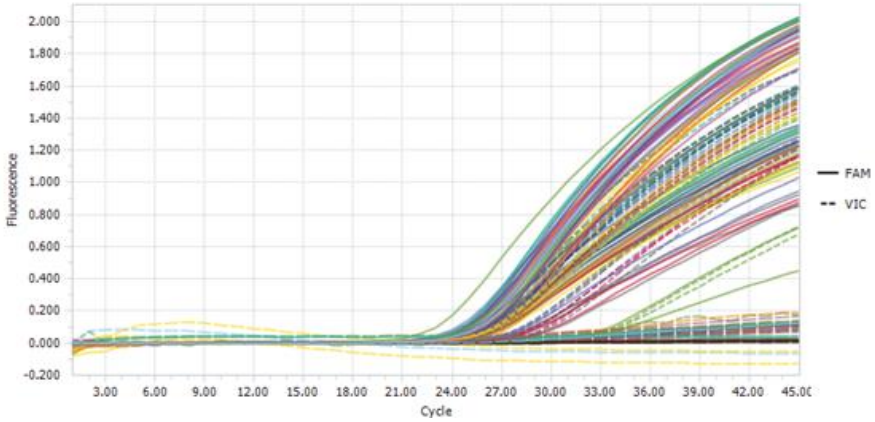


Fig3A5 amplification curve with fluorescence (Vic, FAM and NTC) of the LightCycler 96 instrument

Appendix 6 Method of genotyping (T65058C) using TaqMan

The reagents (master mix and TaqMan), DNA samples (patients and controls) and Negative template control all were duplicate

Table 1A6 Layout of real time PCR plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	**P1**	**P1**	**P9**	**P9**	**P17**	**P17**	**C6**	**C6**	**C16**	**C16**		
B	**P2**	**P2**	**P10**	**P10**	**P18**	**P18**	**C7**	**C7**	**C17**	**C17**		
C	**P3**	**P3**	**P11**	**P11**	**P19**	**P19**	**C9**	**C9**	**C18**	**C18**		
D	**P4**	**P4**	**P12**	**P12**	**P20**	**P20**	**C10**	**C10**	**C19**	**C19**		
E	**P5**	**P5**	**P13**	**P13**	**C1**	**C1**	**C11**	**C11**	**C20**	**C20**	**NTC**	**NTC**
F	**P6**	**P6**	**P14**	**P14**	**C2**	**C2**	**C12**	**C12**	**C21**	**C21**	**NTC**	
G	**P7**	**P7**	**P15**	**P15**	**C4**	**C4**	**C13**	**C13**	**C22**	**C22**		
H	**P8**	**P8**	**P16**	**P16**	**C5**	**C5**	**C14**	**C14**	**C24**	**C24**		

VDR gene polymorphism (T65058C) according to the Layout of real time Lightcycler 96 plate

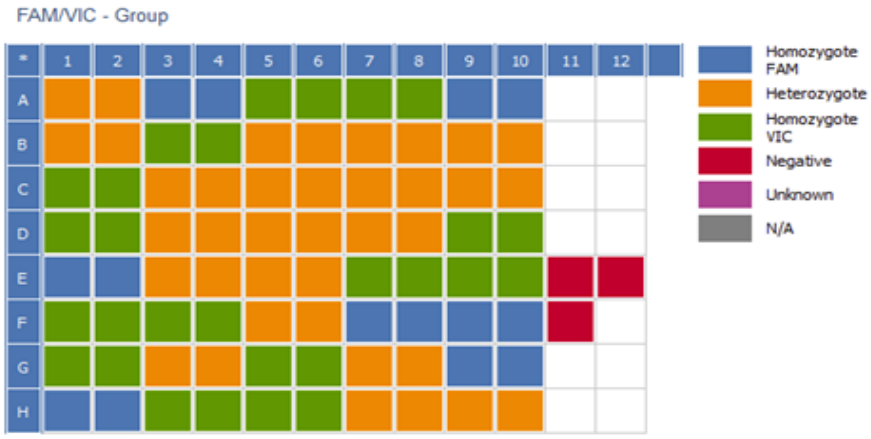


Fig 1A6VDR gene polymorphism (T65058C) were obtained by real time Lightcycler 96 instrument (amplification) and LightCycler 96 application software,

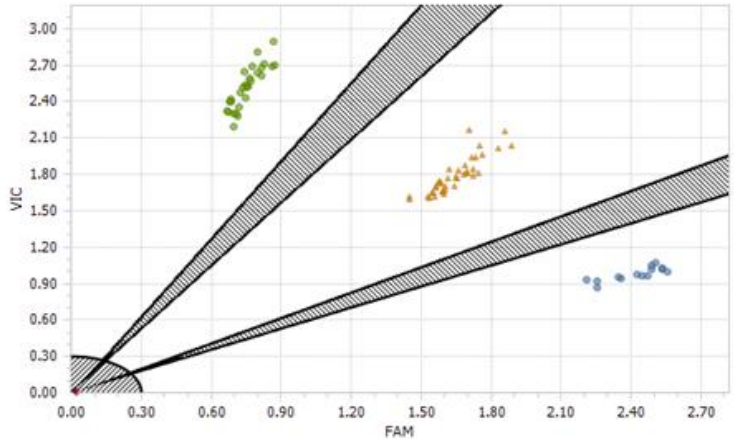


Fig 2A6 scatter plot for genetic polymorphisms separation

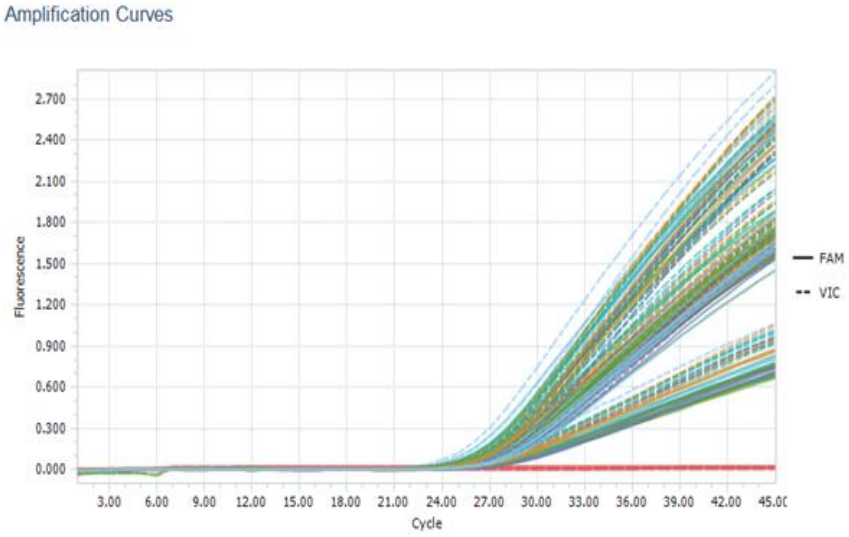


Fig3A6 amplification curve with fluorescence (Vic, FAM and NTC) of the LightCycler 96 instrument

Appendix 7 Measurement of total vitamin D (D3/D2) using UHPLC

clinCal and clinTest (level I and level II) and samples were injected in UHPLC using conditions set (in the protocol) in section (2.9.2.3). The instrument was used in scan mode to identify all the possible molecules (vitamin D2/D3). The results for vitamin D3, vitamin D2 and internal standard are shown in Fig 1A7 and the concentration of vitamin D3 and D2 were calculated by using the equation in the section (2.9.2.4).

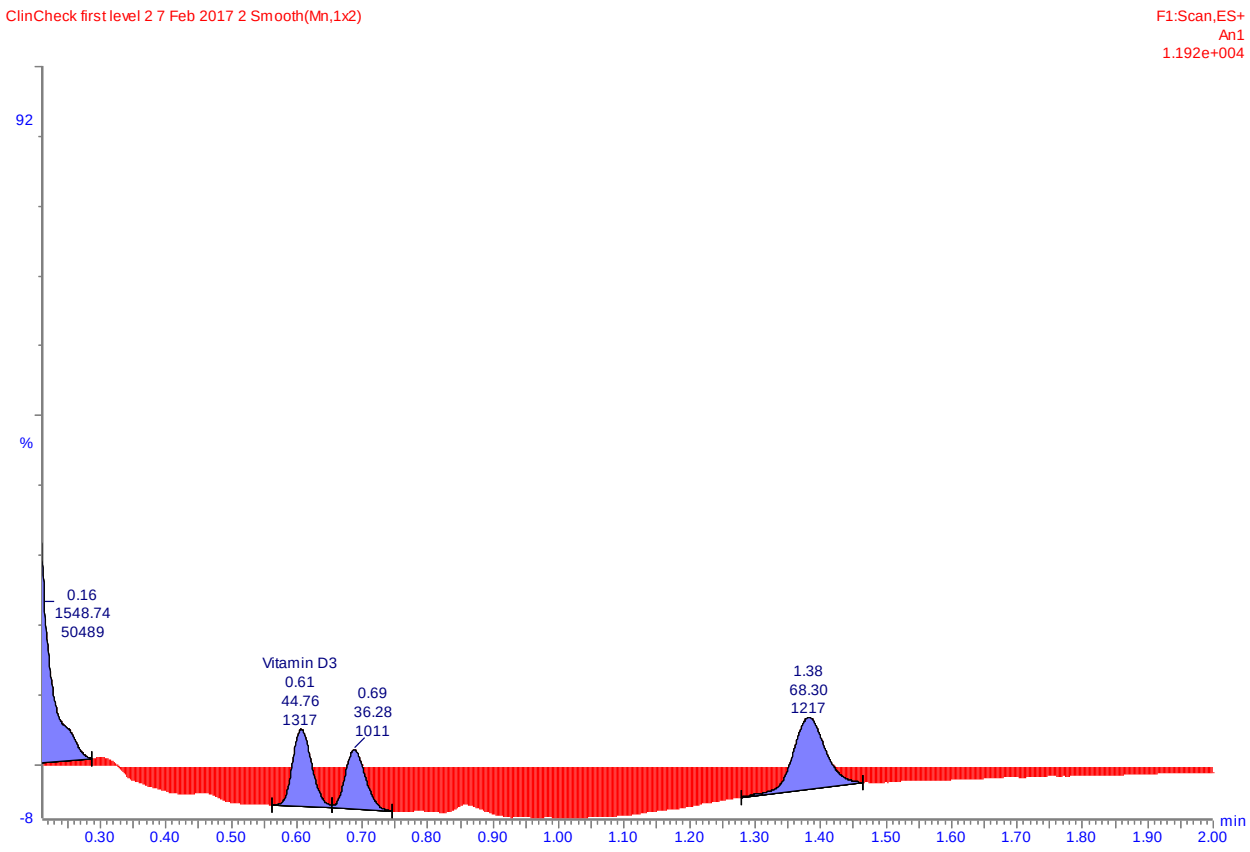


Fig 1A7 ClinCheck Level 1 : at 0.61 peak (vitamin D3), 0.69 (vitamin D2) and 1.38 (IS)

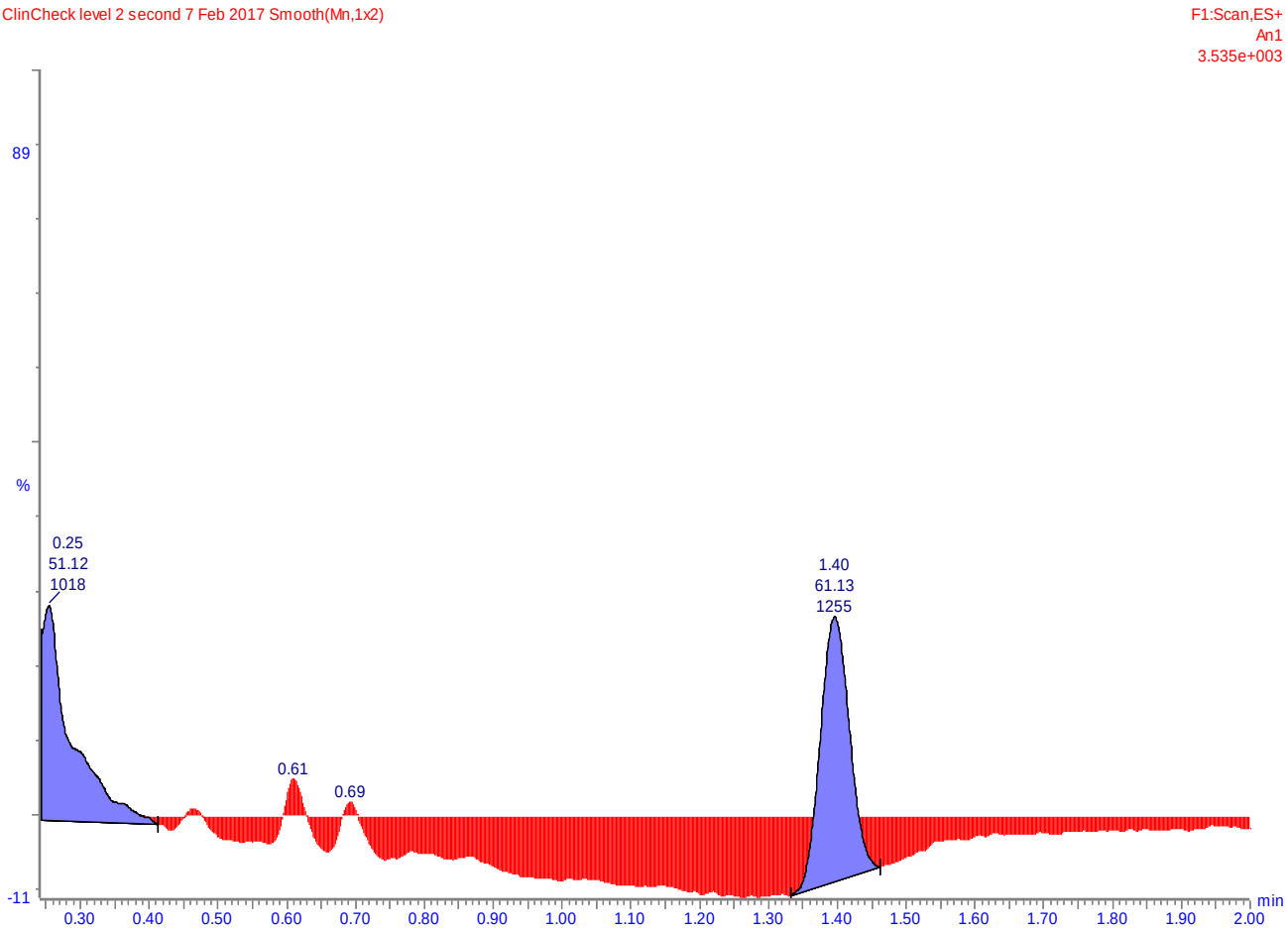


Fig 2A7 ClinCheck Level 2 : at 0.61 peak (vitamin D3), 0.69 (vitamin D2) and 1.38 (IS)

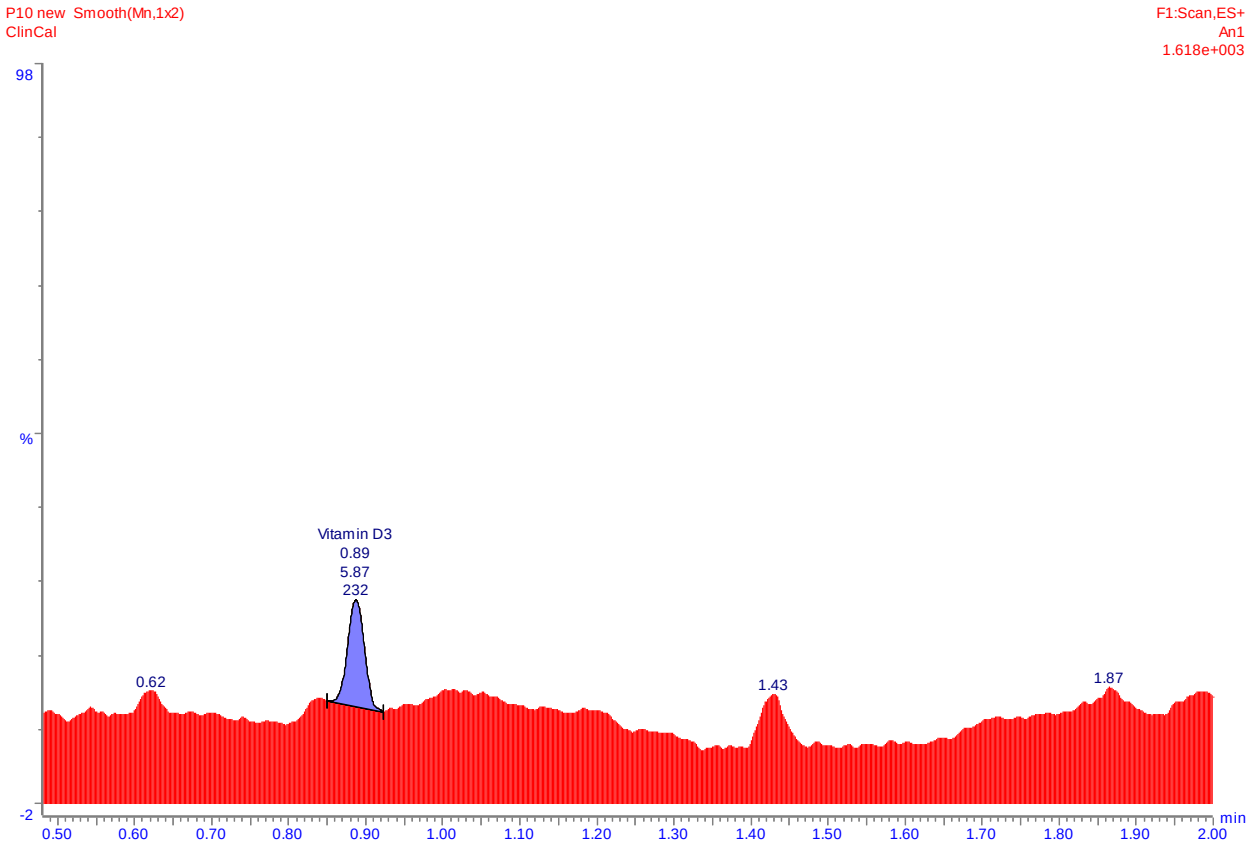


Fig 3A7 sample Patients (P10) : at 0.62 peak (vitamin D3), and 1.43 (IS)

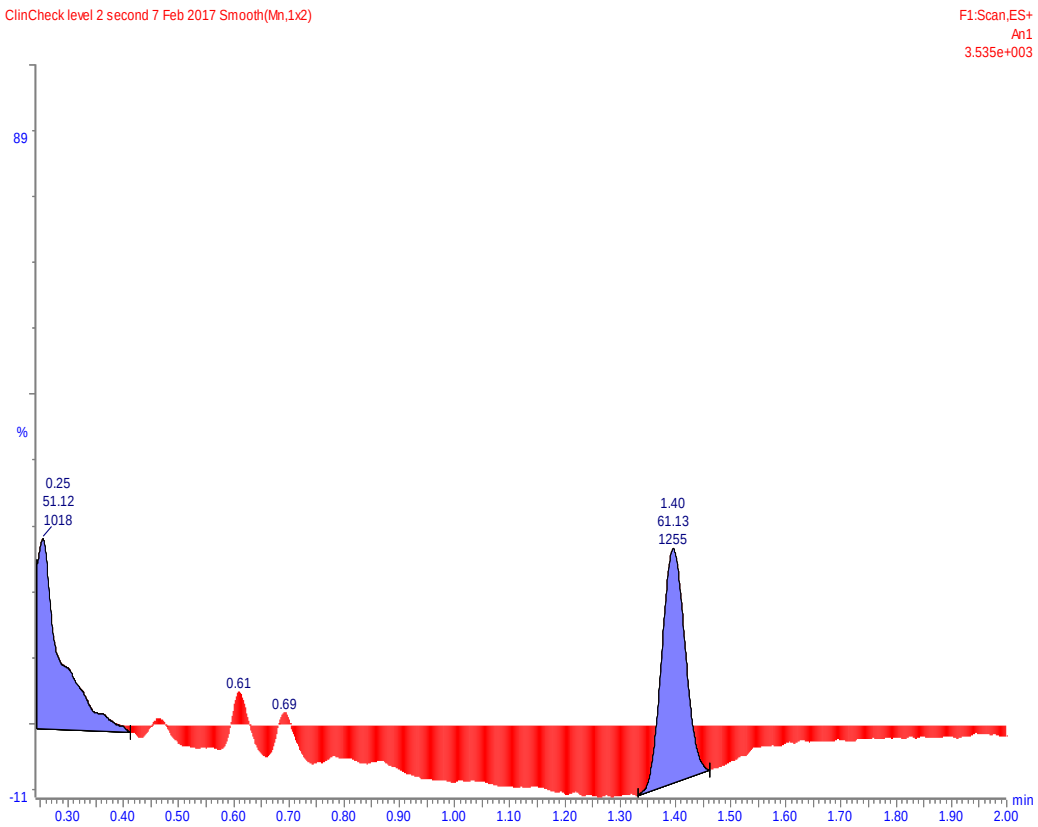


Fig 4A7 ClinCheck Level 2:0.61 peak (vitamin D3), 0.69 (vitamin D2) and 1.40 (IS) (second injection)

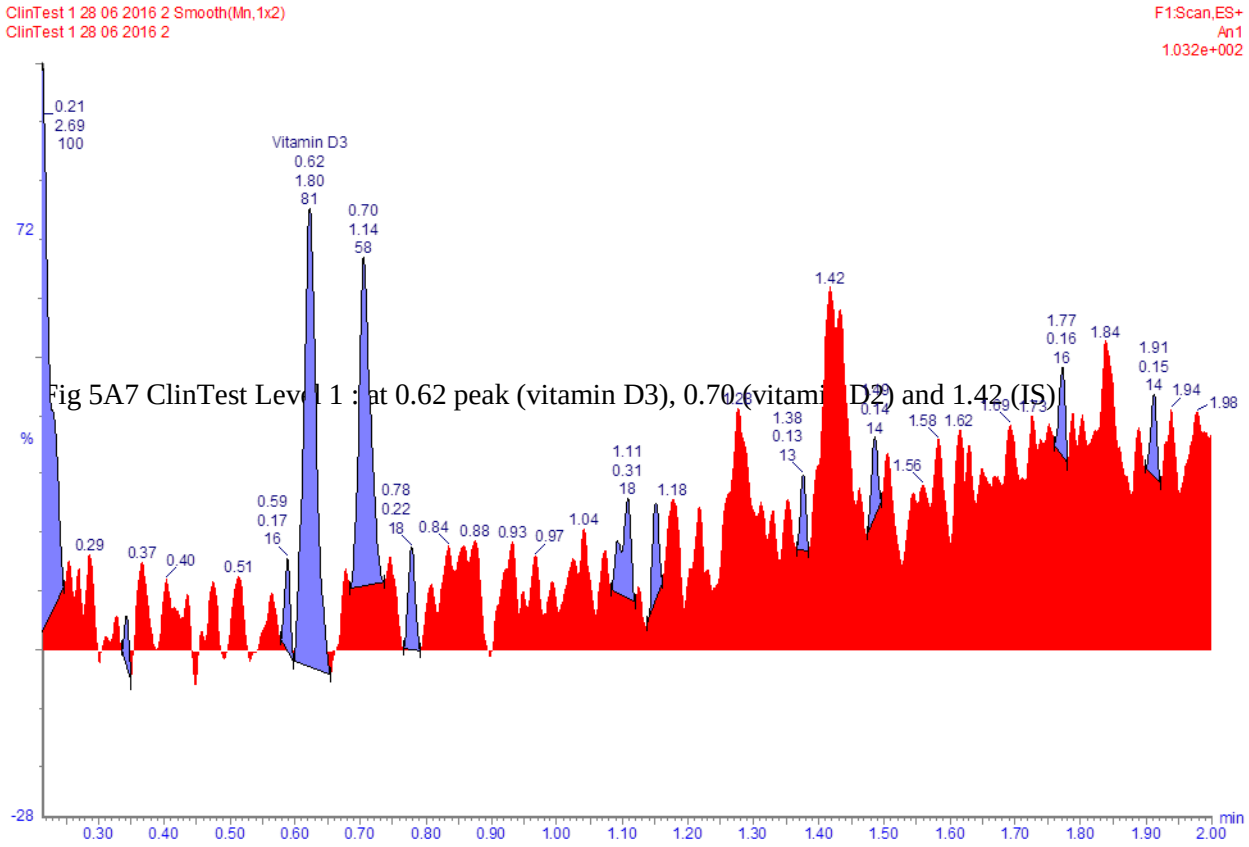


Fig 5A7 ClinTest Level 1 : at 0.62 peak (vitamin D3), 0.70 (vitamin D2) and 1.42 (IS)

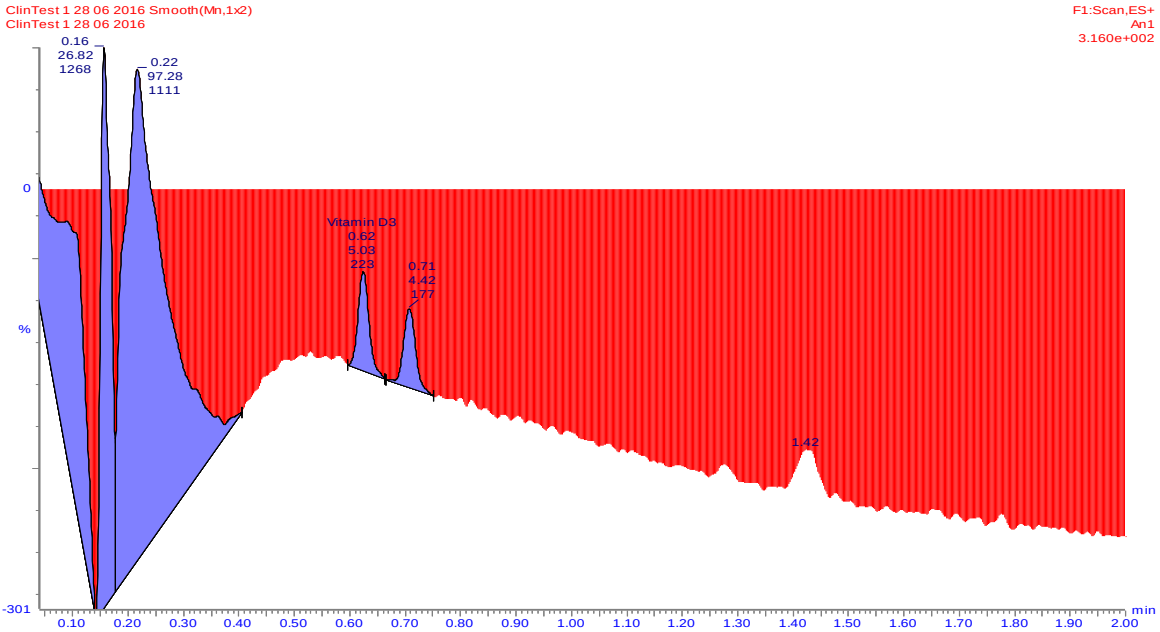


Fig 6A7 ClinTest Level 1 : at 0.62 peak (vitamin D3), 0.71 (vitamin D2) and 1.42 (IS)

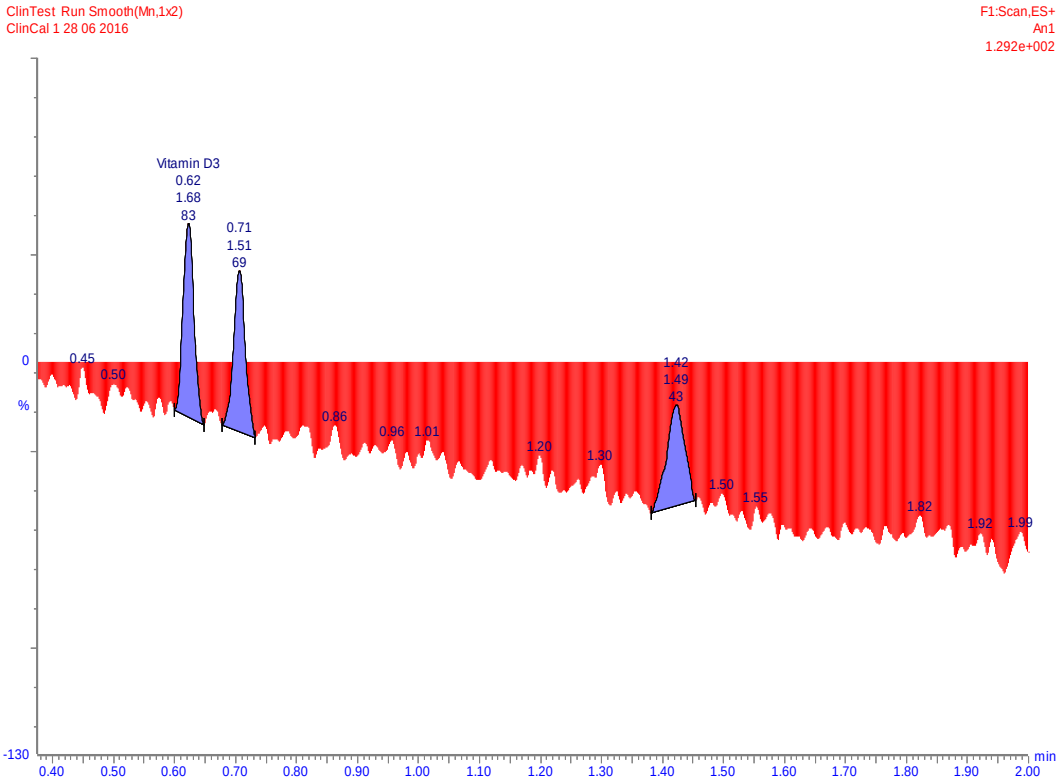


Fig 7A7 ClinCal: at 0.62 peak (vitamin D3), 0.71 (vitamin D2) and 1.42 (IS)

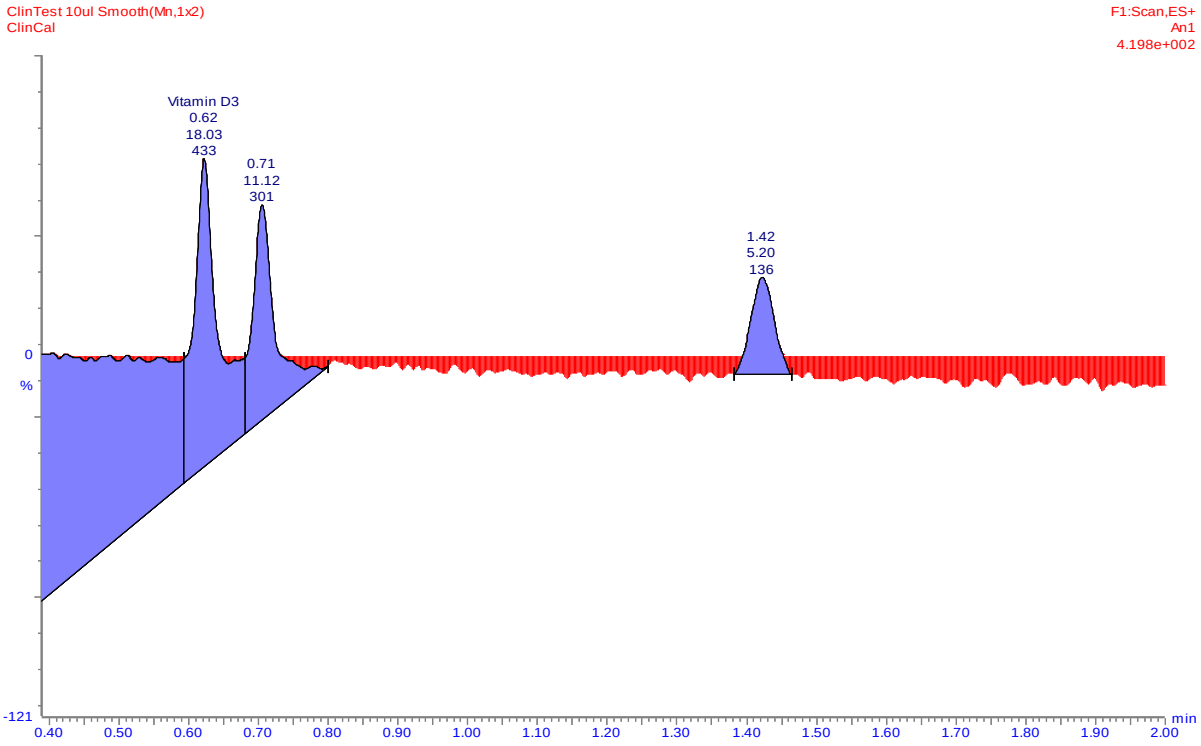


Fig 8 A7 ClinCal: at 0.62 peak (vitamin D3), 0.71 (vitamin D2) and 1.42 (IS)

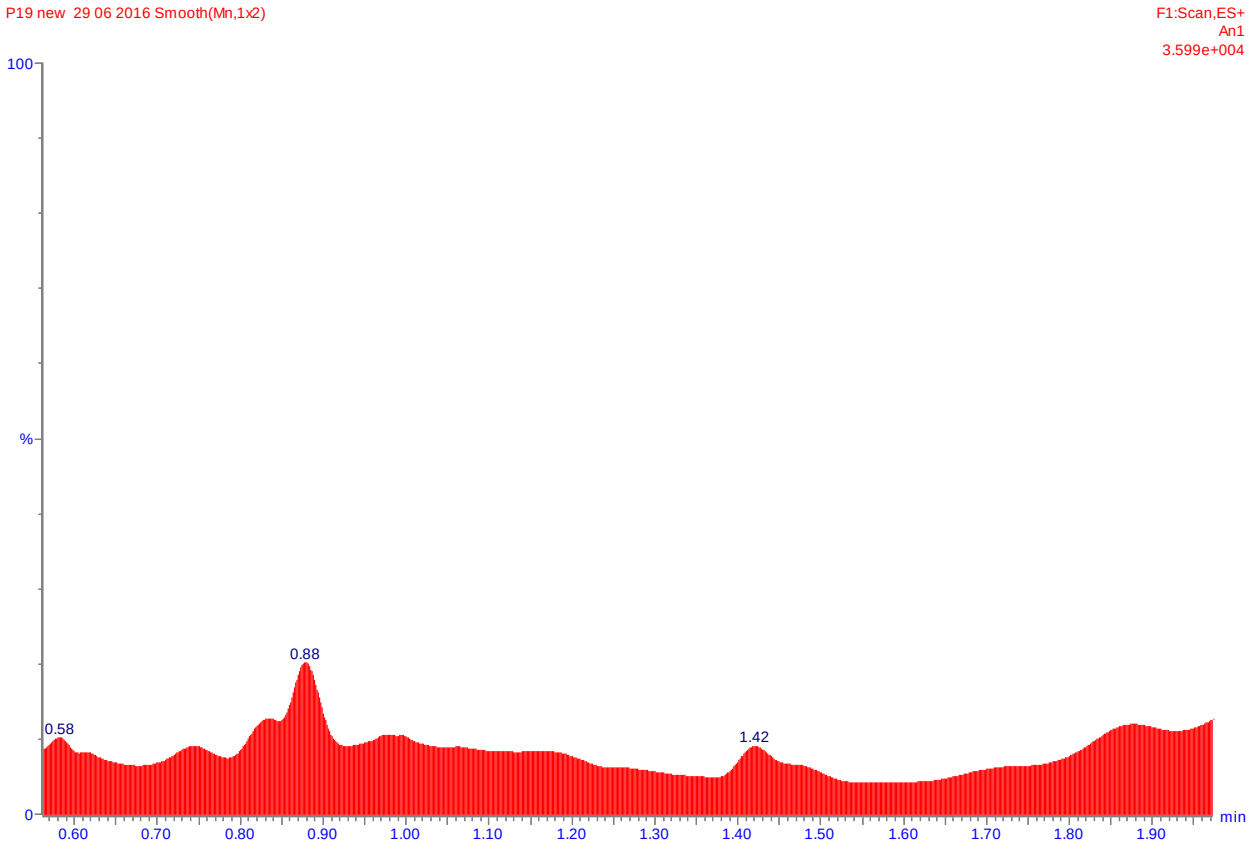


Fig 9A7 Sample patients (P19) : 1.42 (IS)

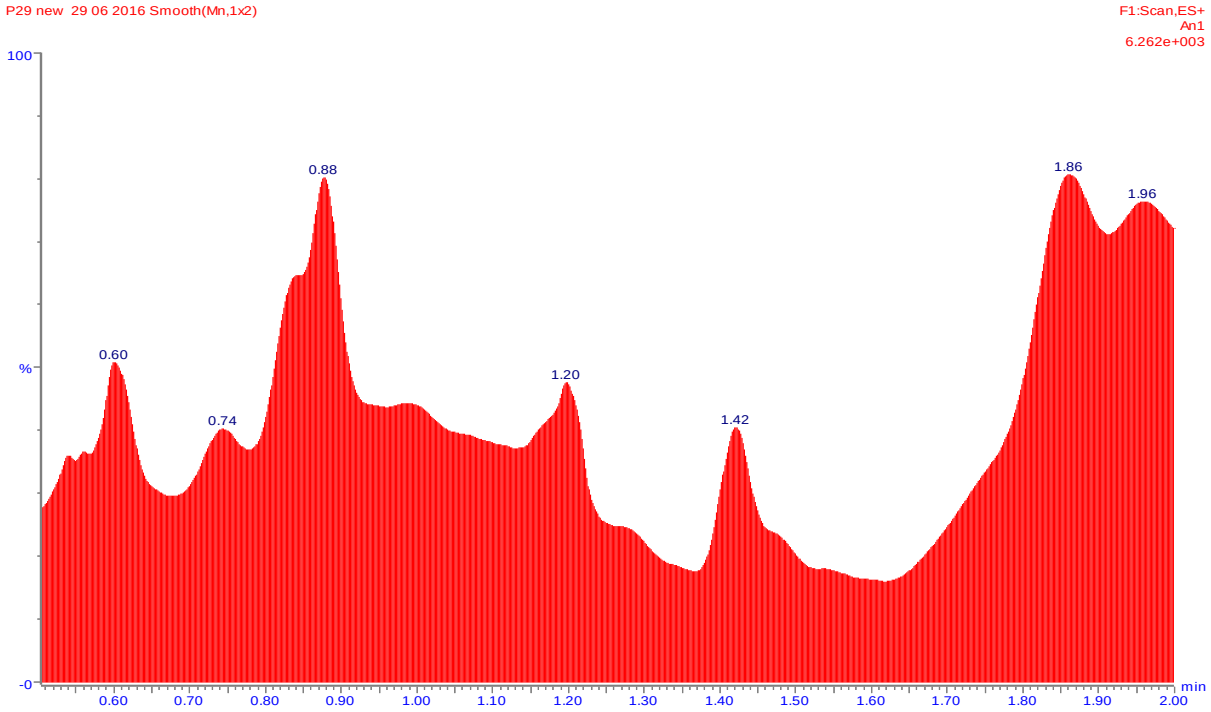


Fig 10 A7 sample patients (P29): at 0.60 peak (vitamin D3), 0.74 (vitamin D2) and 1.42 (IS)

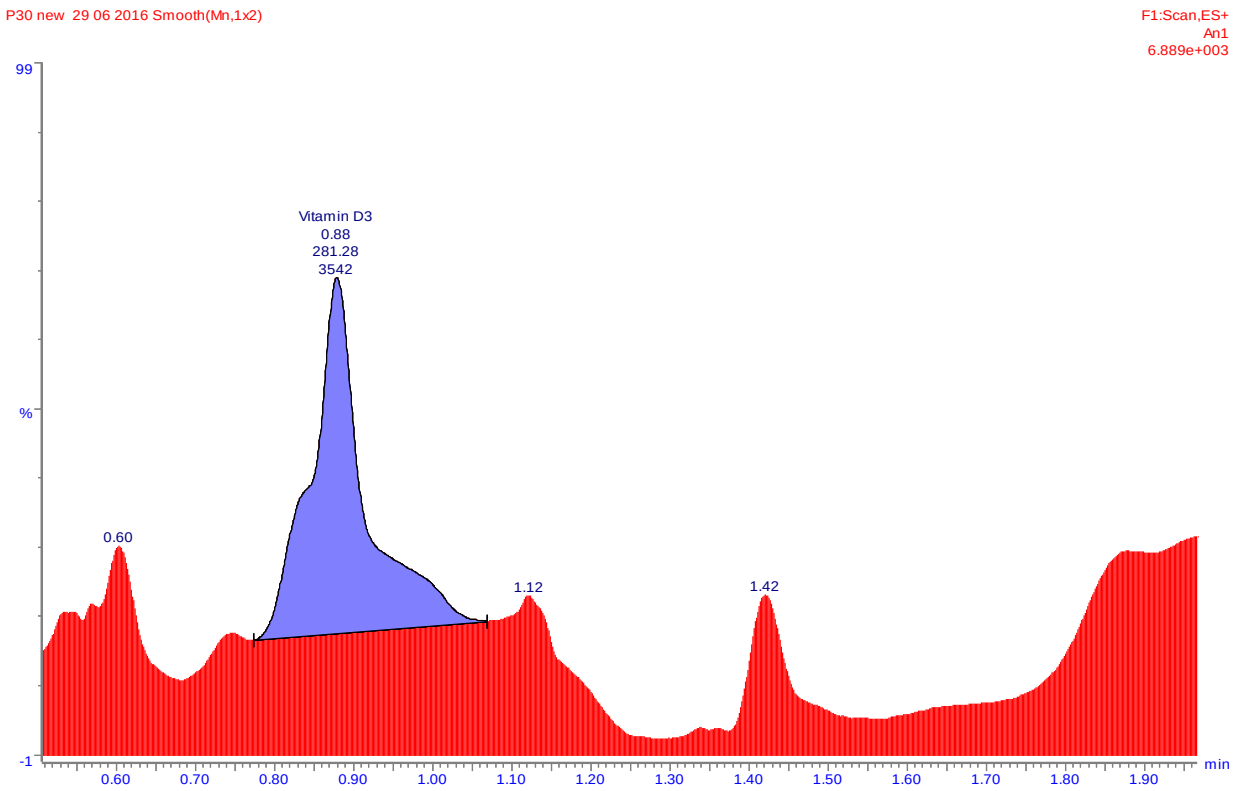


Fig 11 A7 sample patients (P30) : at 0.60 (vitamin D3) and 1.42 (IS)

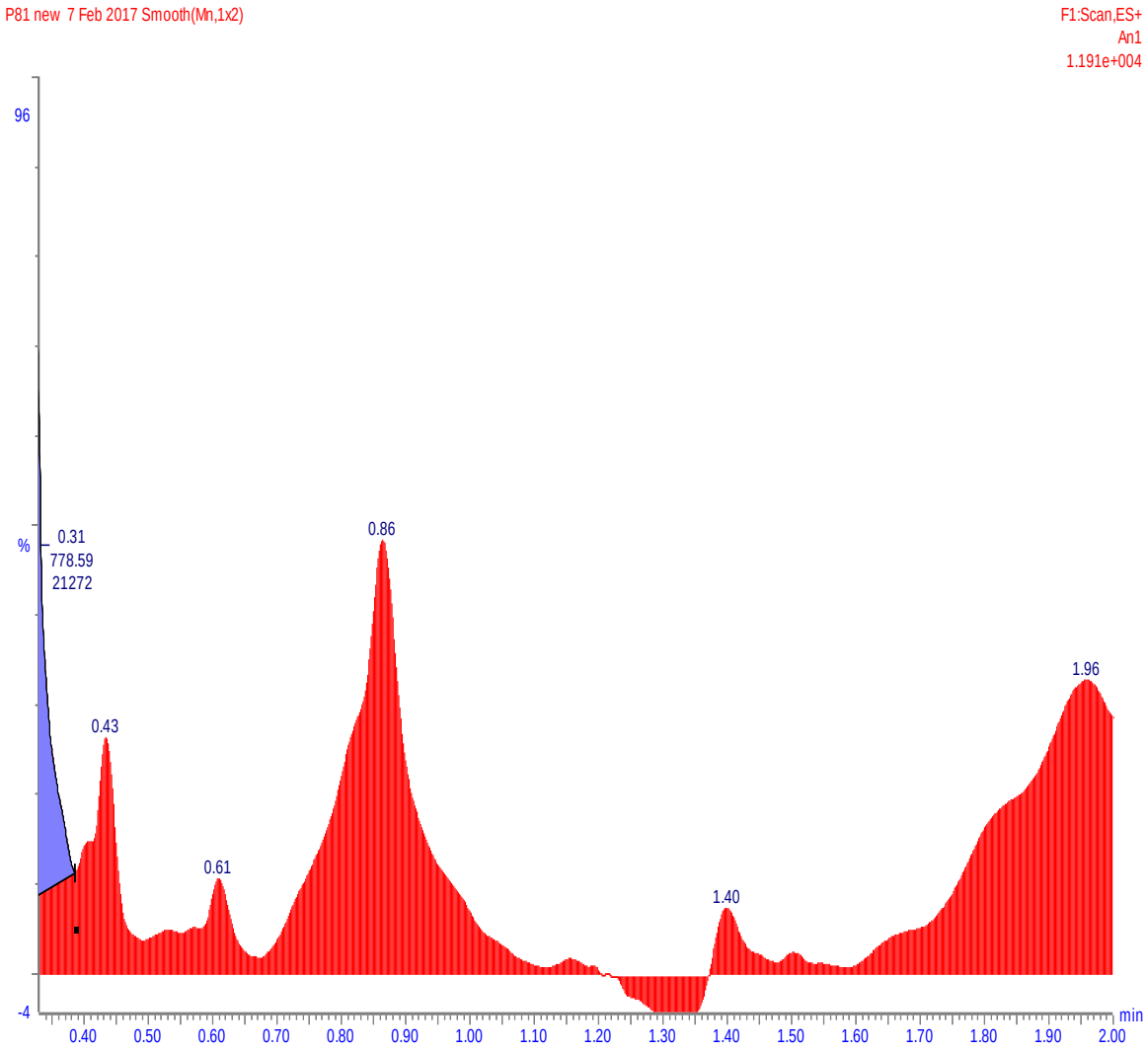


Fig12 A7 (P81)l: at 0.61 peak (vitamin D3), and 1.40 (IS)

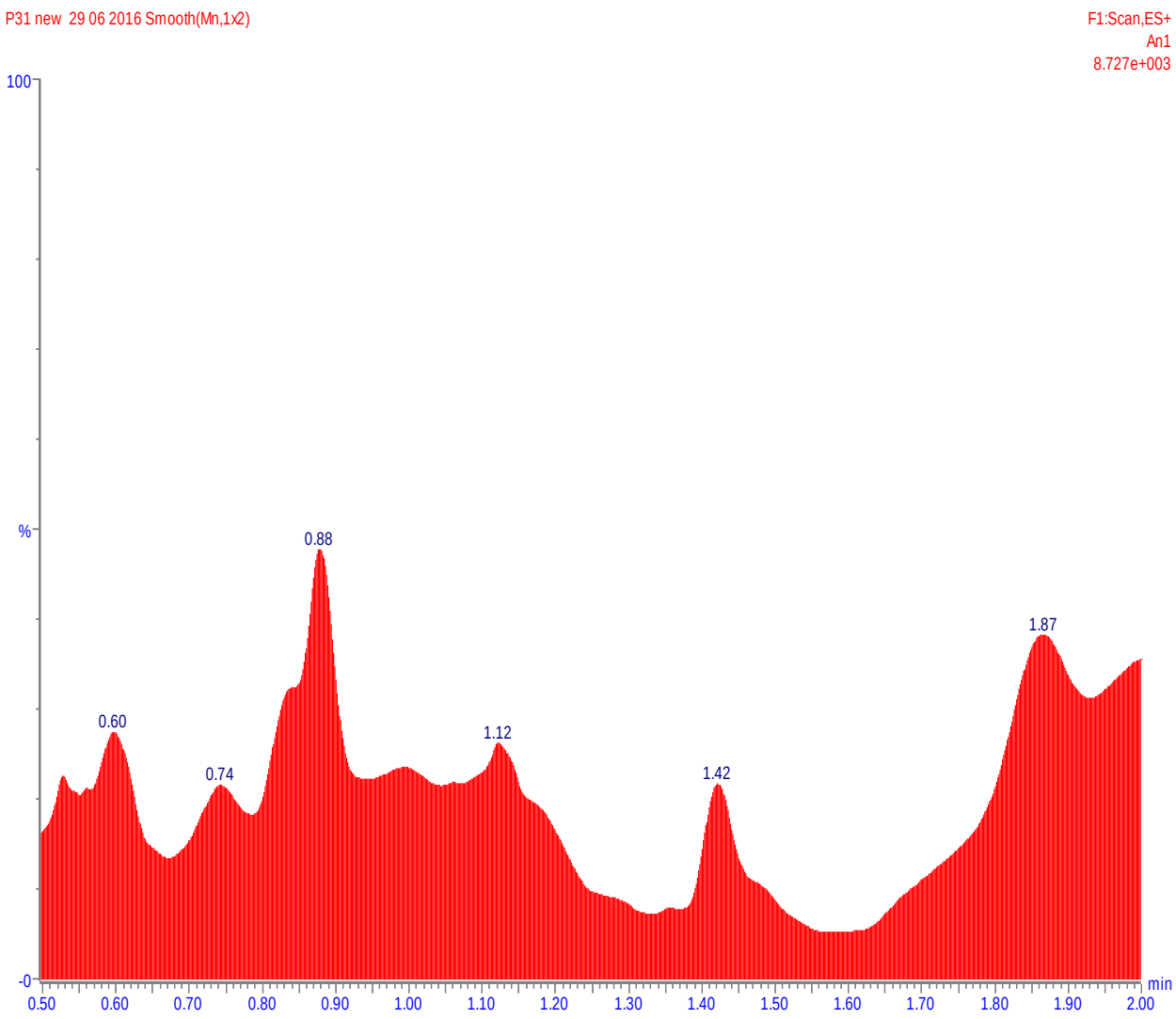


Fig 13 A7 sample patients (P31): at 0.60 peak (vitamin D3), 0.74 (vitamin D2) and 1.42 (IS)

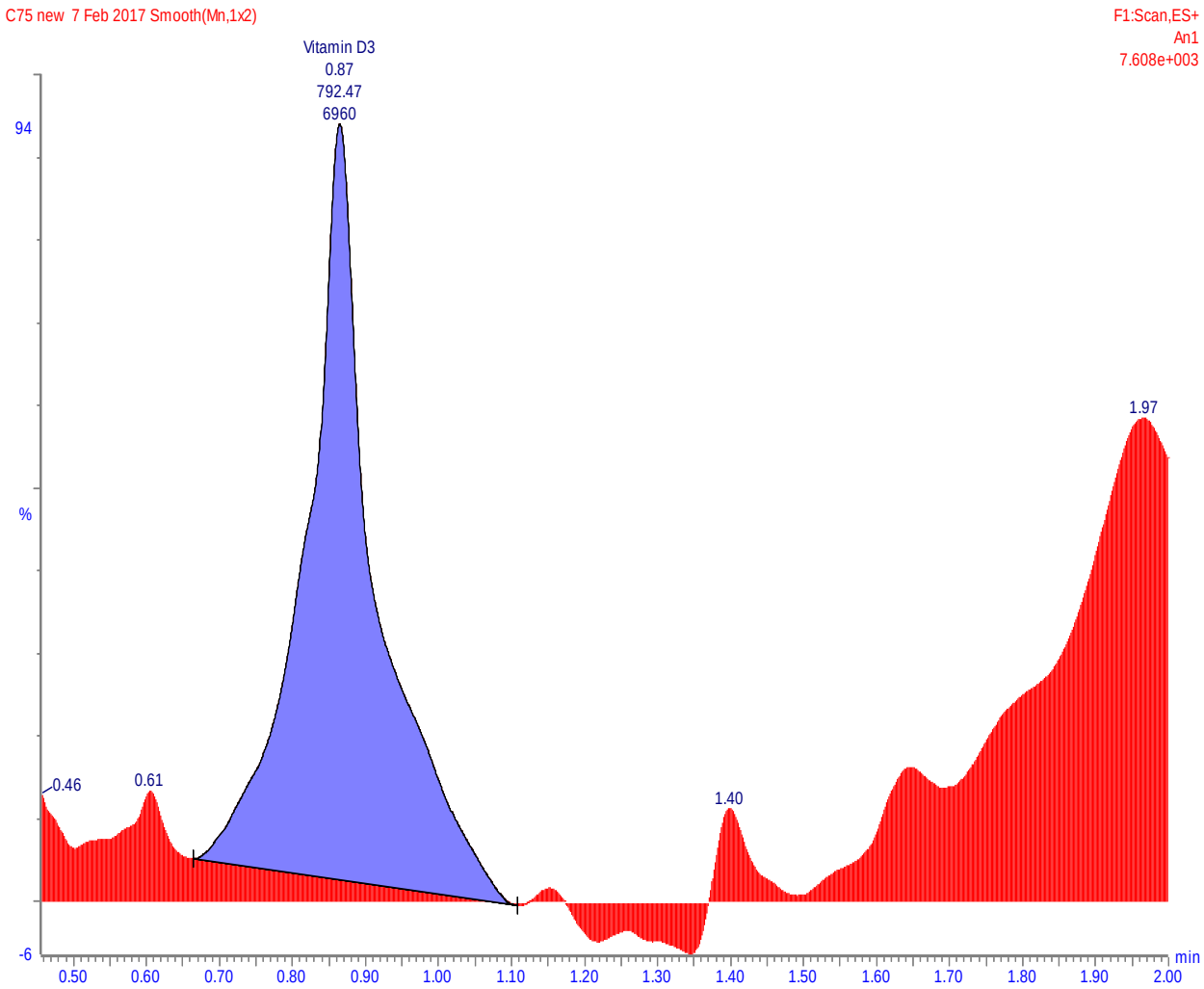


Fig 14 A7 sample control (C75): at 0.61 peak (vitamin D3) and 1.40 (IS)

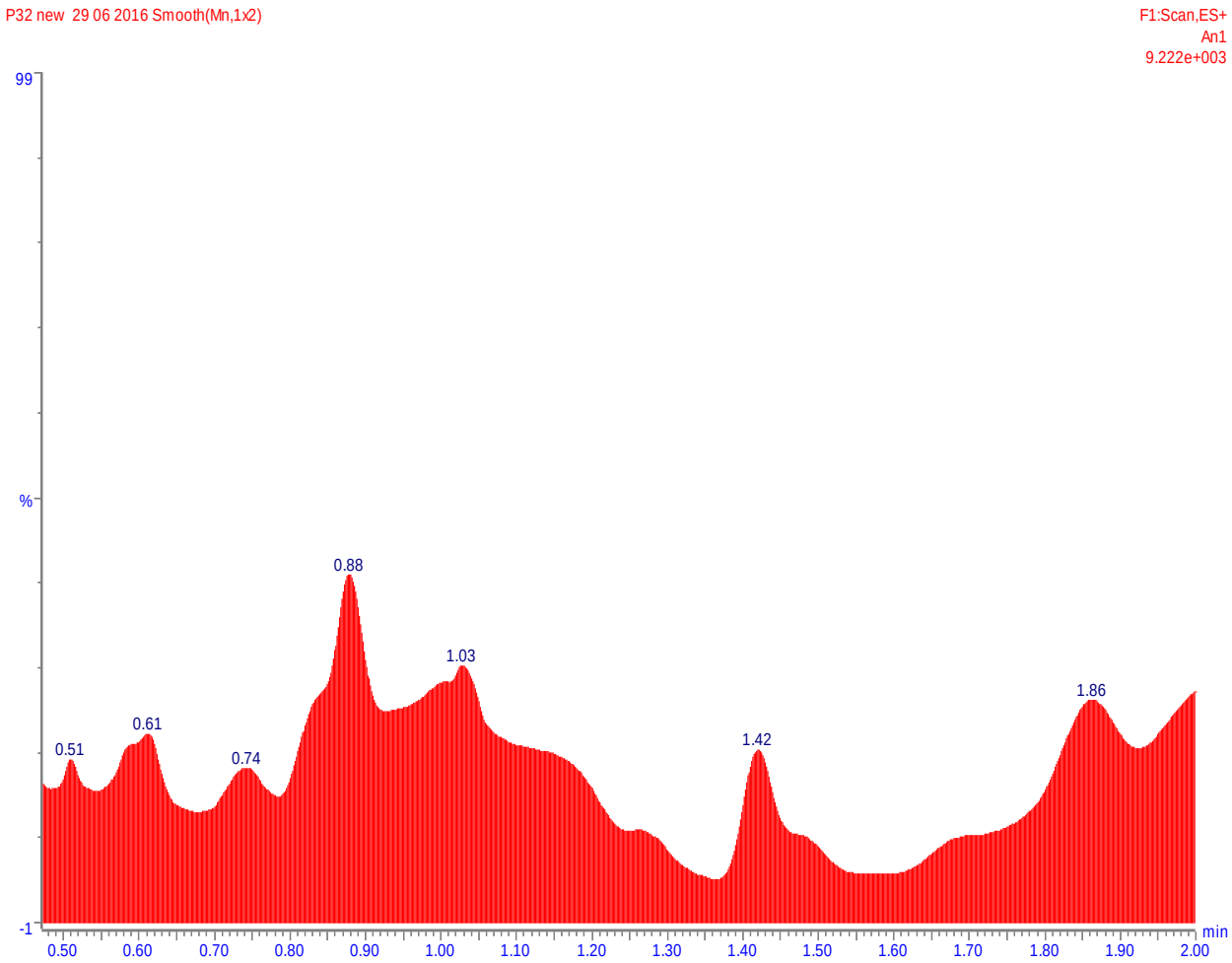


Fig 15 A7 sample patients (P32): at 0.61 peak (vitamin D3), 0.74 (vitamin D2) and 1.42 (IS)

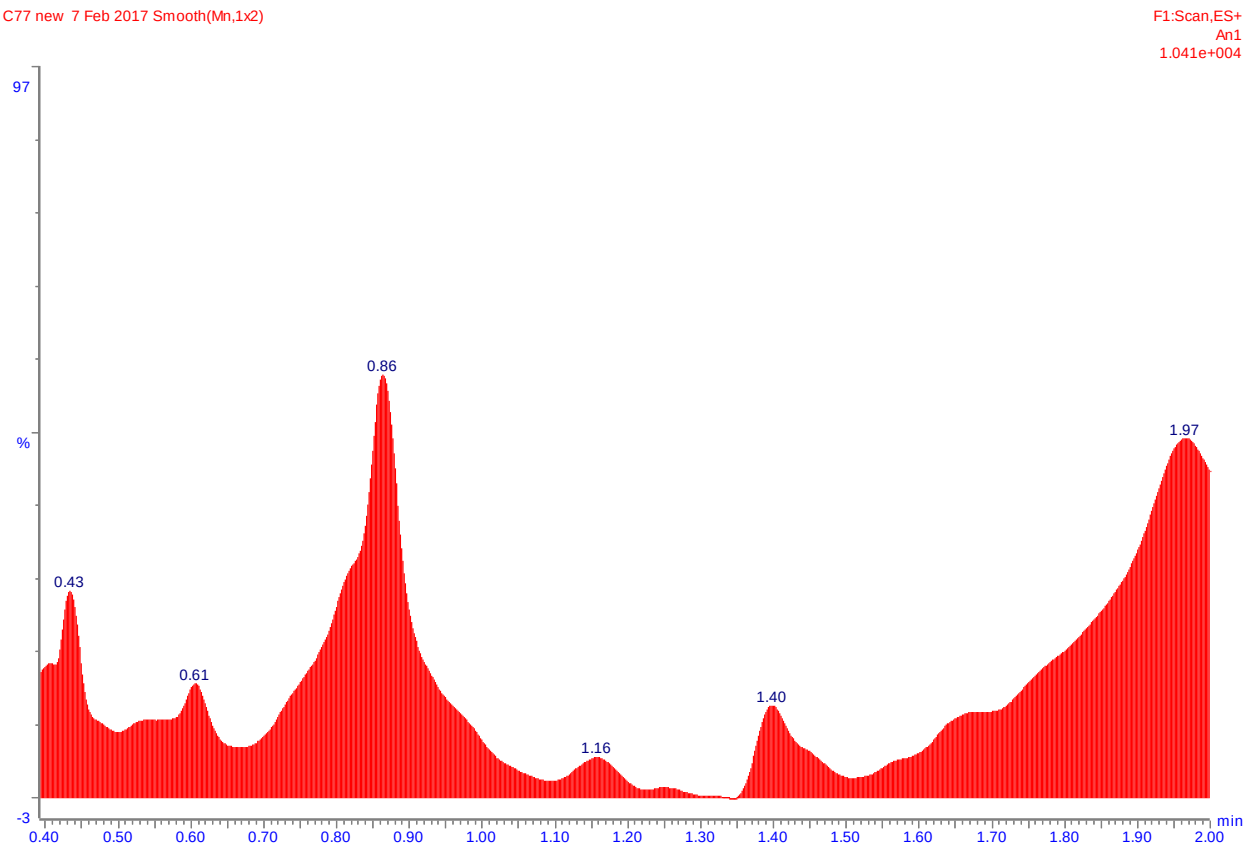


Fig 16 A7 sample patients (P32): at 0.61 peak (vitamin D3) and 1.40 (IS)

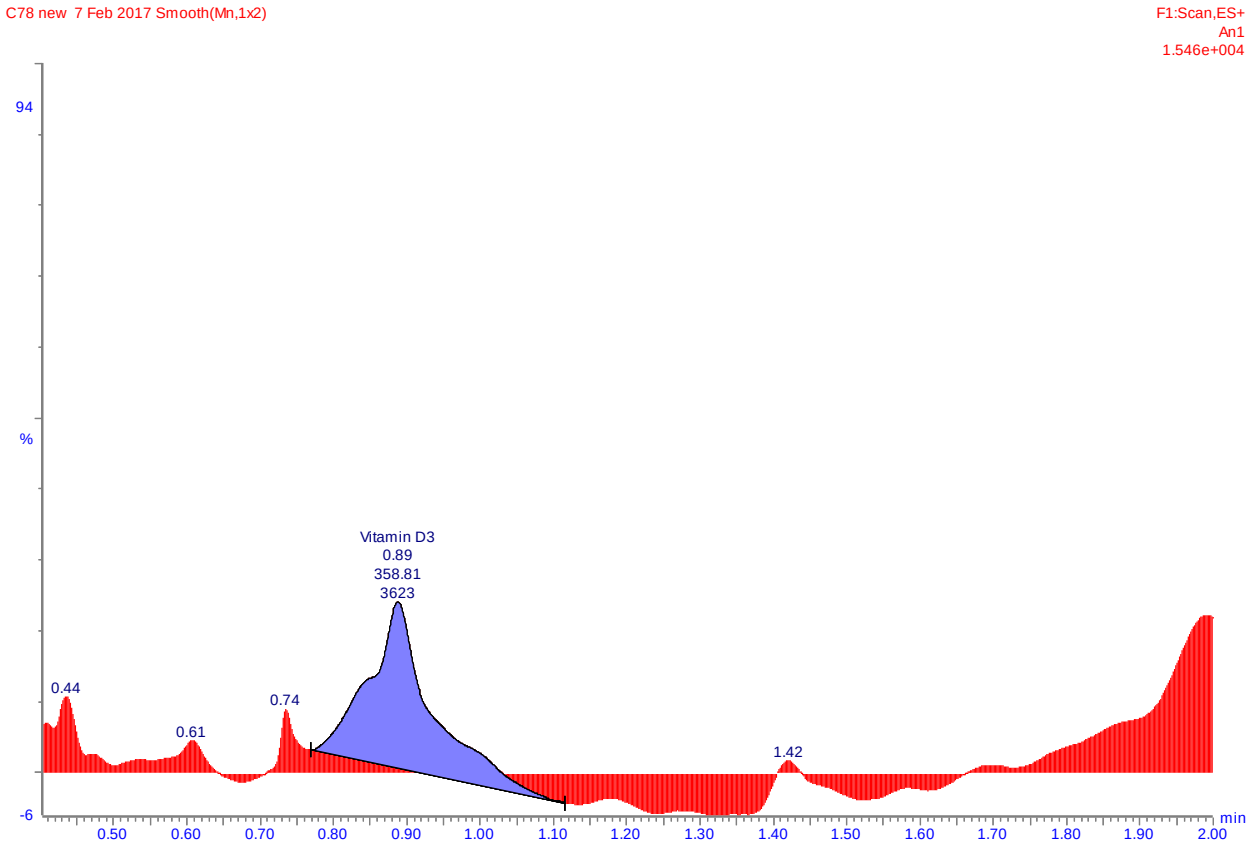


Fig 17 A7 sample control (C78): at 0.61 peak (vitamin D3), 0.74 (vitamin D2) and 1.42 (IS)

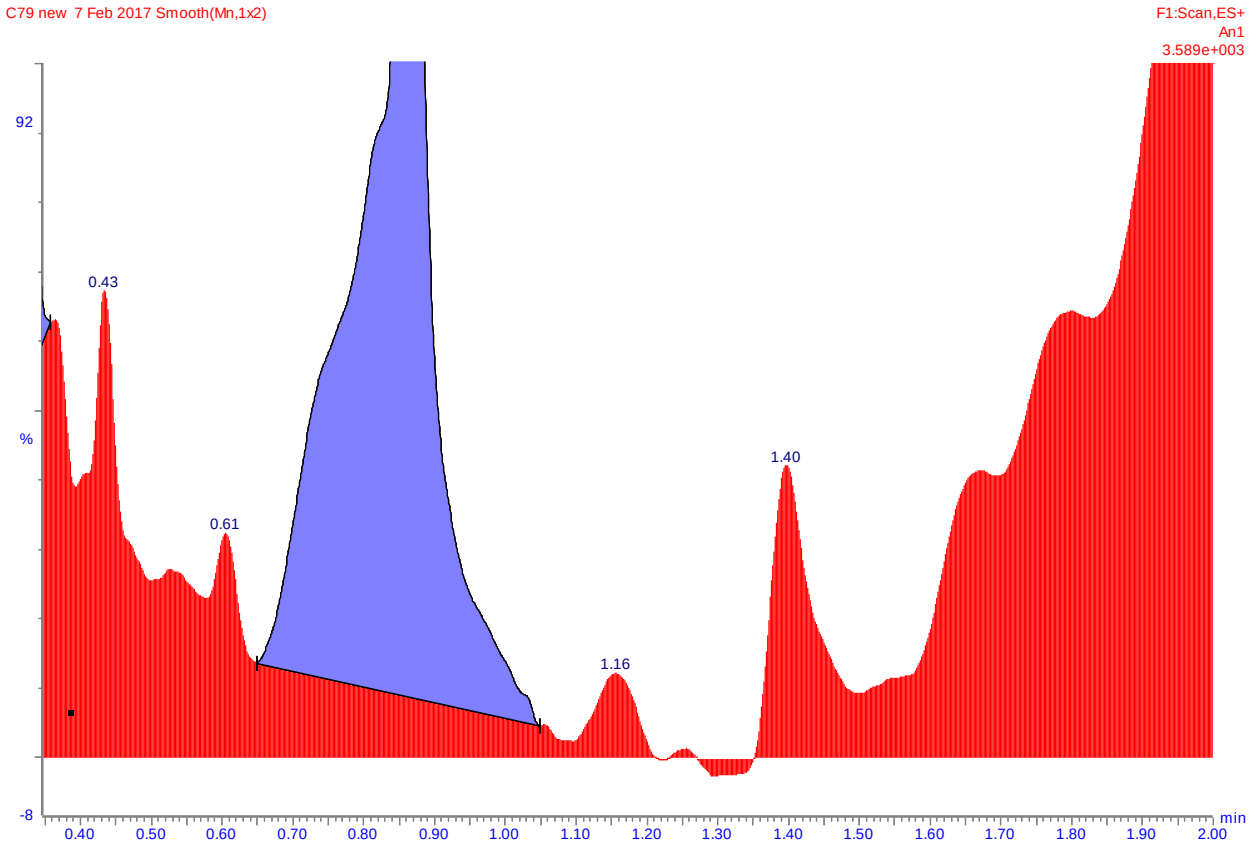


Fig 18 A7 sample control (C79): at 0.61 peaks (vitamin D3) and 1.40 (IS)

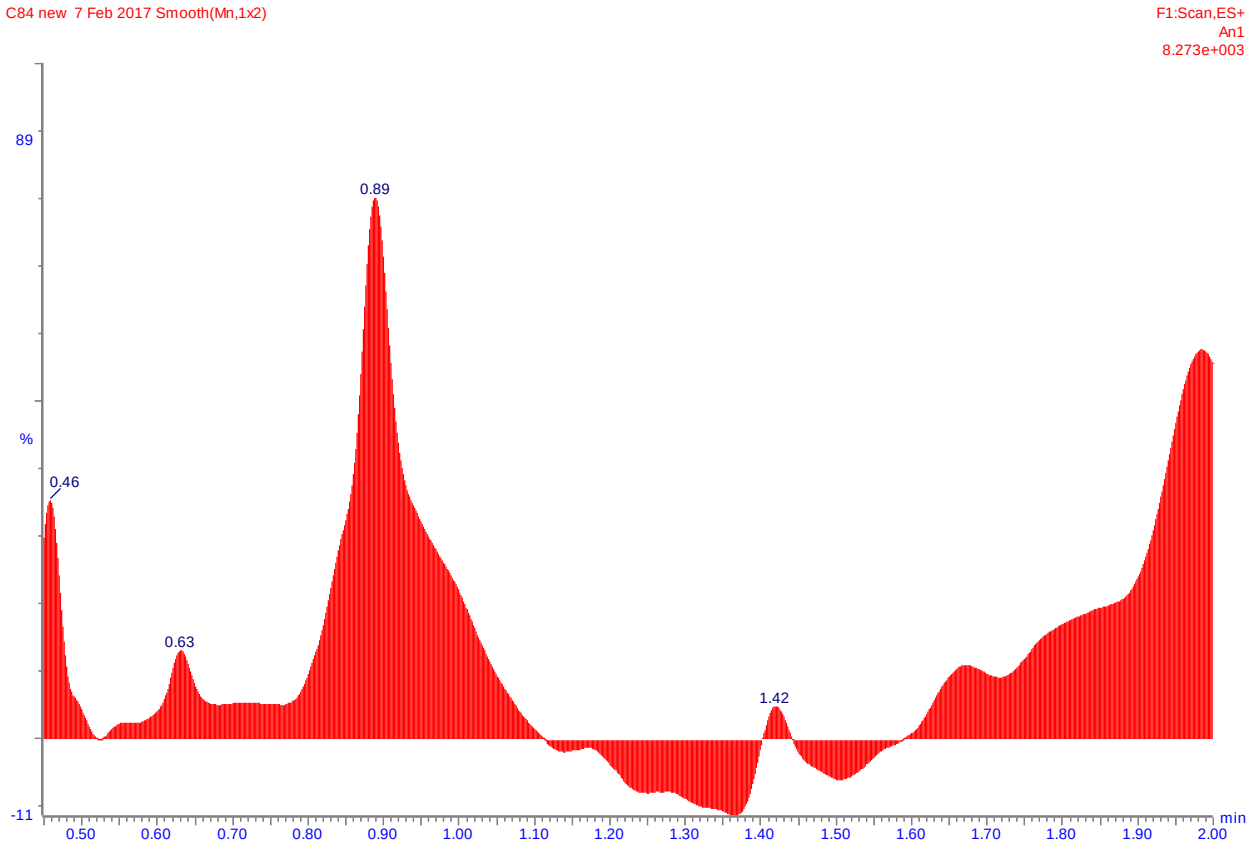


Fig 19 A7 sample control (C84): at 0.63 peak (vitamin D3) and 1.42 (IS)

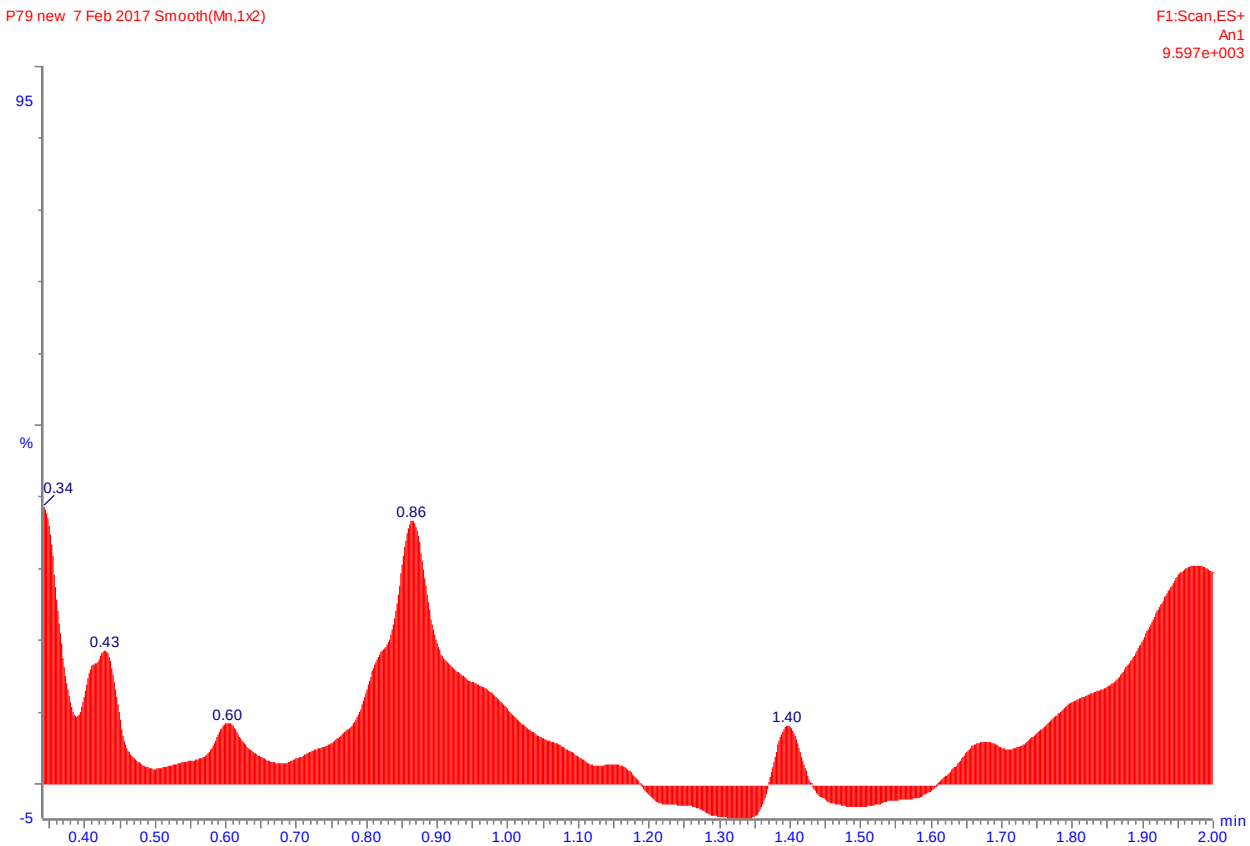


Fig 20 A7 sample patients (P79): at 0.60 peaks (vitamin D3) and 1.40 (IS)