The role of vitamin B12 deficiency on hepatic metabolism of lipids

By

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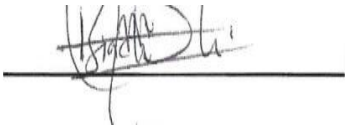
Declaration

I declare that the entire content of this thesis is presented in agreement with the regulations for the degree of Doctor of Philosophy by the High Degree Committee at the University of Warwick. The thesis is the outcome of my own work and has never been submitted previously for any other degree at the University of Warwick or other institutions. Any other information obtained from other sources either unpublished or published has been duly acknowledged and referenced.

Signature: 

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Abstract

Background: Obesity is currently a worldwide epidemic that increases the risk of developing metabolic disorders like diabetes, hypertension and cardiovascular diseases (CVDs) which causes great public health concern. A series of studies involving animal models and epidemiological investigations have demonstrated a relationship between the importance of vitamin B12 (B12) and various components of metabolic syndrome. High prevalence of B12 deficiency has been shown in Europeans (27%) and South Indians (32%) with type 2 diabetes mellitus (T2DM) as well as several T2DM patients on prolonged metformin treatment. Similarly, studies in human adipose tissues showed evidence of dysregulation in lipids by low B12, accounting for higher adiposity and adipocyte dysfunction. However, the liver is the principal storage organ for B12, and hepatic contribution to the lipogenesis of the entire body is also significantly higher compared to adipose tissue. Several clinical studies have shown that metformin, the first drug of choice for T2DM treatment and proposed to reduce lipid levels in patients, had no effect on intrahepatic triglyceride levels. Metformin treatment has also shown an association with low B12 levels. Some studies in the liver have also reported no expression of adiponectin in the liver, unlike its receptors (adipoR1 and adipoR2). Adiponectin is an adipose tissue-derived hormone that regulates glucose and lipid metabolism and is known to upregulate glycolysis and fatty acid oxidation (FAO) as well as reduces gluconeogenesis in liver. Therefore, we hypothesized that low B12 may dysregulate hepatic metabolism of lipids and reduce the lipid lowering effect of metformin in the liver that might be improved by adiponectin treatment**. Methods**: Hep G2 cell line was cultured using custom-made B12 deficient Eagle's Minimal Essential Medium (EMEM) and seeded in different concentrations of B12 media including 500nM (control) and 25pM (low) B12. Oil Red O (ORO) staining, RT-qPCR, total intracellular triglyceride (TG), radioactive flux assay, fatty acid profiling using gas chromatography and extracellular seahorse XF24 flux assay were employed to examine the effect of B12 on lipid metabolism. **Results**: The intrahepatic uptake of B12 was increased in lower circulating B12 concentrations by increased expression of B12 receptors (CD320) and transporters (TCN2), whereas decreased expressions of CD320 and TCN2 accounted for reduced B12 uptake in higher circulating B12 levels. Low B12 increased *de novo* lipogenesis and levels of fatty acid (FA) groups associated with higher CVD risk. There was further decrease in FAO and mitochondrial functional efficiency, accounting for high hepatic lipid accumulation in low B12. Similarly, the lipid lowering effect of metformin was decreased by low B12, but improved *via* adiponectin, in the metformin-treated hepatocyte cell line.

Conclusion: Our data, therefore, provides novel evidence that B12 deficiency dysregulates lipid metabolism leading to hepatic lipid accumulation.

List of abbreviations

A

ACADL - Acyl-CoA dehydrogenase long chain

ACADM - Acyl-CoA dehydrogenase medium chain

ACADS - Acyl-CoA dehydrogenase short chain

ACC – Acetyl CoA carboxylase

ACSL1 - Acyl-CoA synthase

ACLY - ATP citrate lyase

AdoCbl - 5'-Adenosyl cobalamin

AFLP - Acute fatty liver of pregnancy

AGPAT - Acylglycerol-3-phosphate O-acyltransferases

AICAR - 5-Aminoimidazole-4-carboxamide ribonucleotide

AMPD – Adenosine monophosphate deaminase

AMN – Amnionless

AMPK - Adenosine monophosphate-activated protein kinase

AT - Adipose tissue

ATI - Angiotensin I

ATII - Angiotensin I

AWERB - Animal Welfare and Ethical Review Body

B

BMI - Body mass index

C

CACT - Carnitine acyl carnitine translocase

CAD - Coronary artery disease

CBS - Complete blood count

CHD - Coronary heart disease

ChREBP - Carbohydrate responsive element binding protein

CKD - Chronic kidney disease

COMT - Catechol-*O*-methyltransferase

CNS – Central nervous system

CPT1α - Carnitine palmitoyl transferase I alpha

CVD – Cardiovascular diseases

D

DAG - Diacylglycerol

DGAT 1&2 - Diacylglycerol acyltransferase 1&2

DKA - Diabetic keto acidosis

DM - Diabetes mellitus

DMB - 5, 6-dimethylbenzimidazole

DMSO - Dimethyl sulfoxide

DNL - *De novo* lipogenesis

DNMTs - DNA methyl transferases

E

ECL - Enhanced chemiluminescence

EDTA – Ethylene diamine tetra acetic acid

ELOVL6 – Elongation of very long chain fatty acid protein 6

EMEM - Eagle's minimal essential media

ESRD - End-stage renal disease

ESR1 - Estrogen receptor-1

ETC - Electron transport chain

F

$FADH_2$ – Flavin adenine dinucleotide

FAME - Fatty acid methyl esters

FAO - Fatty acid oxidation

FASN – Fatty acid synthase

FCCP - carbonyl cyanide-p-tri-fluoro-methoxy-phenylhydrazone

FFA - Free fatty acids

G

GAD - Glutamic acid decarboxylase

GBD - Global Burden of Disease

GDM - Gestational diabetes mellitus

GLUT4 - Glucose transporter member 4

GIT - Gastrointestinal tract

GPAT – Glycerol-3-phosphate acyltransferase

G3P - Glycerol-3-phosphate

H

HATS - Histone acetyltransferases

HADHB and HADHA - Hydroxyacyl-CoA dehydrogenase trifunctional multi enzyme complex subunits beta and alpha

HB mice – High plasma B12 mice

HCC - Hepatocellular carcinoma

HDACs - Histone deacetylases

HDL-C – High density lipoprotein cholesterol

HFD - High fat diet

Hhcy – Hyperhomocysteinemia

HMGCR - 3-hydroxy-3-methylglutaryl-CoA reductase

HMGCS - 3-hydroxy-3-methylglutaryl-CoA synthase

HoloTC – Holotranscobalamin

HOMA-IR - Homeostasis model of insulin resistance

I

IDF - International Diabetes Federation

IDLs - Intermediate-density lipoproteins

IDI1 - Isopentenyl-Diphosphate delta Isomerase 1

IF - Intrinsic factor

IL1b – Interleukin 1b

IR – Insulin resistance

IVF – In vitro fertilization

ISO – Isoprenaline

K

KHB - Krebs-Henseleit Buffer

L

LB mice – Low plasma B12 mice

LCFAs - Long-chain fatty acids

LC-PUFAs - Long chain polyunsaturated fatty acids

LDL-C – Low density lipoprotein cholesterol

LDLR - Low density lipoprotein receptor

LDs - Lipid droplets

LIPIN1 - Phosphatidic acid phospatase 1

LPA - Lysophosphatidic acid

LXR-α - Liver X receptor alpha

M

MCFAs – Medium chain fatty acids

MCM - Methyl malonyl CoA mutase

MeCbl - Methyl-cobalamin

MetS – Metabolic syndrome

MI - Myocardial infarction

MODY - Maturity-onset diabetes of the young

MMA - Methyl malonic academia

MS - Methionine synthase

MP - Methylation potential

miRNAs – MicroRNAs

MUFA - Monounsaturated fatty acid

MVD - Mevalonic acid dehydrogenase

N

NADH – Nicotinamide adenine dinucleotide hydrogen

NAFLD - Non-alcoholic fatty liver disease

NASH - Non-alcoholic steatohepatitis

NKHS - Non-ketotic hyper-osmolar state

O

OCR - Oxygen consumption rate

ORO - Oil Red O

P

PA - Pernicious anaemia

pACC – phosphorylated acetyl CoA – carboxylase

PhA - Phosphatidic acid

PBS - Phosphate-buffered saline

PDH - Pyruvate dehydrogenase

PPAR-γ - Peroxisome proliferator-activated receptor gamma

Q

QOF - Quality and Outcomes Framework

qRT-PCR - Quantitative Real-Time Polymerase Chain Reaction

R

RIPA – Radio-immunoprecipitation assay

S

SAM – S-adenosyl methionine

SAH – S- adenosyl homocysteine

ScAT - Subcutaneous adipose tissues

SCD1 – Stearoyl CoA desaturase 1

SCFAs – Short chain fatty acids

SDS-PAGE - Sodium dodecyl sulfate-poly acrylamide gel electrophoresis

SFA - Saturated fatty acids

SH - Steatohepatitis

SRC - Spare respiratory capacity

SREBP1c - Sterol regulatory element binding protein 1c

SNS - Sympathetic nervous system

SS - Simple steatosis

stRNAs - Small temporal RNAs

T

TCA - Tricarboxylic acid

TC - Transcobalamin

TCR or TCbIR - Transcobalamin receptor

T1DM - Type 1 diabetes mellitus

T2DM - Type 2 diabetes mellitus

TFAs - Total fatty acids

TG – Triglyceride / Triacylglycerol

TRLs - Triglyceride-rich lipoproteins

TNFα - Tumor necrosis factor-alpha

V

VEGF - Vascular endothelial growth factor

VLDL – Very low-density lipoprotein

W

WAT - White adipose tissue

WC - Waist circumference

WHO - World Health Organisation

WHR - Waist to hip ratio

X

XBP1 - X-box binding protein 1

Chapter one - Introduction

1.1 **METABOLIC SYNDROME**

1.1.1 **Background**:

Metabolic syndrome (MetS), also known as syndrome X or insulin resistant syndrome, is a medical term describing a network of biochemical, metabolic, clinical and physiological factors that upregulate the risk of developing type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) and all-cause mortality [1, 2]. As a result of elevation in cases of obesity, excess consumption of energy, sedentary lifestyle and advancement in urbanization, MetS currently presents a major global clinical and public health problem [2]. As predicted by an earlier study [3], the risks of developing T2DM and CVD may be increased to about 5-fold and 2-fold, respectively, as a result of MetS. Similarly, the risks of incidences of myocardial infarction (MI), stroke and death are about 2-4-fold, 2-5-fold and 2-fold higher respectively in MetS patients [4].

1.1.2 **Epidemiology**

Depending on the particular diagnostic conditions, criteria, sociodemographic and geographic factors, the prevalence of MetS differs across the globe [5]. Evidence shows that the global prevalence of MetS may vary from less than 10% to about 84% depending on the population demographics and syndrome definition applied [6]. The International Diabetes Federation (IDF) estimates that a quarter of the global adult population have MetS [7]. The highest prevalence of MetS has been reported in Mexican-American women [8]. About 35% of adults and 50% of elderly beyond 60 years old in the US were diagnosed with MetS based on the criteria developed by National Cholesterol Education Program Adult Treatment Panel III [9]. In Europe, about 38% of women and 41% of men have been reported based on diagnostic criteria developed by the IDF [10]. In Chinese adults at 60 years and beyond, 58.1% MetS prevalence has been recorded [11]. Finally, MetS prevalence in the Middle East was reported 32.1-42.7% in women and 20.7-37.2% in men based on the criteria of Adult Treatment Panel III [12]. The risk factors aggravating the risk of MetS include obesity [13], decreased physical activity [14], western dietary forms [15], smoking [16], existing family history [17], postmenopausal phase [13], low socioeconomic status [13] and excessive alcohol consumption [18].

1.1.3 **Pathophysiology of metabolic syndrome**

The precise cause of MetS is not entirely understood. However, some probable pathophysiological mechanisms underlying MetS include insulin resistance and hyperinsulinaemia, with a flux of fatty acids [19-21] (Fig 1). A complex interaction between environmental and genetic factors may also contribute to MetS, manifesting as a chronic, low-grade, inflammatory condition. The syndrome constitutes several of the following components: endothelial dysfunction, atherogenic dyslipidaemia, increased blood pressure, prolonged stress, visceral adiposity, insulin resistance and a hypercoagulable condition [2, 22].

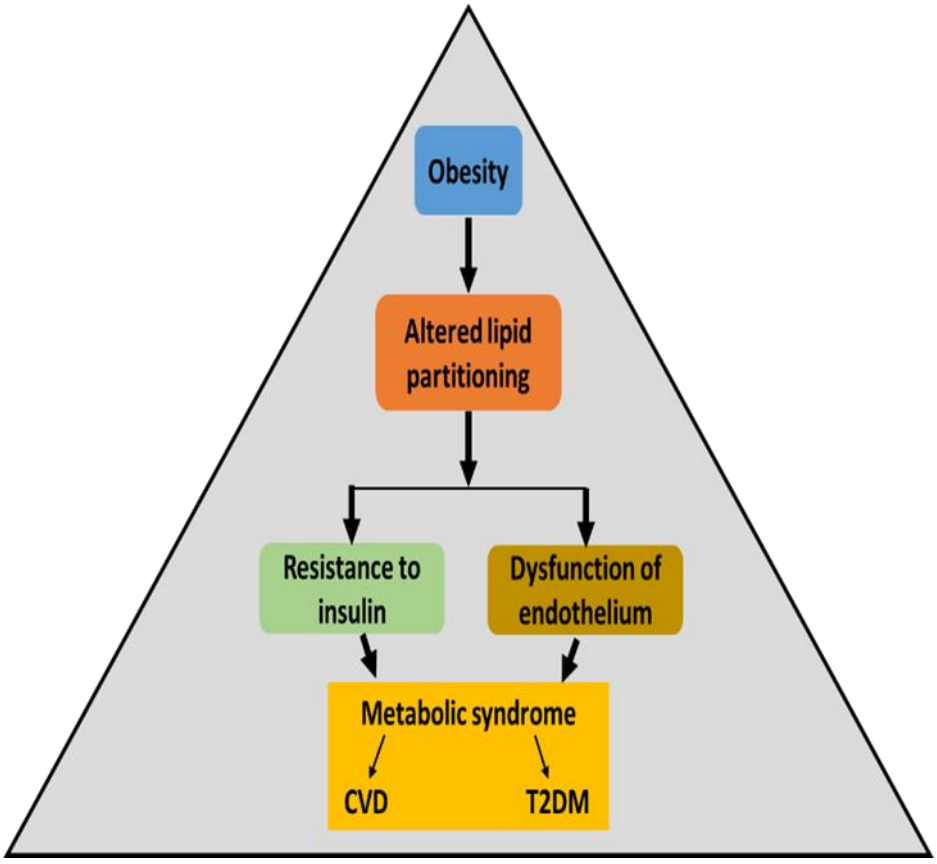

Figure 1: Pathophysiology of metabolic syndrome: Development of resistance to insulin together with endothelial dysfunction are propagated by conditions of dyslipidaemia and or dysregulated partitioning of lipids as observed in cases of central obesity. The resultant effect is the manifestation of metabolic syndrome (MetS) with the subsequent risk of developing cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM).

1.1.3.1 **Insulin resistance (IR)**

MetS is also known as IR syndrome [22]. It is, therefore, hypothesized that IR is the most acceptable factor for defining the pathophysiology of MetS [22]. Clinically, IR is defined as the

failure of insulin to achieve the desired uptake and usage of glucose in tissues of an individual compared with the normal population [23]. This results from a damage in the action of insulin in metabolically active tissues such as the liver, skeletal muscle and adipose tissue [24]. Systemic resistance to insulin could be a measure of decline in the rate of disposal of glucose in response to distinct insulin concentrations [25]. In adipose tissue (AT), IR interferes with the inhibition of lipolysis by insulin which may further elevate the free fatty acid (FFA) pool in circulation [26]. In muscles, insulin-facilitated uptake of glucose is compromised as a result of reduced sensitivity to insulin caused by FFA. Hyperinsulinemia, therefore, develops due to high levels of glucose in circulation promoting pancreatic insulin secretion. Consequently, elevation in the synthesis of triglycerides (TG), glucose and very low-density lipoproteins (VLDL) in the liver due to the high FFA pool is observed. In effect, an ultimate decline in glycogen production from glucose and upregulated accumulation of lipids in the form of TG are evident [22].

1.1.3.2 **Obesity and increased waist circumference**

The build-up of body fat to levels exceeding the suitable range required for normal functioning of the body is termed obesity [27]. Body fat can be broadly categorized into either essential or storage fat. Fat stored in organs such as heart, lungs, bone marrow, liver, muscle and spleen are essential and may facilitate the normal functioning of the body. Total body fat in healthy young adult women and men constitutes 20 to 25% and 15 to 20% of the entire body weight, respectively [27]. Central obesity is basically marked by deposition of fat in the upper torso and abdomen especially is males. However, regional obesity is common in females and may be characterized by deposition of fat in the hips and thighs. Evidence shows that central obesity presents with higher risks of developing T2DM, hypertension, CVD and mortality, compared with total obesity [27]. Waist circumference (WC) measurement, similar to the waist to hip ratio (WHR) for central obesity assessment, provides vital information about body fat distribution and accumulation [28]. Visceral fat has been shown to demonstrate much resistance to metabolic actions initiated by insulin, as compared with subcutaneous fat [29]. The former, likewise, shows much responsiveness to effects of glucocorticoids, lipolytic hormones and catecholamine [29]. Therefore, increased FFAs from lipolysis results in the elevation of hepatic biosynthesis of TGs and VLDL [30].

In spite of the complexity in the pathogenesis of MetS and its concomitant components, central (abdominal) obesity remains a crucial contributory factor, as included in definitions, both by the World Health Organisation (WHO) and ATP III [22, 31]. Central obesity is also indicated as a necessary requirement in the IDF definition of MetS [22, 31]. Excessive build-up of body fat is undoubtedly the commonest contributing factor to the increase in cases of MetS [32]. In

recent times, the global epidemic of obesity is considerably the most significant motivating factor leading to the much higher recognition of MetS [19]. Several definitions of MetS, however, involve utilization of waist circumference [33, 34]. Obesity is known to contribute significantly to conditions of hypertension and hyperglycaemia as well as demonstrating an association with elevation in the risk of developing CVD [35]. Obesity also shows a strong association with several cardiovascular risk factors according to data from epidemiological and clinical studies [35]. Lastly, obesity is associated with metabolic changes in individuals leading to alterations in their metabolism of lipids such as elevation in total cholesterol and triglyceride levels [35]. These obesity-induced metabolic modifications heighten the propensity of developing atherosclerosis in patients [35]. Despite these evidence, the precise mechanisms that account for the association between MetS and abdominal obesity (visceral obesity) are complex and not clearly understood.

1.1.3.3 **Dyslipidaemia**

Atherogenic dyslipidaemia has been shown to be common in patients presenting with MetS [36]. Dyslipidaemia refers to a defect in the metabolism of lipoproteins, which could either be due to insufficiency or overproduction [37]. The development of ischaemic diseases in humans is known to be predominantly caused by dyslipidaemia which, in turn, demonstrates a close association with atherosclerosis [37]. Cerebrovascular and ischaemic cardiovascular events are, however, shown to be the principal causes of mortality and morbidity [37]. Dyslipidaemia presents with three primary components which demonstrate direct linkage with MetS. These include upregulation in fasting and postprandial triglyceride-rich lipoproteins (TRLs), increased small dense LDL-C and reduced HDL-C. Similarly, high levels of triglyceride stores, in the form of very low-density lipoprotein (VLDL) particles, may result from compensatory hyperinsulinaemia status achieved in conditions of IR [38]. Reduction in the production of HDL-C units, together with a decline in the rate of fasting and postprandial TRLs clearance, may be due to a deficiency of the insulin-sensitive enzyme, lipoprotein lipase. Therefore, the principal anomaly of MetS is centred on increased fasting and postprandial TRLs, whereas reduction in HDL with increased LDL-cholesterol may represent consequential events. Individuals resistant to insulin may, therefore, be predisposed to a higher risk of developing CVD due to the defects associated with these lipoproteins [38].

1.1.3.4 **Hypertension**

Hypertension represents a chronic rise in blood pressure that might result in an end-stage organ injury after a prolonged period which, consequently, results in high mortality and morbidity. The blood pressure is a result of the product of both resistance in the systemic vascular system

and cardiac output. It implies, therefore, that patients presenting with arterial hypertension demonstrate resistance in their systemic vascular system, elevated cardiac output or both [39]. Studies have shown that hypertension usually demonstrates an association with diverse metabolic disorders predominated by glucose intolerance, obesity and dyslipidaemia [40]. It is, therefore, hypothesized that both hyperinsulinaemia and hyperglycaemia may facilitate hypertension development in insulin resistant patients [41]. This may result from activation of the renin angiotensin system *via* upregulating the receptors of angiotensin I and II (AT1 and II) and angiotensinogen [41]. Hypertension, resulting from effects of IR and hyperinsulinemia, may subsequently increase renal reabsorption of sodium, cardiac output and vasoconstriction of arteries *via* sympathetic nervous system (SNS) activation [40]. Insulin expresses a potent effect on the endothelium due to its vasodilation property, explained by regulation of complex pathways leading to endothelial stimulation to produce the vasodilator called nitric oxide [42]. Therefore, insulin resistant patients commonly demonstrate a diminished response to vasodilation. Other reasons could be due to enhancement of the reabsorption of sodium by insulin or an increased circulatory pool of fatty acids leading to an indirect vasodilation impairment [42].

1.1.4 **Consequences of metabolic syndrome**

T2DM and CVD are the principal conditions likely to be developed in MetS patients. Studies show that MetS patients present with five- and three-times risks of developing T2DM and CVD respectively [43]. Likewise, disorders of the cardiovascular system such as myocardial infarction (MI), coronary heart disease (CHD) and stroke may subsequently develop as a result of MetS in patients [35]. However, the establishment of a precise algorithm aiding the determination of an individualized relative risk for T2DM or CVD remains to be achieved [44]. Evidence of the possible development of breast [45], pancreas [46], colon and rectal cancers [47], as a result of MetS and its associated abdominal obesity and IR, has also been shown [47]. Furthermore, MetS has been shown to induce the likelihood of sleep disturbance in patients [48]. The health and quality of life of individuals may be grossly affected by MetS due to induction physical incapacity in subjects, hence affecting daily performance of regular activities [49]. Chronic kidney disease, glomerulomegaly, renal microalbuminuria and hypo- and/ or hyper-filtration are all possible renal manifestations in MetS patients [50]. Lastly, liver manifestation of disorders such as cirrhosis, fibrosis, non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD) have been reported in MetS conditions [29].

1.2 **DIABETES MELLITUS (DM)**

1.2.1 **Background**

Diabetes mellitus (DM) is a long-lasting and complex group of metabolic disorders marked by high plasma glucose due to insufficient or defective production of insulin from the β-cells of pancreas and/or elevated resistance of the cells to insulin action [51]. However, 'diabetes' and 'mellitus' were originally derived from the Greek language, signifying 'a passer through' and 'sweet' respectively. The terms were descriptive of an observation in patients who produced large volume of urine that baited bees and flies [52]. It was reported that diabetes alone has accounted for a minimum of 1.5 million mortalities in the past decade [53]. According to the WHO, DM is currently the 7th principal cause of mortality in the US with about 422 million adults cases, a figure that is four-times greater than cases in 1980 [53]. Economically, diabetes care has attracted much attention globally due to the rising epidemic of the disease, imposing pressure in several healthcare sectors to achieve more within limited resources [54]. In the last decade, an assessment of the entire annual budget for diabetes care in the Caribbean and Latin America was US$65.216 billion [55]. Likewise in the UK, the cost of diabetes in the last decade was £23.7 billion, and is further estimated to increase to about £39.8 billion by 2035/36 [56].

1.2.2 **Types of Diabetes**

Diabetes may generally be classified into the following categories [57]:

1.2.2.1 **Type 1 diabetes mellitus (T1DM)**: This type basically results from autoimmune-facilitated deterioration of the beta cells of the pancreas. The consequence is a complete deficiency in insulin; therefore, survival or good health may be dependent on the administration with insulin. Prevalence of T1DM is relatively low, especially in India, although the incidence of the disease is on the rise [58].

1.2.2.2 **Type 2 diabetes mellitus (T2DM)**: T2DM is marked by increase in the resistance of cells to insulin alongside progressive loss of insulin secretion from the pancreatic beta cells, with either of these being predominant. T2DM constitutes more than 90-95% cases of DM and accounts for the emergence of the global epidemic of DM [57]. T2DM is basically a progressive disorder demonstrating an association with metabolic conditions such as dyslipidaemia, central obesity and hypertension [57]. T2DM is predominant in middle- to late-aged individuals presenting with obesity or overweight conditions. However, evidence of increasing emergence has also been noted in younger-aged individuals as well as those presenting with lower body mass index (BMI) [57].

1.2.2.3 **Gestational diabetes mellitus (GDM)**: This refers to diabetes that is first diagnosed in pregnancy, especially within the second or third trimester [59]. However, the development of diabetes, especially in the first trimester is generally classified as T2DM, although it could be GDM or T1DM [59]. According to the latest IDF data, about 14% of global pregnancies, nearly 18 million annual births, are affected by GDM [60]. Conditions such as obesity and being overweight, micronutrient deficiencies, family history of IR or DM and westernized diet are risk factors for the development of GDM [60]. Whereas GDM may induce lasting consequences as well as risk of developing CVD and T2DM in mothers, their offspring are also exposed to risk of developing T2DM, CVD, obesity and GDM later in life [60].

1.2.2.4 S**pecific type**: DM may also be categorized based on any other causes. For instance, monogenic diabetes syndromes may include maturity-onset diabetes of the young (MODY) as well as neonatal diabetes. Chemical or drug-induced diabetes may include those resulting from administration of glucocorticoid following organ transplant or for HIV/AIDS therapy [60].

1.2.3 **Epidemiology of DM**

As early as 1985, the global prevalence of diabetes was about 30 million [61]. In both the US and globally, a significant rise in the prevalence of DM has been recorded in the last four decades. An estimate of about 366 million people globally were diabetic in the past decade, with a further prediction of about 552 million cases by the year 2030 [62]. T2DM accounting for >90% of cases of DM, is expected to increase rampantly as compared to T1DM which is predicted to stay stable. Currently, DM is categorized as the ninth leading cause of death globally and cases of DM have increased fourfold in the last three decades [63]. It is evident that one out of eleven adults, globally, has DM and nearly 90% of these cases are T2DM [63]. India and China are the two topmost countries with high evidence of diabetes incidence. This puts Asia at the global front of the rapid emergence of the T2DM epidemic [63]. Emergence of the current worldwide epidemic of DM is primarily as a result of a more sedentary way of life and unhealthy dietary behaviours, although susceptibility to DM could partly be explained by genetic predisposition in some individuals. Additionally, vulnerability to T2DM later in life could also be due to developmental factors or intrauterine exposure [63]. Adults over 65 years are likely to dominate the prevalence of DM in the industrialized nations [61]. However, persons between 45 to 65 years may predominate DM prevalence in developing countries [61], posing a negative threat on reproduction, fertility and economic productivity [61]. An appreciable fraction of the entire population of the US still presents with undiagnosed DM. About 5.4 million (2.7%) people present with fasting glucose ≥125mg /dL and 13.4 million

(6.9%) individuals also have compromised fasting glucose (110 – 125 mg /dL) [64]. To minimize the risk of individuals being at high risk, screening of individuals is highly recommended [65]. Research shows that the onset of CVD complications of diabetes might precede the clinical diagnosis of T2DM by several years, therefore, early diagnosis is highly essential to minimize this risk [66]. Surprisingly, 7.4% of DM cases in patients between 30 to 74 years in the US were T1DM [67]. Likewise, T2DM cases in adolescents and children are likely to overtake T1DM cases in the next 20 years [68]. Among the Native American, Mexican American and African American ethnic backgrounds, T2DM is disproportionately diagnosed in minority children as well as adolescents [69-71].

1.2.4 **Pathophysiology of DM**

1.2.4.1 **Type 2 Diabetes Mellitus (T2DM)**

Factors such as IR (in the liver and muscles) and impaired secretion of insulin play key roles in the development of the pathophysiological condition of T2DM [72] (Fig 2). Evidence of an association between T2DM and abdominal obesity has been reported, with about 80% of T2DM patients presenting with obesity [72]. Abdominal fat demonstrates more resistance to the action of insulin from achieving an anti-lipolytic effect than subcutaneous fat. In effect, the release of FFAs from abdominal fat is upregulated compared with subcutaneous fat. Elevation in FFAs is the primary reason for development of resistance to insulin by the cells of the liver and muscles. This accounts for the elevation in circulatory glucose due to inhibition of glucose uptake in muscles, mediated by insulin [73]. Similarly, upregulation in hepatic gluconeogenesis occurs leading to hyperglycaemia. Adipocytes, upon expanding, may be unable to store additional fat, resulting in deposition of fat in the liver, muscle and pancreas. The resultant effect is the worsening of cellular resistance to insulin. Development of resistance to insulin and hyperglycaemia induce higher pancreatic release of insulin leading to hyperinsulinemia. Subsequently, maintenance of high insulin concentrations may be compromised affecting the physiology of pancreatic beta cells with gradual degeneration of insulin output. The effect is the dominance of hyperglycaemia underlying the obvious symptoms of T2DM in patients. Loss of about 50% functionality of pancreatic beta cells is usually evident at the time of diagnosis in T2DM patients [73].

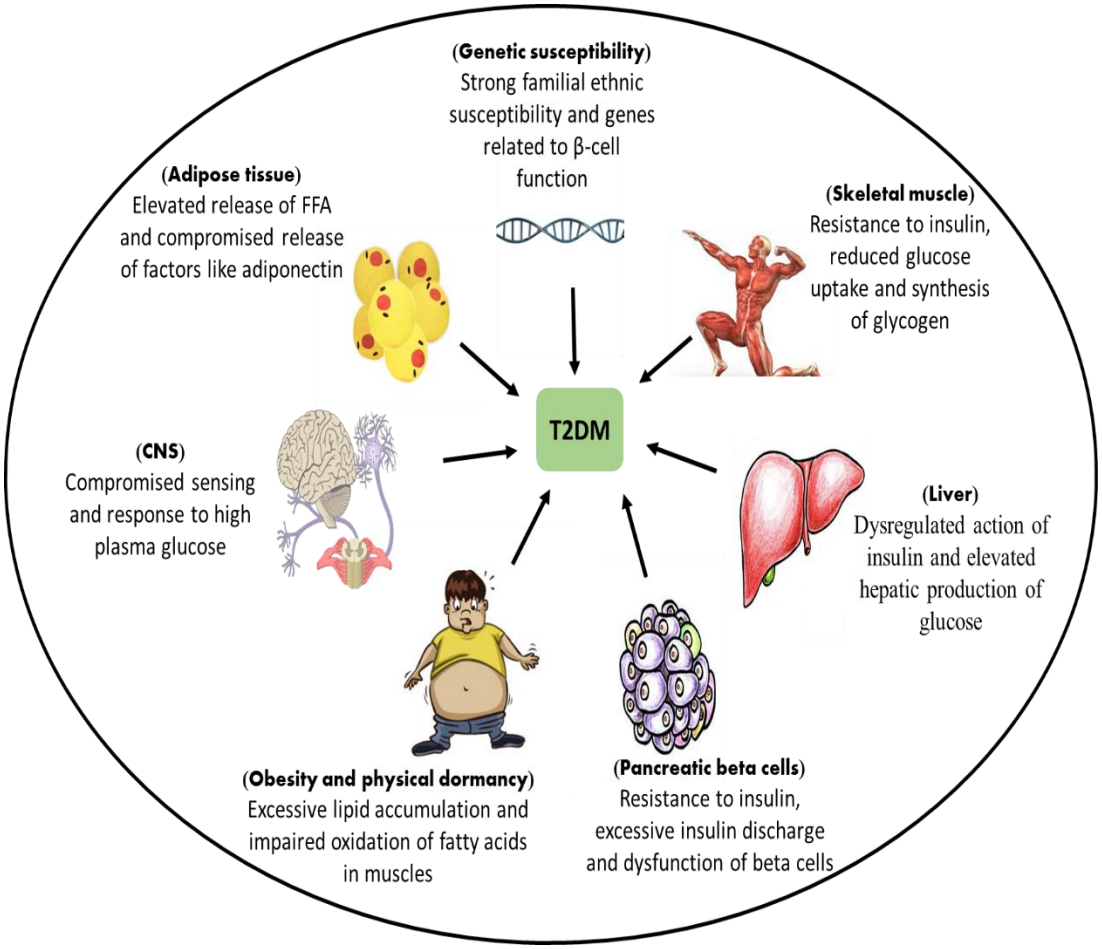

Figure 2: Pathophysiology of T2DM: A history of genetic predisposition in some families could account for defective beta cell functionality leading to the development of T2DM. Development of resistance to insulin accounts for hyperglycaemic conditions that propagate elevated secretion of insulin by the beta pancreatic cells as well as the dysfunction of beta cells. Obesity also presents with excessive accumulation of abdominal fat, with adipose tissues showing elevated resistance to insulin's anti-lipolytic effect, leading to elevation in the circulatory levels of free fatty acids (FFAs) encouraging further resistance to insulin in the liver and skeletal muscles. Hepatic output of glucose is increased boosting hyperglycaemia, whereas CNS detection and response to high circulatory glucose is also reduced.

1.2.4.2 **Gestational Diabetes Mellitus (GDM)**

During pregnancy, the co-existence of chronic resistance to insulin by tissues and deteriorated or dysfunctional beta cells of the pancreas collectively constitute key components involved in the pathophysiology of GDM. Generally, these critical components may be developed earlier before conception and might be progressive, maximizing the risk of T2DM development even after delivery [74]. Beta cells become dysfunctional by their failure to sense effectively precise levels glucose leading to secretion of abnormal insulin levels. However, the precise underlying mechanism accounting for dysfunctional pancreatic beta cells may be complex [75, 76].

Meanwhile, it is believed that a dysfunctional state could be the consequence of dysregulation in the beta cells, leading to prolonged over-secretion of insulin in response to availability of long-lasting excessive fuel [77]. The stages involving pre-biosynthesis of insulin, modulations at post-translational level, storage of granules and circulatory glucose level detection could all be defective. It is evident that the detection of minor deficiencies in the machinery of the beta cells may probably be obvious in the event of pregnancy wherein metabolic stress is inevitable [78]. Development of resistance to insulin by tissues aggravates dysfunction of the beta cells. In the event of hyperglycaemia, the influence of glucose on impaired beta cell functioning is termed glucotoxicity [79]. In effect, GDM resulting from these underlying factors may affect other systems and organs such as adipose tissue, muscle, brain and placenta [78].

1.2.4.3 **Type 1 Diabetes Mellitus (T1DM)**

The underlying mediators include macrophages as well as CD4+ and CD8+ T-lymphocytes that actively penetrate the islets of the pancreas causing autoimmune destruction [80]. Evidence of antibodies to islet cells in the circulation has been detected in about 85% of patients [80]. In addition, the bulk of patients also present with anti-insulin antibodies that are detectable prior to administration of insulin treatment [81]. The majority of anti-islet cell antibodies demonstrate an affinity to glutamic acid decarboxylase (GAD) present in the beta cells of the pancreas [81]. T1DM-associated metabolic disorders are, therefore, a result of the decline in levels of secreted insulin because of the autoimmune-mediated damage of the beta cells of the pancreas. Another observation in patients with T1DM is superfluous levels of glucagon secretion, aside from the deficiency in insulin production, resulting from abnormal or dysregulated function of the alpha cells of the pancreas [81]. Generally, a reduction in the production and release of glucagon are induced by hyperglycaemia. However, the hyperglycaemia-suppressing effect of glucagon is not achieved in patients with T1DM [82]. Insulin deficiency-induced defects of metabolism in T1DM are incongruously increased by circulatory concentrations of glucagon. The resulting effect is the suppression of the metabolism of glucose in other peripheral organs and tissues like the muscles due to dysregulated lipolysis with increased FFA levels [82]. Evidence shows that the insufficiency of insulin and impairment in the utilization of glucose further hamper the expression of key genes that play major roles in the transport of glucose in adipocytes (GLUT4) and hepatocytes (glucokinase) [82]. Generally, various pathways such as lipid, protein and glucose metabolism may be dysregulated as a result of T1DM-associated deficiency of insulin [82].

1.2.5 **Consequences of Diabetes Mellitus (DM)**

Complications associated with DM may be categorized as acute or chronic.

Acute complications associated with T1DM usually involve diabetic ketoacidosis (DKA), whereas T2DM results in the development of non-ketotic, hyper-osmolar state (NKHS). Evidence of total or relatively insufficient insulin, distorted mental condition and volume reduction are commonly observed in both types of DM [82]. The facilitation of metabolic events such as glycogenolysis, gluconeogenesis and development of ketone bodies within hepatocytes with a concomitant elevation in the muscle and adipose tissue release of FFA and amino acids to the liver are promoted by a decline in the insulin : glucagon ratio [83]. Aggravation in FFA discharge from adipocytes due to an elevation in lipolysis results in ketosis. In the event of severe DKA, coma may develop from CNS depression and lethargy. In children, development of serious complications such as cerebral coma is common. Features, usually common in adults with T2DM as a result of NKHS development, may include orthostatic hypotension, polyuria, lethargy and seizures as well as likelihood of coma. The insufficient intake of fluids alongside insufficiency of insulin together account for the condition of NKHS. Osmotic diuresis, principally resulting from hyperglycaemia caused by deficiency in insulin, progresses into exhaustion of the intravascular volume [83].

Chronic complications associated with DM normally account for cases of mortality and morbidity due to several organ systems being affected. Both nonvascular and vascular disorders are the main components of the chronic complications of DM. Similarly, macrovascular abnormalities may comprise cerebrovascular disease, coronary artery disease and retinopathy. On the other hand, microvascular disorders may involve neuropathy, retinopathy and nephropathy. Elevated intracellular glucose concentration, especially at the initial stages of DM, facilitates blood flow complications as well as elevating the permeability of the vessels. This, therefore, highlights the impairment effect on the normal functioning of vasodilators like nitric oxide while facilitating the vasoconstriction effects of endothelin I and angiotensin II. Another vascular defect may involve amplification of vascular endothelial growth factor (VEGF), a potent vascular permeability factor. In the arteries of DM patient, the effects of hyperglycaemia and resistance to insulin, targeted at the pathway of phosphatidylinositol-3-OH kinase, account for dysfunction of the endothelium.

Development of nonvascular abnormalities such as sexual dysfunction, gastroporesis and alterations in the normal skin are also marked in DM. Blindness, usually as a result of retinopathy or cataract in adults, renal disease, amputation, pregnancy complications, cerebral and cardiac abnormalities as well as various devastating neuropathies are collectively the result

of chronic complications that are largely caused by DM [83]. Emergence of severe complications resulting from DM are mostly associated with evolving renal disease [84]. Evidence or signs of diabetic renal involvement are normally seen after a period of 18 years in almost 35% T1DM patients [85]. There is, lastly, evidence indicating that nearly 35% of new renal disease patients, at the time of commencement of dialysis treatment, would have type 2 diabetes [86].

1.3 **CARDIOVASCULAR DISEASE (CVD)**

CVDs indicate a broad range of abnormalities affecting the vascular system (which constitutes the heart and blood vessels), as well as cardiac muscle disorders [87]. CVD involves disorders like stroke, coronary heart disease (CHD), cardiomyopathy and rheumatic heart disease and, collectively, are the principal cause of death globally [88]. CVDs may be primarily caused by underlying conditions of atherosclerosis and/or infectious diseases. Conditions such as physical immobility, diabetes, hypertension, increased levels of LDL- cholesterol, smoking of tobacco and several interconnected metabolic risk factors, collectively, present as probable risk factors to the development of CVD [89].

1.3.1 **Epidemiology of CVD**

An estimate of less than 10% of global mortalities were as a result of CVD at the beginning of the 20th century [90]. However, this estimate increased to about 30% by the year 2001 [90]. Evidence shows that CVD was the second major cause of mortality in the UK in the year 2014 [90]. However, recent data from the Global Burden of Disease (GBD) indicate that the challenge imposed by CVD is decreasing in the UK [91]. For instance, between the years 1990 and 2013, mortalities resulting from CVD in England reduced significantly by 52%, strokes by 46% and CHD by 60% [91]. Evidence from the Quality and Outcomes Framework (QOF) demonstrates that, over a decade in England, the prevalence of CHD was held stable at about 3%, whereas Wales, Scotland and Northern Ireland recorded a prevalence of 4% particularly between the years 2004/2005 and 2014/2015 [90]. However, there is evidence of only a slight elevation in cases of stroke in Scotland, England and Northern Ireland with a prevalence estimated to be about 2%. Evidence demonstrates that men resident in England generally present with a slight elevation in the prevalence of stroke in comparison with women [90]. Similarly, the prevalence of CVD in the year 2008, as estimated by the Scottish Health Survey, declined significantly for women but not men [90]. However, in middle and low-income nations across the world, about 80% of mortalities are a result of CVD [92]. It is, therefore, estimated

that CVD will be the principal cause of disability and mortality, particularly in middle and low-income nations across the globe by the year 2020 [92]. Approximately 9% of global deaths per annum could be attributed to CVD [93].

In India, CVDs are known to be the principal cause of death since the last century [94]. Indians more or less develop CVDs in the course of their midlife ages where productivity is high [95]. When compared with other ethnic origins such as the Europeans, Indians may acquire CVDs at least ten years earlier [95]. Among individuals below the age of seventy, CVDs account for 52% mortalities among Indians compared with about 23% in Europeans [96]. It is predicted that the global rate of CVD may be increased as long as countries, formerly known to be at low-risk, show evidence of a rising prevalence of risk factors for CVD [96]. Evidence recently showed that CVD accounts for about 80% mortality in developing countries [96]. It is further predicted that CVD would become the principal cause of death, exceeding infectious disorders, in several developing countries by the year 2020 [97]. The major cause of worldwide loss of disability-altered years of life, aside being the principal cause of global deaths, is CVD [98].

1.3.2 **Pathophysiology of CVD**

Obesity, marked by excessive accumulation of fat around the waist, is a major risk factor for T2DM development, it strongly aggravates the risk of developing coronary heart disease (CHD). Excessive weight build-up elevates cardiac strain, increases the blood pressure as well as lipids such as TG and cholesterol whilst decreasing levels of HDL cholesterol. As a result, the risk of developing conditions such as atherosclerosis and thrombolytic embolism are increased [99].

Similarly, diabetes (T1DM and T2DM) also independently presents as a major risk factor for various forms of CVDs [86, 99]. Evidence shows that the innate protection against development of CVD in women may be lost due to diabetes [100]. Diabetes can affect muscles of the heart which might result in both diastolic and systolic failure of the heart. Hyperglycaemia is evident in cases of DM and plays a key role in damaging the myocardium following ischaemic events. However, under conditions of normal glucose, other disorders like MetS and pre-diabetic conditions could aggravate the risk of development of major CVDs [101-103]. Efforts to ensure a reduction in the risk of CVD development in patients diagnosed with diabetes by healthcare providers are achieved *via* provision of treatment for conditions such as hypertension and dyslipidaemia, alongside targeting the improvement of glycaemic control [104]. Following the development of CVD in diabetic patients, prognosis for survival is normally worse as compared with non-diabetic patients [105].

In CVD-mediated defects of the macro-vasculature in patients, atherosclerosis is the principal underlying cause independent of diabetes mellitus. Clinical evidence demonstrates a high association between atherosclerosis and dyslipidaemia with about 97% of diabetic patients additionally presenting with dyslipidaemia [106]. Not only this but also dysfunction of the endothelium, presents as an underlying mechanism through which atherosclerosis could be promoted by DM. Other conditions such as deficiency and resistance to insulin also enhance the development of dyslipidaemia together with other processes such as glycosylation, decreased oxidation as well as augmentation of TGs within lipoproteins. All these factors adding up to endothelial dysfunction, therefore contribute towards the development of atherogenicity as is evident in patients with DM and CVD. Likewise, DM demonstrates an effect on smaller blood vessels in the whole body such as those found in the heart, brain and the vasculature in the circulation. The condition of myocardial damage, devoid of macrovascular defects such as coronary artery disease (CAD), demonstrates a greater probability of resulting from microvascular dysfunction. Conditions such as fibrosis, myocardial injury and hypertrophy, as observed in cases of cardiomyopathy in diabetic patients, could be as a result of microvascular damage in DM patients [107].

The amount of fat in the liver has similarly been shown to be prognostic of the risk of developing MetS and CVD [108]. Non-alcoholic fatty liver disease (NAFLD), comprising simple steatosis (SS), non-alcoholic steatohepatitis (NASH) and liver cirrhosis, is a well-known chronic liver disease which is commonly associated with IR and MetS. Substantial evidence has been shown in the past years suggesting that NAFLD may be significantly associated with CVDs including subclinical carotid atherosclerosis and CAD [108]. Likewise, elevations in pulse wave velocity, development of endothelial dysfunction as well as increased thickness of the carotid intima-media are associated with NAFLD and have been similarly shown to be potential markers for CVD [109]. CVD and NAFLD have also been proposed to be bi-directionally associated, such that one condition could potentially potentiate the other, although the causal relationship between them has not been completely proven [110]. Moreover, studies have reported higher rate of CVD incidence in NAFLD patients and the former may furthermore be accountable for increased mortality in NAFLD patients [110]. To buttress this, a previous meta-analysis reported that the risk of fatal and/or non-fatal CVD development in NAFLD patients was about 64% greater compared with patients without NAFLD [111].

1.3.3 **Consequences of CVD**

Coronary atherosclerosis in diabetic patients usually develops into myocardial ischaemia even without any manifestation of prior symptoms [112]. This subsequently results in the emergence of multi-vessel atherosclerosis earlier, before the manifestation of ischaemic symptoms or commencement of treatment. In effect, a worsening prognosis for survival may be observed as a result of delayed recognition of the diverse groups of coronary heart disease (CHD) in diabetic patients [112]. Similarly, diabetic patients with a diagnosis of ischaemic heart disease tend to demonstrate a poor prognosis as a result of the advanced state of myocardial dysfunction with higher chances of developing cardiac failure [113-115]. There is about three-fold higher likelihood of death resulting from stroke in diabetic patients [116]. Lastly, the development of carotid emboli leading to irreversible damage to the brain is most likely to be observed in diabetic patients diagnosed with CVD.

1.4 **ENVIRONMENTAL FACTORS AND METABOLIC RISK**

Humans, in the course of evolution, have adapted geographically to different ecosystems for the principal purpose of survival. Humans, therefore, do inhabit complex microenvironments that were self-generated as well as socially networked and moulded by culture and history. Unlike humans, other animals dwell strictly within only their natural environment. It is, therefore, essential for critical evaluation of the human environment (constituting natural, personal and social domains) in order to appreciate the entire settings governing humans. These domains of the environment may, therefore, play a crucial role in balancing both the pathogenic and salutogenic impacts on humans as exposed by the natural environment. However, unlike the other environmental domains, the personal environment, which is characteristically flexible and proximal to humans, is a major contributing factor to human health.

The personal environment, as in the perspective of the social environment, is usually fashioned by both the preferences of an individual and adoption of a personal lifestyle. Some key factors that constitute the personal environment may include nutrition or dietary behaviour, physical activity, alcohol consumption and smoking.

It is obvious that a person's life does not necessarily commence at the time of birth but from the onset of conception, wherein all the information required for growth into a new individual is contained within the genome of the conceptus [117]. The flow of the information originates from DNA, through RNA and ends up in proteins as explained by the central dogma of molecular biology. This flow may, however, be subjected to regulation by external factors,

hence influencing the growth and differentiation of the systems, organs, tissues and cells that make up the phenotype of the person. The exact environmental processes connecting the components of the genotype to that of the phenotype at birth are termed epigenetics [118]. In the event of unfavourable conditions or adversity *in utero*, efforts are made by the foetus to adapt to this unconducive environment. The foetus is, therefore, subjected to go through predictive, adaptive programming by way of lessening the influence of the incompatible environment *in utero*. However, the *in utero* adaptations in the foetus normally persist even after birth and could probably be helpful if the environmental conditions *in utero* do not alter even after birth. Meanwhile, environmental orientation in postnatal life is normally different and, therefore, adaptation to the new postnatal environment by the individual becomes unrealistic. Some factors that may adversely affect the foetal environment may include smoking, alcohol intake, maternal undernutrition, DM, uterine artery blockage, as well as drugs and pharmaceutical products [118].

Evidence from numerous studies showed that the risk of developing CVD during the late stages of life may be determined by the uterine environment [118] (Fig 3). Similarly, it was evident that stimulation of atherosclerotic lesions during the developmental stages of the foetus could be as a result of poor uterine environment [118]. This is further confirmed by the identification of fatty streaks, build-up of oxidized lipids as well as signs of inflammation within the aorta of foetuses of premature humans, with further thickening of the intima of their coronary arteries [119]. Again, an underprivileged environment *in utero* normally accounts for low birth weight and has been shown to demonstrate a strong negative association with ischaemic cardiac disease [119]. Upon further adjustment for duration of gestation, the negative relationship continues, therefore, highlighting that restrictions to foetal growth, though not premature delivery, was accountable for the elevated risk of CVD [120]. Exposure of parents to small volumes of smoke derived from tobacco within the environment, in a study involving mouse models, accounted for an effect on the weight gain of the offspring [121]. This also induced lipid profiles capable of altering the risk of CVD in the offspring later in life [121]. Similarly, mothers who normally smoked in the course of pregnancy had infants who presented with elevated risk of developing obesity [122]. In the UK population, the maternal level of vitamin B12 was shown to demonstrate an association with increased BMI and the risk of developing GDM and it is also an autonomous determinant of macrosomia [123]. The authors further recommended B12 assessment in early pregnancy by longitudinal studies to explore this association, due to the rising burden of GDM and maternal obesity [123]. Similar studies have also shown a

relationship between low maternal plasma B12 and increased IR and obesity in a White British, non-diabetic population [124].

In conclusion, parental exposure to pollutants and other hostile conditions of the environment can elevate the risk of CVD development in the mother and could also trigger reprogramming of the foetus that may cause an alteration in the risk of future CVD.

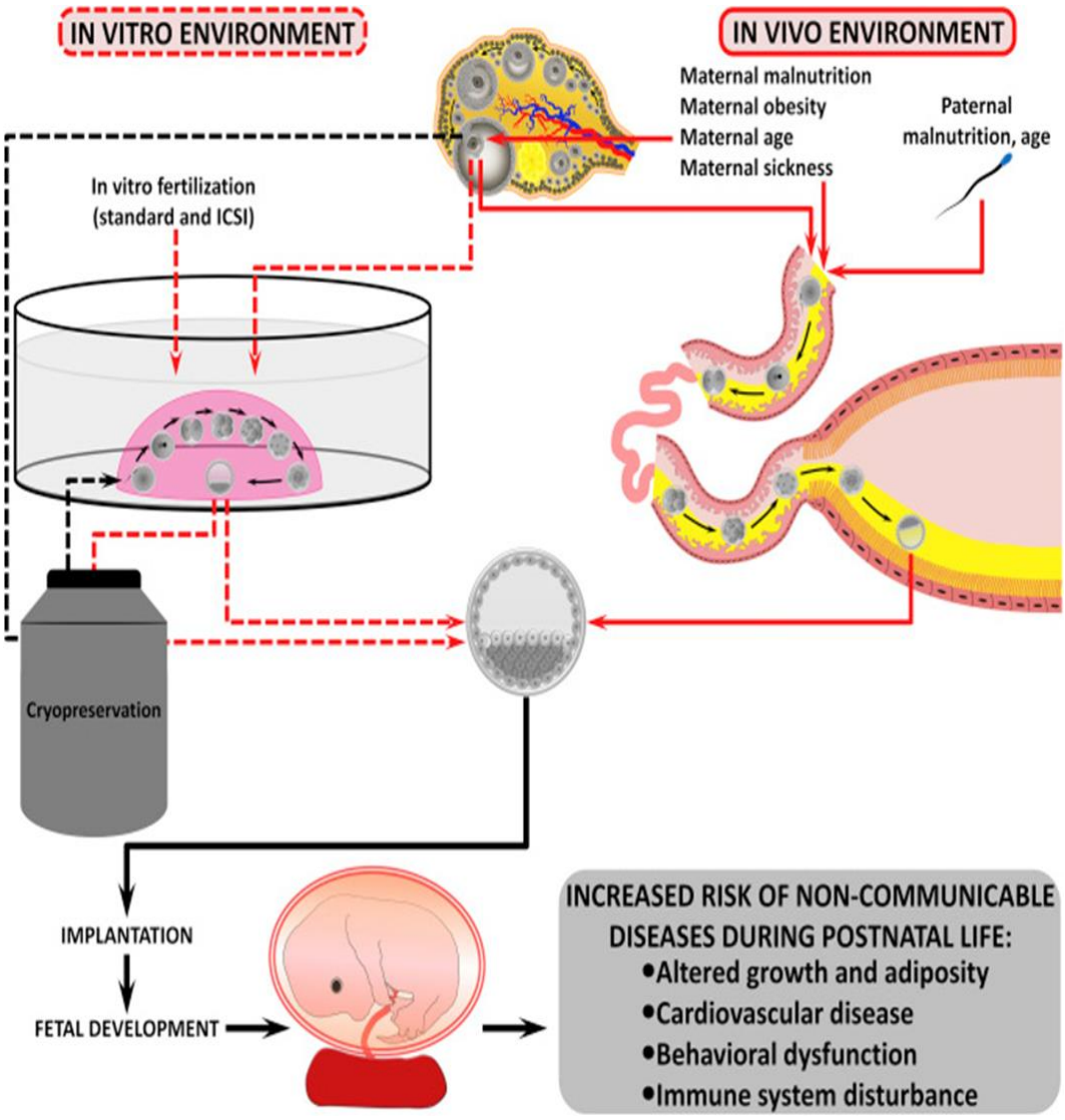

Figure 3: Developmental origins of health and disease (DOHaD) and the periconceptional period [125]: The periconceptional phase of a mouse originating from fertilization, following initial maturation of the oocyte, with subsequent formation of the blastocyst after cleavage as well as isolation of lineages of embryonic / extraembryonic cells leading to foetal development until late gestation. *In vivo*, maternal health, metabolism and dietary conformation can collectively, or separately, result in periconceptional programming. Similarly, probable conditions *in vitro* including cryopreservation, culture of embryo, fertilization (IVF) as well as other treatments supporting conception may also induce related effects [125] (p. 36).

1.5 **VITAMIN B12: ROLE IN METABOLIC DISEASES**

1.5.1 **Vitamin B12 deficiency**

1.5.1.1 **Background**

Description of deficiency in vitamin B12 (B12) was first reported in 1849 as a result of its deadly consequences observed in patients [126]. However, in 1926, the remedy for slowing the fatal effects of B12 deficiency was recognized *via* ingestion of B12-rich liver diet [126]. So far, two key aspects of nutrition involving B12 prevail as a great challenge for human nutrition in the past century. First, the incidence of pernicious anaemia (PA), an exclusive autoimmune disorder of humans and second, the challenge of the sources of B12 entirely restricted to animal-rich protein diets [126]. It was formerly assumed that developing B12 deficiency was a gradual process requiring several years for its manifestation and that the vulnerable groups of individuals were only strict vegetarians and patients with pernicious anaemia. However, it is currently evident that dietary insufficiency and malabsorption of B12 may result in a condition of sub-clinical deficiency which could additionally account for some disease consequences in patients [126]. PA is normally a result of an autoimmune impairment of intrinsic factor (IF) secretion that consequently leads to inception of a severe deficiency of B12. Patients with B12 deficiency resulting from PA clinically present with macrocytic anaemia and an impairment of the bone marrow efficacy affecting all cell lines. It was initially challenging to distinguish B12 deficiency-induced megaloblastic anaemia from that of folate deficiency because the anaemia tends to respond to pharmacologic therapy with folic acid [127]. About 30% of individuals diagnosed with PA present with the signs and symptoms associated with demyelination of the cerebral part of the brain, the spinal cord and peripheral or cranial nerves. This impairment of the central nervous system (CNS) is inversely associated with the severity of the megaloblastic anaemia and usually presents with the challenge of adequately finding a deficiency of B12 when elucidation of the deficiency is based on the megaloblastic anaemia [128]. Defects associated with movement are usually evident in younger children and infants with B12 deficiency. Irritability, difficulties in feeding, unusual reflexes, likelihood of coma following obtundation and the possibility of long-lasting disabilities resulting from poor brain development are all symptoms noticeable in infants [129]. Adequate B12 replacement in cases of PA facilitates improvement of disorders of the central nervous system (CNS) as well as reversing the condition of megaloblastic anaemia. Therefore, it suggests that identification and treatment of PA is essential. Likewise, milder forms of B12 deficiency also require early detection and treatment. This may help alleviate the associated hyper-homocysteinaemia presenting as a key risk factor for development of cognitive impairment as well as disorders of the vascular system.

Evidence of high rates of B12 sub-clinical deficiency have been shown in aged individuals, population of vegetarians and inhabitants of several developing countries [126]. Although the detailed long-term outcomes associated with B12 deficiency remain quite unclear, the proposed implications may involve possible effects on ageing and on the aftermath of pregnancy [126].

1.5.1.2 **Epidemiology**

Studies from the Global Hunger Index reported that around 2 billion humans are affected by micronutrient deficiency. The WHO is specifically concerned about the levels of vitamin B12 (B12) and folate due to the increasing prevalence of deficiency across populations [130]. Epidemiological studies have clearly shown an association of these nutritional metabolites and manifestations of metabolic risk [131-134].

Global epidemiological assessment of the prevalence of total B12 deficiency is currently missing [135], however, evidence suggests a probable higher prevalence among vegetarians and the high prevalence in the vegan population found in Southern part of Asia [136]. Manifestation of B12 deficiency could be evident in various groups of individuals, such as actively growing children, in pregnancy and adolescence, where high B12 demand is observed. Similarly, a significant reduction in B12 intake leading to deficiency may be observed in other groups of individuals including the elderly, strict vegetarians as well as those on poor nutrition. Among people younger than 60 years in the UK and US, the prevalence of B12 deficiency is about 6% [137]. However, almost 20% prevalence has been reported in the elderly over 60 years of age [137]. Almost 40% of children as well as adults living across Latin America present with subclinical and clinical deficiencies of B12 [137]. Countries of both Asian and African origin have recorded higher prevalence of B12 deficiency. For instance, 80% prevalence of B12 deficiency was reported among preschool children in India, 70% in school children of Kenya and also 70% prevalence in adults resident in India [138]. The rates of prevalence may, however, differ among vegan and vegetarian populations. In the UK, for instance, prevalence of B12 deficiency among vegans was 11%, whereas about 62% of pregnant vegetarian women were B12 deficient in Ethiopia. [139].

1.5.1.3 **Diagnostic markers and risk factors**

Investigation of B12 deficiency is ideal for patients presenting with suspected clinical indicators and screening may now be recommended for patients presenting with the known risk factors [137, 140, 141]. However, performance of routine screening among individuals presenting with an average risk of deficiency of B12 is generally not recommended [142]. Laboratory tests such

as complete blood count (CBC) and serum B12 are recommended for patients who may be suspected of deficiency in B12 [143]. Deficiency of B12 may be non-dependent on an established 'cut-off' value internationally but serum B12 levels lower than 150 pmol/l are termed deficient (Lower limit: 120-200 pmol/l, normal range: 150-850 pmol/l) [140, 144]. There is evidence that other parameters such as levels of B12-bound-transcobalamin II complex (holotranscobalamin, holoTC), serum methyl malonyl CoA (MMA) and homocysteine give a more reliable basis for the diagnosis of B12 deficiency [131]. Currently, there are limited data on the global prevalence of B12 deficiency based on MMA and homocysteine levels [145]. The risk of developing B12 deficiency is higher in vegetarian populations such as in India [146]. However it is not uncommon (10-30% prevalence) in other populations, and is higher in pregnant populations [144]. Some causes of B12 deficiency are shown in Table 1.

Table 1: Causes of B12 deficiency [137].

a). **Reduced B12 intake**

- i. Malnutrition
- ii. Decreased ingestion of animal-rich products
- iii. Strict vegan diet

b). **An impairment of B12 bioavailability via gastric wall damage.**

- i. Due to total and or partial gastrectomy
- ii. Zollinger-Ellison syndrome
- iii. Pernicious anaemia

c)**. Impairment of absorption via the intestines**

- i. Blind loop syndrome
- ii. Infections including overgrowth of bacteria and giardiasis
- iii. Ileal resection
- iv. Luminal disturbances

d). **Inherited (Congenital) conditions of B12 deficiency**

- i. Defect of the intrinsic factor receptor such as in ImerslundGräsback syndrome
- ii. Juvenile pernicious anaemia – A congenital intrinsic factor (IF) deficiency
- iii. Cobalamin mutation (C-G-1 gene)

iv. Deficiency in Transcobalamin (TC)

e). **Increased B12 requirements**

i. Haemolytic anaemic conditions

ii. HIV infection

f). **Drugs**

i. Nitrous oxide

ii. Alcohol intake

iii. Inhibitors of proton pump

iv. Blockers of H_2 receptor

v. Metformin

1.5.2 **B12: Biochemical structure and synthesis**

B12 (cobalamin) classified as an essential vitamin, is entirely obtained from the diet. Although the vitamin is synthesized naturally by the large intestine-resident bacteria in humans, the human B12 uptake system is located in the small intestine so this source is generally less bioavailable to humans [127, 147]. B12 was first isolated in 1948 [148] following the discovery of liver extract as a source of therapy for pernicious anaemia [149]. Using X-ray crystallography, B12 was structurally described as a massive organometallic compound with size ranging between 1300 to 1500Da [150]. The vitamin is uniquely composed of a central cobalt atom linked to six ligands, four of which are structurally reduced to form a corrin ring that encircles and is linked to the cobalt atom through direct nitrogen linkages (Fig. 4). Directly below the central cobalt atom is an α-axial 5,6-dimethylbenzimidazole (DMB) ligand which, through phosphoribosyl moiety, links to the corrin ring and confers a high specificity on the vitamin for intrinsic factor (IF) binding in the lower gastrointestinal tract [151]. The β-axial ligand (R-ligand) positioned above the corrin ring may vary from a methyl-, 5'-deoxyadenosyl, hydroxo-, acquo- or cyano- group. These are named as methylcobalamin, deoxyadenosyl cobalamin, hydroxycobalamin, acquocobalamin and cyanocobalamin, respectively.

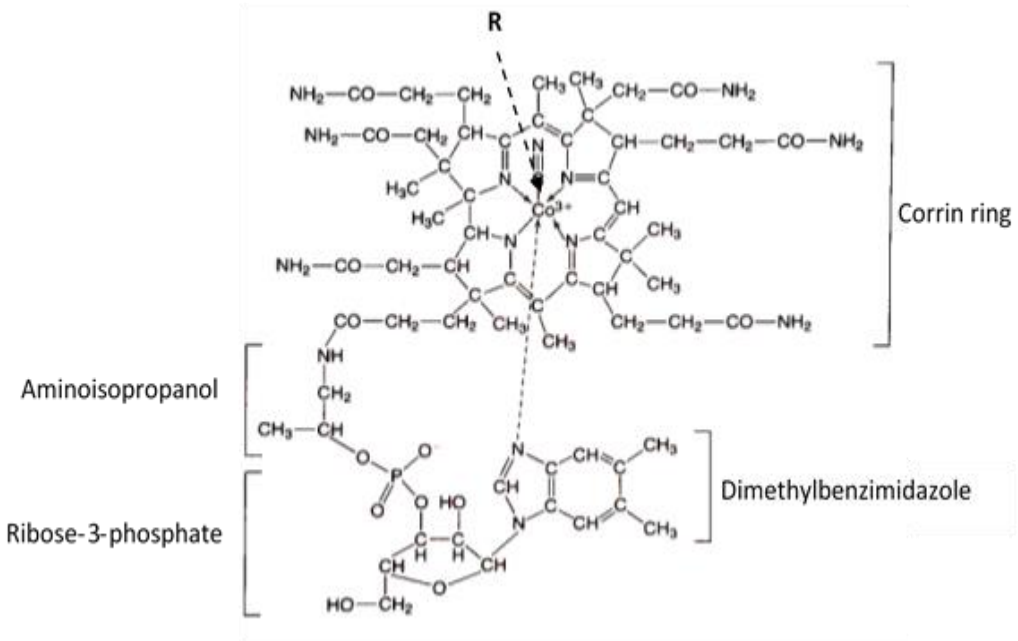

Figure 4: Structure of vitamin B12 [150]: The central point of the vitamin is a cobalt atom to which a corrin ring is formed from four of the six ligands directly linked to the cobalt, encircling through nitrogen atom linkages. The R-group at the β-axial (upper) position may be a methyl-, 5'-deoxyadenosyl-, hydroxo- or a cyano- group whereas the α-axial (lower) ligand is a 5,6-dimethylbenzimidazole (DMB) linked through phosphoribosyl moiety to the central cobalt atom.

1.5.3 **Sources, bioavailability, transport and hepatic uptake of B12**

B12 is absent in plants but natural sources predominate in animals and their products such as eggs and milk. In addition, the gastrointestinal (GIT)-resident microorganisms possess the enzymes needed for B12 biosynthesis [152, 153]. The absorptive capability of the GIT of an individual undergoes adverse alteration with age which determines the bioavailability of B12 in humans [154]. However, the absorption of B12 is aided by availability of calcium and takes place at the terminal ileum where precise receptors, such as cubam, are expressed on microvilli on the intestinal endothelium. The receptors bind to the intrinsic factor (IF) part of the IF-B12 complex and then delivers the B12 into the peripheral blood [154, 155]. While in circulation, plasma protein such as transcobalamin (TC) II plays a major role in transporting B12 to various tissues but predominantly in the liver (Fig 5). Therefore, significant amounts of the water soluble B12 are stored in the liver of healthy humans. An in-depth study using samples obtained from liver biopsies showed an average healthy liver B12 content of 1.94µg (range: 1.41 to 2.58µg) per gram of wet liver tissue [156]. However, B12 content of the liver may be lower in cases of cirrhosis, viral hepatitis, fatty liver and obstructive jaundice [157]. The human body is

known to eliminate a total of about 2 to 5µg of vitamin B12 daily, primarily *via* faeces with minimal amounts in urine [158].

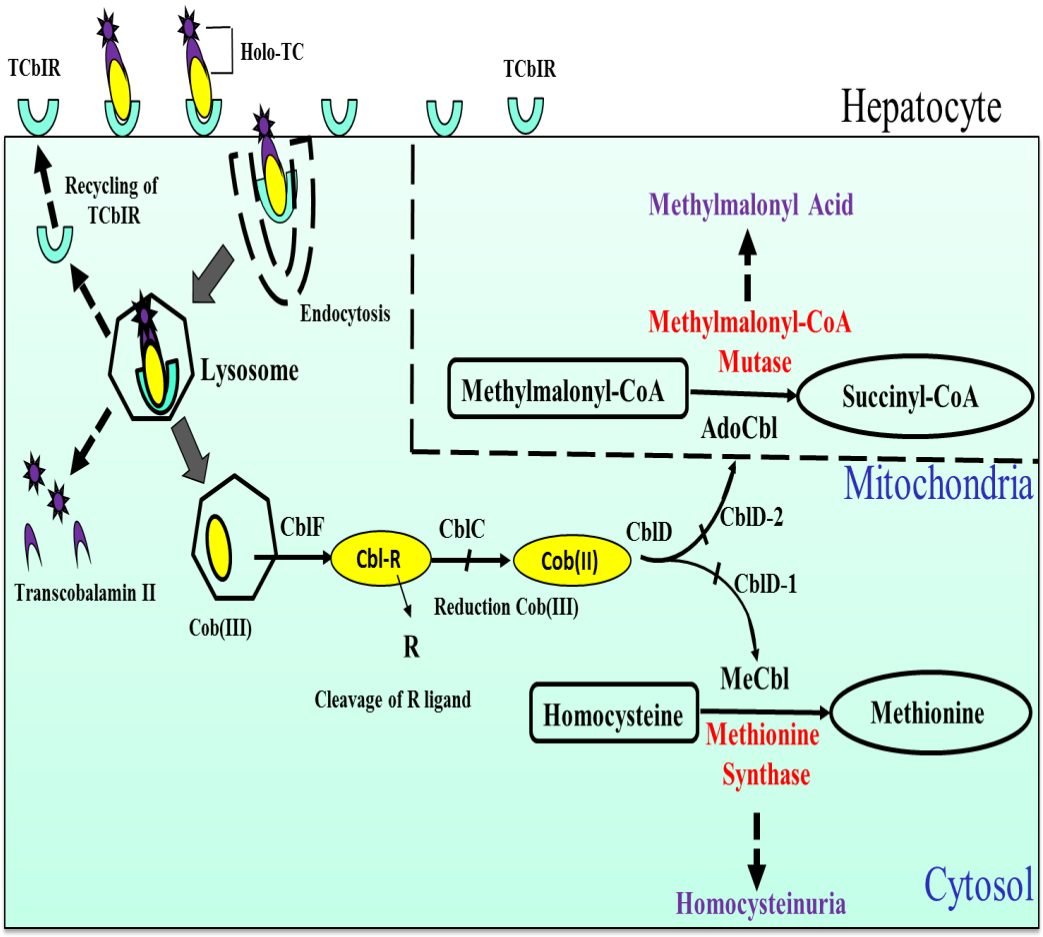

Figure 5: Hepatic uptake and metabolism of B12: Hepatocytes internalize B12-bound transcobolamin (holo-TC) with the aid of transcobalamin receptors (TCR) TCbIR / CD320 *via* endocytosis and it is fused into lysosomes. Within this organelle, B12 is liberated from the TC with the free TC (Apo-TC) subjected to degradation while the former (B12) is transported to the cytosol and further processed to its catalytic forms: 5'-adenosyl cobalamin (AdoCbl) and methyl-cobalamin (MeCbl). These serve either in the mitochondria or cytosol as co-enzymes in the Methyl malonyl CoA mutase (MCM) and methionine synthase (MS) pathways, respectively. The transcobalamin receptors (TCbIR) are, however, recycled back to the surface of the hepatocyte.

1.5.3.1 **Hepatic metabolism of lipids**

Apart from being the principal storage organ for B12, the liver is also paramount in the regulation of lipid metabolism in humans. During the stabilization of blood sugar levels, the liver, as a result of responding to the energy requirements of the body, induces modifications in the hepatic metabolism of lipids and carbohydrates [159]. Following the hepatic uptake of glucose and complete restocking of the liver stores of glycogen, facilitated by the insulin-

dependent liver enzyme glycogen synthase, a further mechanism to facilitate a prolonged storage of energy is adopted by the liver *via de novo* lipogenesis which makes use of the excess glucose available [160]. Lipids such as triglycerides (TG) are synthesized from fatty acids and glycerol and undergo further packaging into very low density lipoprotein (VLDL) which are suitable for delivery *via* the circulation to the adipose tissue for storage [161, 162]. Hepatic lipid metabolism normally undergoes modulations by key hepatic nuclear transcription factors including the carbohydrate responsive element binding protein (ChREBP) [163, 164], sterol regulatory element binding protein 1c (SREBP 1c), peroxisome proliferator-activated receptor gamma (PPAR-γ), liver X receptor alpha (LXR-α) as well as the x-box binding protein 1 (XBP1) [165, 166]. Regulation of hepatic metabolism of lipids by these transcription factors is achieved *via* their interaction with diverse transporters and lipogenic enzymes present in the liver (Fig 6). However, abnormal lipid accumulation in the liver may result from dysregulation of the hepatic metabolism of lipids. The lipid accumulation usually begins with simple steatosis (SS) and further progresses into steatohepatitis. Possible further progression may end up in deadly conditions such as fibrosis of the liver, cirrhosis and even hepatocellular carcinoma (HCC). All these conditions, collectively referred to as non-alcoholic fatty liver disease (NAFLD) [160], have shown a significant association with hyperlipidaemia, obesity, IR and T2DM [167].

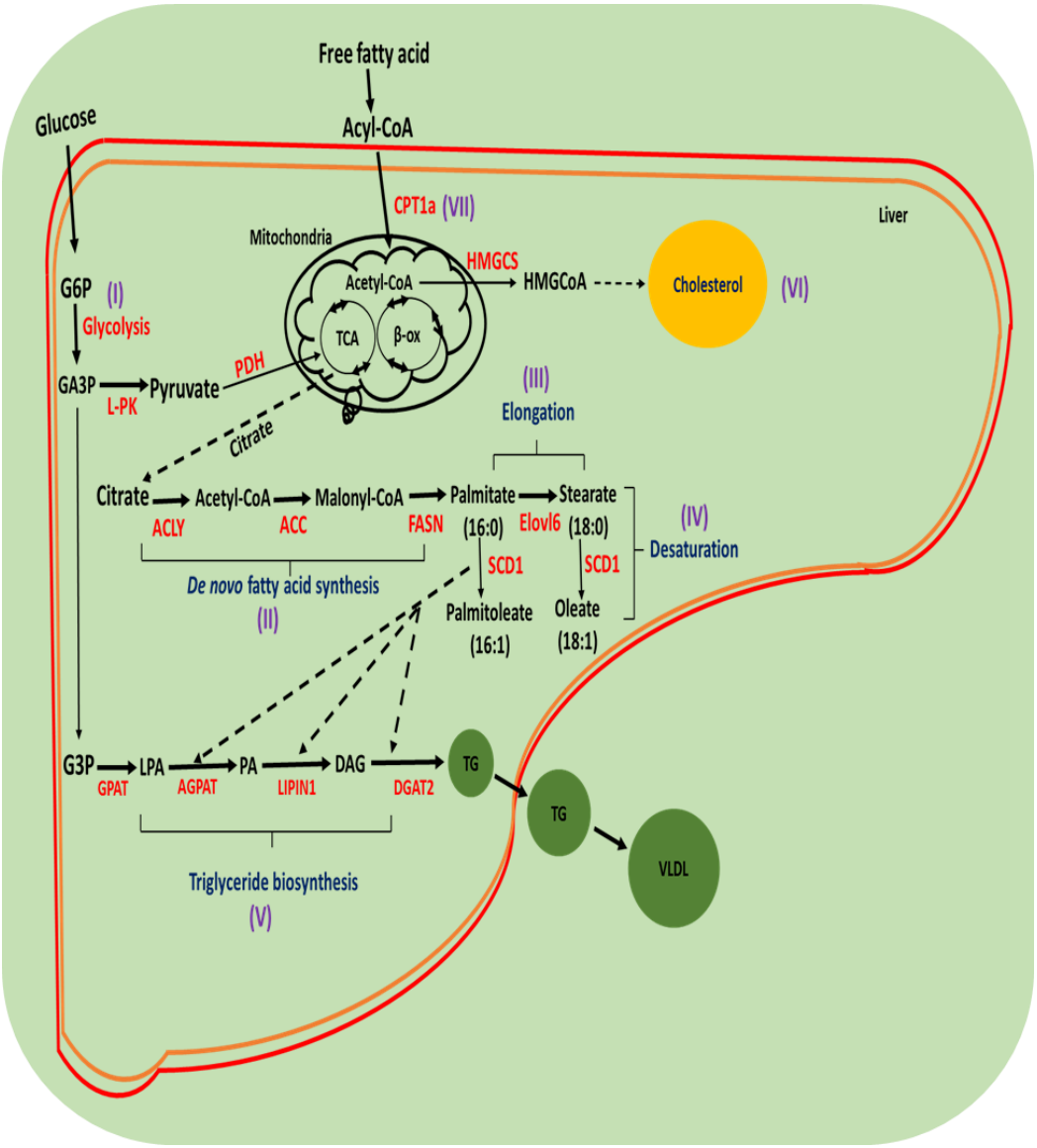

Figure 6: **Hepatic lipid metabolism**: Lipogenesis constitutes fatty acid, triglyceride and cholesterol biosynthesis. *De novo* fatty acid synthesis **(II)** begins with the utilization of citrate, a product from the tricarboxylic acid (TCA) cycle in the mitochondria using pyruvate from glycolysis **(I)**, which is further converted to acetyl co-enzyme A in the cytosol using the enzyme ATP citrate lyase (ACLY). Palmitate is first synthesized followed by elongation (**III**) into stearate, with both subjected to desaturation (**IV**) to produce palmitoleate and oleate, respectively, by collectively utilizing the enzymes ACC, FASN, ELOVL6 and SCD1.

Esterification of fatty acids onto a glycerol-3-phosphate (G3P) substrate using enzymes such as GPAT, AGPAT, LIPIN1 and DGAT2 leading to triglyceride biosynthesis (**V**). TGs are further packaged into very low-density lipoprotein (VLDL) for storage in adipose tissues. Acetyl CoA derivation from the TCA or Krebs cycle in the mitochondria is utilized for cholesterol synthesis (**VI**) using rate limiting enzymes such as 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS). In the fatty acid oxidation (FAO) pathway, long chain fatty acyl CoA derived from long chain fatty acids is delivered into the mitochondria with the aid of carnitine palmitoyl transferase I alpha (CPT1α) (**VII**) for subsequent breakdown into small units of acetyl CoA.

1.5.4 **Vitamin B12 deficiency is associated with obesity**

i. Evidence from pre-clinical studies

Given the vastness of observational and epidemiological evidences on the effects of B12 on metabolic risk, it is expected that animal studies based on these hypotheses provide cues on the underlying mechanisms. Studies in adult Wistar rats with restricted B12 levels in Wistar rats' maternal or postnatal period, predicted higher visceral adiposity and resulted in alterations in the metabolism of lipids in the offspring [168]. Severe, not moderate, deficiency of B12 also induced higher adiposity in C57BL/6 mice [169]. Separate and/or combined exposure to B12 and folate deficiency for a period of three months in pre-pregnant Wistar rats resulted in an increase in body weight in both B12-deficient and folate deficient cases rather than in controls. Observation of higher total body fat was evident in B12-deficient animals whereas folate-deficient rats presented with elevations in visceral fat mass [168, 170].

ii. Evidence from clinical studies:

Among adults in the US, an inverse association was recently shown between serum B12 levels and obesity [171]. In a cross-sectional and primary care-based study that assessed the probable correlation of B12 with obesity, IR and MetS, low level of B12 was associated with overweight and obesity, but not with gender [172]. The study further concluded that B12 had a stronger negative association with BMI than IR and MetS [172]. Low concentration of B12 was reported to be associated with higher risk of obesity in adolescents and children [173]. In another study, mean B12 concentrations were observed to be significantly less in obese children compared with healthy volunteers [174] and B12 was also negatively associated with the severity of obesity [175]. The inverse relationship between maternal BMI at early stage pregnancy and plasma B12 and folate, according to a recent observational study, was observed in obese women compared with normal BMI women [176]. In a recent systematic review of clinical data [177], lower concentration of B12 was observed among individuals with higher BMI, according to the evidence from direct pairwise subnetworks such as; overweight versus control, obesity versus overweight and obesity versus control [177]. Sukumar *et al*. reported that pregnant women presenting with insufficiency of B12, at the first trimester, had increased BMI compared with those with normal B12 levels [178]. In India, low maternal circulatory levels of B12 were suggestive of contributing to an increased likelihood of developing higher adiposity as well as the risk of T2DM in pregnancy [179].

1.5.5 **Vitamin B12 is associated with metabolic disorders**

1.5.5.1 **Vitamin B12 and insulin resistance**

i. Evidence from pre-clinical studies:

Increased resistance and elevated blood pressure in models such as lambs (sheep offspring) due to restriction of maternal B12/folate/methionine at conception was explained by induction of epigenetic modulations [180]. In addition, the adult male offspring were observed to have higher adiposity and altered functioning of their immunity. This evidence was explained by observation of male-specific demethylation of the affected loci, therefore, providing concrete reasons for the observation of these differences in the phenotypes of the various sexes of the offspring [180]. Further, this result of decreased methylation of DNA could be explained by lower availability of the methyl donor SAM, affecting the epigenetic mechanisms that account for the increased resistance to insulin [180, 181].

ii. Evidence from clinical studies:

Among obese children presenting with IR, the levels of B12 were observed to be significantly decreased [182]. Likewise, in other population groups such as non-pregnant adults, polycystic ovarian syndrome women and obese adolescents, the evidence of higher insulin resistance has been shown to be a result of insufficiency of B12 levels in these subjects [172, 183-185]. In obese adolescents with low or borderline levels of B12, there was a demonstrable association between low B12 status and insulin resistance as well as obesity [186]. In an observational study based in Pune, India, cases of either B12 deficiency in mothers, particularly at the eighteenth week of gestation, or high folate in red blood cells at the twenty-eighth week of gestation, independently predicted an increased risk of resistance to insulin in their children between the ages of 6 to 7 years [179]. The highest resistance to insulin was observed in children born to mothers with both deficiency in B12 and elevated folate in pregnancy [179]. Low levels of B12 in the plasma of mothers at early, not late, pregnancy was linked with a significant increase in the resistance to insulin, expressed by homeostasis models of insulin resistance (HOMA-IR), in their children [187]. However, there was no association established between folate status in the mothers and insulin resistance in their children [187]. In White Caucasian pregnant women without GDM, evidence of an inverse relationship existing between B12 and resistance to insulin has been reported [183]. Children, after six years of age, born to mothers with low circulatory B12 levels had higher adiposity as well as high insulin resistance, according to the Pune maternal nutrition study [179]. Similarly, within school children of Nepal,

high insulin resistance was tangible as a result of low maternal levels of B12 [188]. Though these clinical studies provide clear evidence of association between B12 and IR, a study from our group further demonstrated that altered, circulating, micro-RNAs derived from adipose tissues could possibly mediate adiponegic and insulin resistant phenotypes as a precursor to obesity in low B12 pregnant women [189].

1.5.5.2 **Vitamin B12 and dyslipidaemia**

i. Evidence from pre-clinical studies:

Mother Wistar rats, deficient in B12, produced offspring which had an increased level of adiposity, triglycerides and total cholesterol, as well as decreased leptin and adiponectin compared with control offspring, showing dysregulated metabolism of lipids [168]. Similarly, offspring of mice presenting with a severe deficiency in B12 during pregnancy, portrayed with altered lipid profiles as well as elevation in body fat percentage [190]. Additionally, rats of B12-deficient, folate-deficient and combined B12-folate-deficient groups had increased plasma TG, cholesterol and pro-inflammatory markers including interleukin-6, tumor necrosis factor-alpha (TNFα), IL-1b as well as lower adiponectin concentrations [170]. B12 deficiency inhibited hepatic beta oxidation as well as lipolysis in rat offspring born to B12 deficient mothers whereas supplementation during parturition resulted in restoration as in controls [191]. Similarly, 21-day old offspring of methyl donor deficient rats with NAFLD displayed dysregulation of genes involved in the beta-oxidation of fatty acids [192]. An adipocyte model study from our group targeting the elucidation of the cellular mechanism induced by low B12 in human adipocyte cell line (Chub-S7), exhibited elevated levels of cholesterol as well as hyper-homocysteinaemia in low B12 cells compared with controls. This was explained by a reduction in the methylation potential (MP), SAM: SAH ratio [193]. Validation of the above findings was further endorsed in primary human adipocyte models demonstrating that B12-deficient, primary human adipocytes had significantly elevated levels of total cholesterol, homocysteine and mRNA expression of genes involved in the biosynthesis and regulation of cholesterol compared with controls [193].

ii. Evidence from clinical studies:

A deficiency of B12 was observed to be significantly associated with both low HDL and hyperhomocysteinemia, whereas following control for co-founders homocysteine was found to be negatively correlated with all lipid indices with the exemption of HDL [194]. A negative correlation was established between serum B12 levels and the prevalence of metabolic

syndrome, marked by any three of the following biomarkers: high BMI, fasting blood glucose, high triglyceride levels, low HDL and high blood pressure in erythroid participants [195]. Our clinical studies involving three independent cohorts of women (i) in early pregnancy (ii) at child-bearing age (iii) at delivery, revealed that insufficient levels of vitamin B12 were associated with elevated levels of LDL-cholesterol, total-cholesterol and cholesterol-to-HDL ratio [196]. The study also showed that the women who were due childbirth had high prevalence of serum B12 deficiency (39.6%), increased BMI, triglycerides, homocysteine, total cholesterol and LDL cholesterol compared with mothers with normal serum B12 levels [196]. To further validate this evidence, maternal subcutaneous adipose tissues (ScAT) obtained from mothers with low B12, showed that the expression of genes involved in regulation and biosynthesis of cholesterol like HMGCR, SREBF2, LDLR and SREBF1 was significantly upregulated compared with controls [193]. Further evidence have shown that babies born to B12 deficient mothers are 'thin-fat', a term describing phenotypically lean individuals with increased fat accumulation in their bodies as well as decreased lean mass [179, 197]. This may consequently lead to elevated resistance to insulin as well as increased risk of developing CVD in adulthood [179, 197]. Thus, these studies implicate low B12 status at child-bearing age, in mothers at early pregnancy and pregnant mothers at delivery, with a link to increased LDL cholesterol, total cholesterol, cholesterol-to-HDL ratio and subsequent insulin resistance. Studies in patients with NAFLD and NASH showed that deficiency in B12 significantly increased the levels of triglycerides, cholesterol and blood glucose levels in these patients [198]. A similar study by Setola *et al* [199] demonstrated that folate and B12 treatment in patients with MetS improved IR and endothelial dysfunction as well as decreased homocysteine levels, suggesting the beneficial effects of these vitamins on CVD risk factors.

1.5.5.3 **Vitamin B12 and cardiovascular diseases**

i. Evidence from pre-clinical studies:

B12 deficiency resulting from prolonged stress, a condition associated with disruption in the intrinsic factor release from gastric parietal cells of the stomach, in adult male Sprague-Dawley rat models, was shown to be significantly interconnected with peripheral cortisol and androgen testosterone levels as well as demonstrated an association with vascular dysfunction [200]. A study delivered simultaneous therapy with 10mg/kg folate and 500 µg/kg B12 for exactly four weeks aiming to assess the effect of B12 and folate supplementation on isoprenaline (ISO)-

induced myocardial infarction (MI) in hyperhomocysteinemic (Hhcy) rats. It demonstrated significant reductions in the elevated heart rate and blood pressure as well as attenuation of severe cardiac histopathological alterations [201]. It is, therefore, recommended that aggravation of MI results from hyperhomocysteinemia (Hhcy). Therefore, B12 and/or folate administration reduces MI risk and Hcy levels accounting for reduced harmful consequences associated with Hhcy [201].

ii. Evidence from clinical studies:

B12 is involved in the remethylation of homocysteine leading to generation of methionine [202]. Therefore, deficiency of B12 is associated with an elevation of circulatory homocysteine which is known to be an independent risk factor for cardiovascular diseases (CVD) [203]. In Chinese subjects, a recent study showed a correlation between the highest circulating levels of B12 and the lowest Hcy levels in patients presenting with CVD only [204]. However, in Japanese subjects, evidence of an inverse association was observed between dietary intake of folate and B6 and the incidence of heart failure mortality in men as well as mortality resulting from stroke, CAD and total CVD in women, though no association was evident between B12 ingestion and risk of mortality [205]. This was similar to the evidence in an American population study in which a follow-up on subjects after 7.3 years therapy with B12, B6 and folate combination pill resulted in no significant reduction in a joint end stage of total CV events among women at high risk, although evidence of a significant decrease in homocysteine was observed [206]. Other studies suggested that supplementation with 250µg B12 and 5mg folate resulted in 32% reduction of fasting plasma total homocysteine (tHcy) level following 12 weeks of therapy in patients with coronary artery disease (CAD) [207]. Recent evidence has shown that elevated tHcy and decreased circulatory B12 levels in women, demonstrated a strong association with higher risk of all-cause and CVD deaths in the aged [208]. Meta-analysis of several prospective studies showed reliable evidence of a correlation existing between plasma total homocysteine (tHcy) and elevated CVD risk [209]. For instance, twelve (12) RCTs targeting an established renal disease or CVD with B12 supplementation (3 studies) using a dosage range 0.4 to 1.0 mg B12 per day, accounted for a significant reduction in the risk of developing stroke [209]. Hyperhomocystenemia and the incidence of cardiovascular risk and CV events are associated with chronic kidney disease (CKD), end-stage renal disease (ESRD) and dialysis [210-212]. Although CKD patients demonstrate an impairment in tissue uptake of B12 resulting in functional deficiency [213], the current evidence remains unclear to consider

altered B12, folate and elevated homocysteine levels as markers for CVD and CV mortality risk in ESRD and CKD individuals [214].

1.5.6 **Molecular mechanisms regulating B12 deficiency and lipogenesis**

Environmental factors such as nutrition may affect lipid metabolism by targeting at the transcriptional level and, thereby, regulating the expression of genes as well as the phenotype without altering the sequence of nucleotides by the process of epigenetics [215]. B12 plays a crucial role in humans by acting as a cofactor in the reaction pathways essential for maturation and preservation of cells [216, 217]. In the cytosol, B12 is a cofactor for methionine synthase (MS), which is actively involved in the reaction resulting in generation of methionine from homocysteine and further used for synthesis of s-adenosyl methionine (SAM) that acts as a methyl donor for methylation of several biochemical processes. Currently, the well-known markers of epigenetic mechanisms involved in the regulation of lipid metabolism and insulin resistance are DNA methylation, microRNAs, chromatin remodelling as well as modification of histones [218, 219]. B12, acting as a co-enzyme for a source of methyl groups (SAM), regulates these epigenetic mechanisms and several transcriptional as well as post-translational factors involved in the process of *de novo* lipogenesis. In the mitochondrial-based propionate metabolism pathway, B12 serves as a cofactor in the conversion of methyl malonyl-CoA to succinyl-CoA by methyl malonyl-CoA mutase (MCM) [220]. Succinyl-CoA is utilized as substrate in the Krebs cycle for ATP synthesis essential for the sustenance of cellular metabolisms (reviewed by [221]) and utilized for haemoglobin synthesis in red blood cell production. Deficiency of B12, therefore, results in a reversible increase in methyl malonyl-CoA leading to methyl malonic acidaemia (MMA). MMA, in turn, acts as a potent inhibitor of the rate limiting enzyme CPT1, critical for the breakdown of long chain fatty acids in the beta oxidation pathway. This results in dyslipidaemia due to the build-up of FAs and triglycerides (reviewed by [222]), accounting for higher lipogenesis and IR [223] (Fig 7).

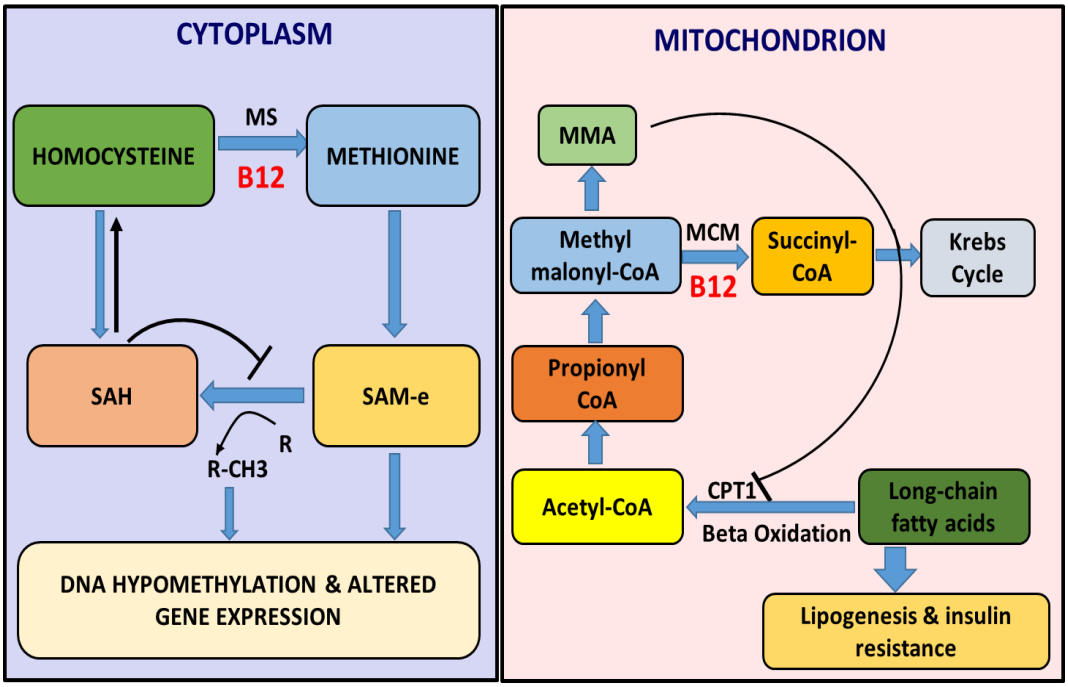

Figure 7: The Cellular role of B12 in lipogenesis. B12 deficiency in the cytosol results in the decline of methionine synthesis leading to reduced production of methyl donor SAM-e, accounting for hyperhomocysteinaemia with reciprocal elevation in s-adenosyl homocysteine (SAH) level, which is a potent inhibitor of DNA methyl transferases (DNMTs). The inhibition of DNMTs, together with low levels of SAMe, results in hypomethylation of DNA and altered gene expressions. Again, beta oxidation of fatty acids is grossly impaired resulting from the inhibition of the rate limiting enzyme CPT1 by build-up of MMA from MM-CoA inside the mitochondria due to insufficiency of the B12 cofactor required in the propionate metabolism pathway for conversion of methyl malonyl CoA to succinyl-CoA.

1.5.7 **Epigenetic mechanisms underlying B12 deficiency in lipid metabolism:**

There is, currently, emerging evidence suggesting that abnormalities, associated with one-carbon metabolites due to B12 deficiency, might possibly exert their modulations through epigenetic mechanisms. Some preliminary findings have indicated that induction of alterations in normal methylation of DNA is due to changes in levels of one-carbon metabolites. However, it was suggested that, in order to elucidate the molecular mechanism underlying these effects, there is a need for future studies to target combinations of gene expression assays with epigenetic studies [221]. Studies in animals might be initially informative since there is a likelihood of confrontations in the elucidation of molecular mechanisms in humans due to tissue-specificity orientation of epigenetic phenomena [221].

1.5.7.1 **Regulation of DNA methylation and lipid metabolism through B12 deficiency**

Methylation generally involves the process of transferring a methyl group to substrates such as enzymes, proteins, amino acids and DNA of various cells and tissues in the body [224]. In the process, guanine is normally bound with 5-methylcytosine following its development from cytosine [225]. Usually, hypermethylation of global DNA and CpG islets, located within the promoters of genes involved in lipid synthesis, accounts for the stability of the genome and silencing of the lipogenic genes, respectively [226]. This is greatly dependent on the availability and sufficiency of SAM synthesized with the aid of methyl donors such as B12 and folate [218]. B12 deficiency independently impairs the methionine synthase action [227] affecting SAM/SAH ratio which is a key determinant of the methylation potential of cellular DNA, thereby inducing dysregulation of gene expressions [228, 229]. Demethylation, associated with DNA of the genome, persists as one of the earliest evidence of epigenetic modifications induced by methyl donor-deficient diets contributing to hepatocarcinogenesis in animals [230].

Study of zebrafish showed that parental micronutrient deficiency affects liver DNA methylation and gene expression of lipogenesis leading to lipid accumulation in the F1 offspring [229]. High global methylation of DNA and promoter region methylation of lipid genes are critical to the maintenance of normal physiology of metabolism [192, 231] by ensuring stability of the genome and silencing of abnormal lipogenic genes [226]. The adipocyte model showed that hypomethylation in promoter regions led to higher expressions of LDLR and SREBF1 genes and cholesterol biosynthesis under low B12 conditions [193]. Increased genome incorporation with uracil subsequent to hypomethylation, due to low B12, was observed in rats [232], whereas fatty acid supplementation in low B12 conditions could not restore global methylation [233]. Evidence of hypomethylation, as observed in low B12, was observed in the CpG sites of some liver-derived genes involved in the pathogenesis of T2DM due to folate deficiency [234]. Methyl donor supplementation in rat obesogenic models ameliorated hypomethylation near the promoter of genes such as AGPAT3, SREBF2 and ESR1 (estrogen receptor-1) resulting in the reversion of higher fatty acid, triglyceride and cholesterol accumulation in the liver [235]. These studies, thus, implicate that methyl donor deficiency compromises methylation capacity expressing a direct consequence in the dysregulation of lipid metabolism [229, 236, 237] and this can be reversed by supplementation of methyl donors.

1.5.7.2 **Regulation of microRNAs (miRNAs) and lipid metabolism through B12 deficiency**

MicroRNAs (miRNAs), formerly termed as small temporal RNAs (stRNAs), are the class of small RNAs comprising 21-25 nucleotides of single-stranded RNA which are highly conserved and engaged in regulating the expression of genes at the transcriptional and post-translational levels [238]. MiRNAs physiologically express diversified patterns and are involved in the regulation of genes in lipid metabolism and inflammation [239, 240]. MiRNAs are proposed to be involved in modulation of adipocyte differentiation accounting for the development of T2DM, insulin resistance and dyslipidaemia [189, 241, 242]. Genes involved in the hepatic metabolism of fatty acids as well as insulin signalling may be repressed by some miRNAs regulating the levels of HDL, TG and insulin [243]. About 100 different miRNAs are expressed differentially in cases of NASH in humans [244] and alterations in miRNA-29c, miRNA-34a, miRNA-200b and miRNA-155 are due to methyl-donor deficiencies [245]. In humans, miRNA-122 is largely expressed in hepatocytes but under-expressed in NASH [245] and, therefore, it is proposed to be the potential target for treatment of dyslipidaemia [246] and high cholesterol [247]. Using adipocyte models, our group reported that twelve different adipocyte-derived miRNAs targeting PPARγ (miRNA-31, miRNA-130b and miRNA-23a), adipocyte differentiation (miRNA-143, miRNA-145, miRNA-146a, miRNA-125b, miRNA-222 and miRNA-221), CEBPα (miRNA-31) and pathways of insulin resistance (miRNA-107 and miRNA-103a) were identified to be significantly altered as a result of low B12, thereby modulating adipocyte differentiation and physiology [189]. Assessing the relationship between obesity, serum B12 and miRNAs, maternal BMI was inversely correlated with B12, with the latter further showing a significant positive correlation with seven different circulating miRNAs (miRNA-27b, miRNA-130, miRNA-103a, miRNA-107, miRNA-125b, miRNA-23a, miRNA-221 and miRNA-222) [189]. Finally, following the adjustment for confounding variables like age, insulin, glucose, smoking, parity and usage of supplement, four different miRNAs within the circulation (miRNA-107, miRNA-27b, miRNA-23a and miRNA103a) in addition to circulating B12 levels were found to be independently associated with BMI [189].

1.5.7.3 **Regulation of hepatic lipogenesis through modifications of histone by B12**

The mechanistic role of B12 in the regulation of hepatic lipid metabolism *via* modulation of histones is primitive. However, mechanisms including methylation, acetylation, phosphorylation as well as ubiquitylation are proposed to be involved in ubiquiltylation modification of histones that contribute to regulation of lipid metabolism in the liver [248]. The most intensively understood mechanism directly involved in dysregulation of hepatic lipid metabolism is the acetylation of histones which is normally catalysed by histone acetyltransferases (HATS) and histone deacetylases (HDACs). In female C57BL/6 mice, chronic vitamin B12 deficiency showed altered expression of histone-modifying enzymes in the brain and abnormal behavioural anomalies [249]. Recent evidence from pilot data indicated that folate deficiency in the liver had an association with modulations presenting with higher levels of methylation in H3K4 [250]. Reduced methylation of histone has also been shown in liver X receptor-alpha (LXRα) in rats, following consumption of a high-fat diet after the third generation in rats [251]. Histones such as H3 lysine 4 (H3K4) and H3 lysine 9 (H2K9) are known to be subjected to regulation by methylation of the histones in association with DNA methylation [252]. Evidence of deficiency in the methyl donor, choline, in C57BL/6 mice at the 12^{th} -17^{th} days of gestation resulted in alterations in the methylation of histone H3, whereas choline, after undergoing conversion to betaine, enhanced re-methylation to homocysteine [253]. Profound evidence showing the link between NAFLD [254], resistance to insulin and obesity, and modification of histone such as demethylation of H3 at lys9, has been shown [255]. Conditions of hyperlipidaemia and obesity were observed in mouse models following the functional loss of Jhdm2a, the H3K9-specific demethylase, which implies that the status of methylation of H3K9 is very essential and tends to regulate the expression of metabolic genes [256]. The need for further studies is, however, recommended for elucidating the role of B12 on the metabolism of lipids *via* modulation of histones.

1.5.8 **Impact of one carbon micronutrient supplementation on hepatic lipid metabolism**

In recent times, although agents that potentially resolve insulin resistance appear promising, the general therapy for correcting dysregulation of lipogenesis has not been established. However, current treatment may be reliant on alterations in lifestyle such as diet, weight reduction and/or exercise [257]. It is generally suggested that interventions involving fortification of foods, supplementation with B12 during preconception period and provision of adequate education

could, collectively, be the most effective ways to enhance improvement in the levels of B12 in infants as well as mothers [258]. Supplementing with diet rich in methyl donors such as vitamin B6, B9 and B12, is the principal way to significantly influence methylation of DNA because of their contribution to the synthesis of SAM [259]. A similar micronutrient, bataine, obtained from food, functions as a methyl donor and has been identified as a potent alleviator of fatty livers. Bataine was shown to facilitate the export of triglycerides from the liver as a way of attenuating steatosis of the liver, especially in cases of NAFLD [260]. Post-supplementation with only B12 was associated with significant alteration in the methylation of DNA in which 589 CpGs demonstrated differential methylation in addition to 2892 regions. Supplementation with both B12 and folate resulted in differential methylation of 169 CpGs and 3241 regions, thereby influencing the expression of genes associated with type 2 diabetes [261]. Evidence of higher percentage of transmethylation to methionine and reduced transsulfuration to cysteine were observed as a result of supplementation with both B12 and milk protein compared with only milk supplementation in women. Therefore, among Indian women presenting with low B12 in early pregnancy, supplementation with B12 and energy-protein balance plays a crucial role in enhancing the optimum function of the methionine cycle especially at the third trimester of pregnancy [262]. Similarly, B12, folate, choline and betaine-rich methyl donor supplements were used to regress the accumulation of fats in the liver induced by high-fat-sucrose (HFS) probably by changing the levels of methylation of CpG areas in the promotor regions of SREBF2, oestrogen receptor 1 (ESR1) and/or acylglycerol-3-Oacyltransferase-3 (AGPAT3) [237]. Hypermethylation of DNA in FAS gene expression was estimated after supplementation with methyl donors that apparently boosted the retrogression of NAFLD induced by high fat diet (HFD) [235]. Normalization of histochemical indices in hepatic lobules was achieved in an animal-model study where the accumulation of lipids towards the centre of hepatic lobules was brought under control subsequent to treatment with methionine in combination with vitamin B12 rather than B15 [263]. Depreciation of circulatory levels of homocysteine and methyl malonic acid was attained following the upregulation of plasma B12 levels in infants whose mothers were supplemented orally with B12 during pregnancy [264].

1.6 **Conclusion and prospects**

In summary, the global incidence of metabolic disorders is increasing with advancement in urbanization alongside several environmental and genetic factors. The pathogenesis of MetS, T2DM and CVD have been associated with deficiencies in micronutrients such as B12 and folate. The role of B12 deficiency in the pathogenesis of several metabolic disorders especially T2DM, CVD, IR as well as obesity, has been studied at the pre-clinical and clinical levels. Clinically, B12 deficient subjects such as children, adolescents and pregnant mothers presented with increased adiposity and higher lipids, as well as increased risk of T2DM, CVD and IR later in life. Babies born to B12 deficient mothers *via* maternal programming have a high accumulation of fats at birth with higher risk of developing CVDs later in life. However, there is no evidence, so far, whether dyslipidaemia and increased adiposity due to B12 deficiency are directly linked with regulation in the hepatic metabolism of lipids. Therefore, exploring the effect of B12 deficiency on the hepatic lipid metabolism in humans, as well as elucidating the mechanisms underlying the modulations, will help develop novel biomarkers for the diagnosis of MetS and will further enhance the development of possible therapeutic targets.

1.7 **Hypothesis and Aims**

The liver is foremost in the storage and metabolism of about 50% of the entire body's B12. Following uptake and processing into its co-enzyme forms, B12 plays a crucial role as a cofactor to rate limiting steps and/or enzymes in two key metabolic pathways such as the MS and the MCM pathways in the hepatocellular cytoplasm and mitochondria, respectively. Evidence has shown that B12 deficiency is associated with attenuation of these pathways leading to accumulation of metabolites such as homocysteine and MMA which are known to be associated with metabolic risk including CVD. Similarly, the liver is again paramount in the regulation of lipid metabolism including *de novo* fatty acids (FA), TG and cholesterol biosynthesis, as well as fatty acid oxidation (FAO). Dysregulation in the hepatic metabolism of lipids is normally associated with the accumulation of lipids in the liver in the form of simple steatosis (SS), steatohepatitis (SH), non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD). These disorders are also known to be associated with other metabolic disorders including dyslipidaemia, obesity, MetS, T2DM and CVD. There is evidence that low B12 accounts for dysregulation of normal adipocyte integrity and function in the storage of lipids, thereby leading to the accumulation of higher levels of TG and cholesterol. We, therefore, hypothesize that B12 deficiency may affect hepatic metabolism of lipids accounting for dysregulation in the lipogenesis (FA, TG and cholesterol biosynthesis) and FAO pathways.

In order to investigate this, we aimed:

1. To assess the liver regulation of B12 uptake and storage in various circulatory B12 concentrations ranging from deficient to high levels;
2. To assess whether B12 deficiency affects hepatic *de novo* lipogenesis;
3. To assess whether deficiency in B12 affects the hepatic FAO pathway;
4. To assess the effect of B12 deficiency on the lipid lowering influence of metformin in human hepatocyte cell line;
5. To elucidate the underlying mechanisms regulating the lipid metabolism in B12 deficient state in human hepatocyte cell line.

Chapter Two – Materials and Methods

2.1 List of Materials and Reagents

2.1.1 Reagents / materials for culture of Hep G2 cell line (ATCC, Manassas, Virginia, USA)

Eagle's minimal essential media (EMEM) (Custom-made, School of Life Sciences, University of Warwick, UK)

Eagle's minimal essential medium powder (Fisher Scientific, Loughborough, UK)

Vitamin B12 (5g- powder) (Sigma Aldrich, UK)

Penicillin / Streptomycin (100X) (Fisher Scientific, Loughborough, UK)

Foetal Bovine Serum, heat inactivated (South American origin) (Fisher Scientific, Loughborough, UK)

L-Glutamine (100X) (Invitrogen, Fisher Scientific, Loughborough, UK)

Seahorse XF Base Media (Agilent Technologies, Stockport, UK)

Bovine Serum Albumin (Sigma-Aldrich, Gillingham, Dorset, UK)

0.05% Trypsin-EDTA (Invitrogen, Fisher Scientific, Loughborough, UK)

1, 1-Dimethylbiguanide hydrochloride (Metformin) (Sigma-Aldrich, Gillingham, Dorset, UK)

Krebs-Henseleit Buffer (Sigma-Aldrich, Gillingham, Dorset, UK)

Adiponectin human, recombinant (Sigma-Aldrich, Gillingham, Dorset, UK)

AICAR (Sigma-Aldrich, Haverhill, UK)

2.1.2 Reagents for protein characterization studies

Primary antibodies

Anti-CD320 Antibody-C-Terminal (ab171634) (ABCAM PLC, Cambridge, UK)

Anti-TCN2 antibody (ab189871) (ABCAM PLC, Cambridge, UK)

AMPK Alpha (D63G4) Rabbit mAb (Cell Signalling Technology, London, UK)

Acetyl-CoA Carboxylase (C83B10) Rabbit mAb (Cell Signalling Technology, London, UK)

Phospho-AMPK Alpha (Thr172) (D795E) Rabbit mAb (Cell Signalling Technology, London UK)

Phospho-Acetyl-CoA Carboxylase (Ser79) (D7D11) Rabbit mAb (Cell Signalling Technology, London, UK)

Beta-actin antibody (49675) (Cell Signalling Technology, London, UK)

Secondary antibodies

Anti-rabbit IgG HRP-linked Antibody (Cell Signalling Technology, London, UK)

Reagents for gel preparation

10% Resolving Gel (two plates): Resolving buffer (5.2ml), Acrylamide (6.6ml), distilled water (7.8ml), 10% APS (150µl) and Temed (15µl)

10% Stacking Gel (two plates): Stacking buffer (2.5ml), Acrylamide (1.3ml), distilled water (6.1ml), 10% APS (50µl) and Temed (10 µl)

General western blot reagents

Protein Assay Dye Reagent Concentrate; Bradford reagent (Bio-Rad Laboratories LTD, Hemel Hempstead, UK)

Protein Ladder; Page ruler plus prestained; Nine proteins spanning 10 to 250kDa (Thermo Scientific Pierce, Fisher Scientific, Loughborough, UK)

Ponceau S stain (Sigma-Aldrich, Gillingham, Dorset, UK)

Whatman filter paper (Sigma-Aldrich, Gillingham, Dorset, UK)

PVDF membrane (Sigma-Aldrich, Gillingham, Dorset, UK)

Enhanced chemiluminescence (ECL) (BIO-RAD Laboratories LTD, Hemel Hempstead, UK)

I-Block(tm) Protein-Based Blocking Reagent (Invitrogen, Fisher Scientific, Loughborough, UK)

Temed (Sigma-Aldrich, Gillingham, Dorset, UK)

10% Ammonium per sulphate (APS) (Sigma-Aldrich, Gillingham, Dorset, UK)

Acrylamide (Sigma-Aldrich, Gillingham, Dorset, UK)

Running Buffer (Sigma-Aldrich, Gillingham, Dorset, UK)

Stacking Buffer (Sigma-Aldrich, Gillingham, Dorset, UK)

Methanol (Sigma-Aldrich, Gillingham, Dorset, UK)

2.1.3 **Reagents and materials for RNA, cDNA and gene expression studies**

Qiazol (Thermo scientific, UK)

Chloroform (Sigma-Aldrich, Gillingham, Dorset, UK)

Isopropanol (Fisher Scientific, Loughborough, UK)

RNASe-free water (Invitrogen, Fisher Scientific, Loughborough, UK)

Enzyme mix (Thermo scientific, Loughborough, UK)

5X Reaction buffer (Thermo scientific, Loughborough, UK)

10mM nucleotides dNTP Mix (Applied Biosystems, Warrington, UK)

Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems, Warrington, UK)

TaqMan Universal PCR Master Mix (Applied Biosystems, Warrington, UK)

Reverse transcriptase RevertAid H Minus MMLV (Applied Biosystems, Warrington, UK)

Random hexamer primer (Applied Biosystems, Warrington, UK)

RiboLock RNase Inhibitor; Recombinant (Applied Biosystems, Warrington, UK)

Frosted and semi-skirted, thin wall 96 x 0.2ml high profile plate (Geneflow Ltd, Elmhurst, UK)

Amplification grade DNase 1 Kit (Sigma-Aldrich, Gillingham, Dorset, UK)

100x ROX reference dye (Sigma-Aldrich, Gillingham, Dorset, UK)

2.1.4 **Genes for RT- qPCR**

2.1.4.1 **Genes for Human**

SREBF1 - Hs01088691_m1 (Applied Biosystems, Warrington, UK)

ACACA - Hs01046047_m1 (Applied Biosystems, Warrington, UK)

ATP citrate lyase (ACLY) - Hs00982738_m1 (Applied Biosystems, Warrington, UK)

FASN - Hs01005622_m1 (Applied Biosystems, Warrington, UK)

SCD – Hs01682761_m1 (Applied Biosystems, Warrington, UK)

DGAT2 - Hs01045913_m1 (Applied Biosystems, Warrington, UK)

Adipo R2 - Hs00226105_m1 (Applied Biosystems, Warrington, UK)

Adipo R1 - Hs00360422_m1 (Applied Biosystems, Warrington, UK)

CPT-1α Hs00912671_m1 (Applied Biosystems, Warrington, UK)

ACADM - Hs00936575_m1 (Applied Biosystems, Warrington, UK)

LDLR - Hs01092524_m1 (Applied Biosystems, Warrington, UK)

IDI1 - Hs00743568_s1 (Applied Biosystems, Warrington, UK)

CaMMKK2 - Hs00198032_m1 (Applied Biosystems, Warrington, UK)

DGAT1 - Hs01020362_g1 (Applied Biosystems, Warrington, UK)

LKB1/ STK11 - Hs00975988_m1 (Applied Biosystems, Warrington, UK)

HMGCR - Hs00168352_m1 (Applied Biosystems, Warrington, UK)

ChREBP or MLXIPL - Hs00975714_m1 (Applied Biosystems, Warrington, UK)

ATP citrate lyase (ACLY) - Hs00982738_m1 (Applied Biosystems, Warrington, UK)

ELOVL6 - Hs00907564_m1 (Applied Biosystems, Warrington, UK)

GPAT1 (GPAM) - Hs00326039_m1 (Applied Biosystems, Warrington, UK)

AGPAT2 - Hs00944961_m1 (Applied Biosystems, Warrington, UK)

Lipin1 - Hs00299515_m1 (Applied Biosystems, Warrington, UK)

ACSL1 Hs00960561_m1 (Applied Biosystems, Warrington, UK)

CACT or SLC25A20 Hs00386383_m1 (Applied Biosystems, Warrington, UK)

ACADL Hs00155630_m1 (Applied Biosystems, Warrington, UK)

ACADS Hs00163506_m1 (Applied Biosystems, Warrington, UK)

HADHA Hs00426191_m1 (Applied Biosystems, Warrington, UK)

HADHB Hs01027271_m1 (Applied Biosystems, Warrington, UK)

2.1.4.2 **Primers for mouse genes**

L19: GGAAAAAGAAGGTCTGGTTGGA–Forward, TGATCTGCTGACGGGAGTTG-Reverse (Sigma-Aldrich, UK)

CD320: GGTCCAAGTCTCCGGCTCTA-Forward, AGCACATGACTCAATCCTACAGT-Reverse (Sigma-Aldrich, UK)

TCN2: CTTTGCTGGATCTTCCTTGG-Forward, TCCTGGGGTTTGTAGTCAGC-Reverse (Sigma-Aldrich, UK)

2.1.5 **Reagents and materials for Radioactive flux assay**

Glass TLC Plate (Thermo scientific, Loughborough, UK)

^{14}C 50µCi (1.85MBq) Oleic Acid (Perkin Elmer Las LTD, Seer Green, UK)

Oxygen-free nitrogen gas (BOC LTD, Middlesbrough, UK)

100mM L-Carnitine inner salt (Sigma-Aldrich, Gillingham, Dorset, UK)

43mg Glyceryl tri-palmitate (Sigma-Aldrich, Gillingham, Dorset, UK) in chloroform (500µl)

70mM Sodium oleate (Sigma-Aldrich, Gillingham, Dorset, UK)

Iodine, ACS reagent (Sigma-Aldrich, Gillingham, Dorset, UK)

Rectangular TLC developing tanks (Sigma-Aldrich, Gillingham, Dorset, UK)

Flat Head Borosilicate Glass (Sigma-Aldrich, Gillingham, Dorset, UK)

0.88 % (w/v) Potassium Chloride (Sigma-Aldrich, Gillingham, Dorset, UK)

Hexane (Sigma-Aldrich, Gillingham, Dorset, UK)

Glyecerol (Sigma-Aldrich, Gillingham, Dorset, UK)

Diethyl ether (Sigma-Aldrich, Gillingham, Dorset, UK)

Formic acid (Sigma-Aldrich, Gillingham, Dorset, UK)

Chloroform (Sigma-Aldrich, Gillingham, Dorset, UK)

Methanol (Sigma-Aldrich, Gillingham, Dorset, UK)

2.1.6 **Reagents and materials for seahorse flux assay**

Seahorse XF24 FluxPak (Agilent Technologies, Stockport, UK)

Oligomycin D (Sigma-Aldrich, Gillingham, Dorset, UK)

Rotenone (Sigma-Aldrich, Gillingham, Dorset, UK)

Antimycin A (Sigma-Aldrich, Gillingham, Dorset, UK)

Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma-Aldrich, Gillingham, Dorset, UK)

Palmitate (Sigma-Aldrich, Gillingham, Dorset, UK)

Bovine Serum Albumin (Sigma-Aldrich, Gillingham, Dorset, UK)

Sodium Pyruvate (Sigma-Aldrich, Gillingham, Dorset, UK)

L-Glutamine (Sigma-Aldrich, Gillingham, Dorset, UK)

Sodium Chloride (Sigma-Aldrich, Gillingham, Dorset, UK)

Glucose (Sigma-Aldrich, Gillingham, Dorset, UK)

2.1.7a **Media formulations**

Media for seahorse assay (oxygen consumption rate - OCR)

- 1x Krebs-Henseleit Buffer (KHB) (Sigma-Aldrich, UK), 10mM Hepes, 0.1% BSA, 2mM Glutamine, Sodium Pyruvate, 2.5mM Glucose, pH 7.4, Filter sterilize

Eagle's minimal essential media (EMEM) for cell culture

- Custom-made B12-deprived-EMEM media supplemented with compounds such as 1% v/v Penicillin/ Streptomycin, 1% v/v L-Glutamine, 10% v/v FBS and various concentrations of B12.

2.1.7b **Buffers**

5X Krebs-Henseleit Buffer (KHB) (Sigma-Aldrich, UK) (1 Litre)

- 32.53g Sodium Chloride (NaCl), 1.752g Potassium Chloride (KCl), 1.204g Magnesium Sulphate (MgSO4) and 0.852g Disodium Hydrogen Phosphate (Na2HPO4) dissolved in 1 litre of distilled water, filter sterilized and stored at 4^{o}C.

Stop solution for DNase step (Sigma-Aldrich, Gillingham, Dorset, UK)

- Constitutes 50mM Ethylenediamine tetraacetic acid (EDTA)

Reaction buffer of 10x DNase I (Sigma-Aldrich, Gillingham, Dorset, UK)

- Made of 200mM Tris-HCl and 20mM Magnesium Chloride ($MgCl_2$) at pH 8.3

Phosphate Buffered Saline (PBS)

- 2.5mM Potassium Chloride (KCl), 10mM Disodium hydrogen Phosphate (Na_2HPO_4), 140mM Sodium Chloride (NaCl) and 1.5mM Potassium Dihydrogen Phosphate (KH_2PO_4) at pH 7.2

RIPA lysis buffer (Merck Millipore, UK) (with cocktail of protease and phosphatase inhibitors)

- 1x RIPA lysis buffer, 2 tablets of Roche Complete-Mini protease inhibitor cocktail (Sigma-Aldrich, Gillingham, Dorset, UK), 20mg sodium vanadate (Sigma-Aldrich, Gillingham, Dorset, UK) and 8mg Sodium Fluoride (Sigma, UK)

Buffers for western blot

Running Buffer: 100ml 10x Tris-Glycine (SDS) (Bio-Rad Laboratories Ltd, Hemel, Hempstead, UK) in 900ml distilled water

Transfer Buffer: 100ml 10x Tris-Glycine (Bio-Rad Laboratories Ltd, Hemel, Hempstead, UK), 200ml Methanol (Sigma-Aldrich, Gillingham, Dorset, UK) and 700ml distilled water

PBS-Tween: 1ml Tween (Sigma-Aldrich, Gillingham, Dorset, UK), 100ml 10x PBS and 900ml distilled water

I-Block buffer: 0.2% (w/v) I-Block (Invitrogen, Fisher Scientific, Loughborough, UK) in PBS-Tween, slightly warmed and stirred overnight in a cold room.

2.1.8 **Solutions**

Stock solution of Oil Red O (Sigma-Aldrich, Gillingham, Dorset, UK): 0.5g of ORO powder dissolved in 100ml of isopropanol (absolute, >99.7%), stir overnight for complete dissolution at room temperature

Oil Red O working solution: Prepared by dissolving ORO stock with distilled water at 3:2 ratio (Stock: water), Leave at room temperature for 10 minutes followed by filtering through a 0.45µm filter unit (or Whatman No 1 filter paper)

4% Paraformaldehyde in 1x Phosphate buffered saline (PBS) (Affymetrix, Buckinghamshire, UK)

Calibrant solution for seahorse (Agilent Technologies, Stockport, UK)

75% Ethanol (Sigma-Aldrich, Gillingham, Dorset, UK)

2.1.9 **Miscellaneous materials and reagents**

Triglyceride Quantification Kit (ab65336) (ABCAM PLC, Cambridge, UK)

Sterile six-well cell culture plates (Thermo scientific, Loughborough, UK)

Seahorse XF24 microplates for cell culture, with cartridges (Agilent Technologies, Stockport, UK)

0.22µm filters for Syringes

Sarstedt Scintillation Vial 58mm x 27mm Screw Cap (Sarstedt Ltd, Leicester, UK)

Sterile filter unit, Vacuum Stericup; Filter diameter of 73mm with 500mL funnel and 500mL receiver; Membrane: PVDF; Pore Size: 0.22µm (Thermo Scientific, Loughborough, UK)

RNase-free Eppendorf tubes (Sigma-Aldrich, Gillingham, Dorset, UK)

Plain adhesive seals (Appleton Woods)

Pipette tips (Sigma-Aldrich, Gillingham, Dorset, UK)

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Gillingham, Dorset, UK)

Disposable Plastic BioSafe Pipette – 10ml, 25ml (Sigma-Aldrich, Gillingham, Dorset, UK)

50ml Falcon tubes (Sigma-Aldrich, Gillingham, Dorset, UK)

10ml Falcon tubes (Sigma-Aldrich, Gillingham, Dorset, UK)

Disposable latex gloves (Sigma-Aldrich, Gillingham, Dorset, UK)

2.2 **Methods**

In this section, the general descriptions of principal methods performed in the study are outlined, however, brief descriptions with specific modifications in each method are stated accordingly in the respective chapters.

2.2.1 **Cell culture**

2.2.1.1 **Hep G2 cell line:**

Hep G2 cell line (ATCC, Manassas, Virginia, USA) is mainly an epithelial, continuous cell line which is characteristically adherent to cell culture plates. The cells were originally developed subsequent to isolation from liver biopsy of a 15-year-old male Caucasian American who was diagnosed with a well-differentiated hepatocellular carcinoma [265, 266]. Under suitable conditions of culture environment, the Hep G2 model is highly suitable for *in vitro* experiments targeting the examination of metabolism of lipids in the liver of humans as a result of its capacity to grow on a large scale and furthermore demonstrating a high level of differentiation both physiologically and morphologically *in vitro*. All cell cultures in this study were therefore done using the Hep G2 cell line.

2.2.1.2 **Thawing of Hep G2 cells**:

Hep G2 cells, formerly cryopreserved within cryovials and stored in the liquid nitrogen, were thawed at 37^{o}C using the water bath. The resultant suspension of cells was steadily added to an initially warmed (37^{o}C) 10ml EMEM media in a clean and sterile 50ml falcon tube, followed by centrifugation at 250g for 5 minutes. The supernatant was then discarded whereas the pellet of cells was carefully suspended in 20ml of fresh pre-warmed (37^{o}C) custom-made B12-deficient-EMEM medium supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/

streptomycin in a 50ml falcon tube. The entire suspension of cells was then transferred into a sterile T75 (75cm^2) cell/tissue culture flask (Sigma, UK) and incubated at 37^{o}C at 5% CO_2 saturation, with routine changing of media every 48-hours until reaching confluence on the10th day.

2.2.1.3 **Passaging of Hep G2 cells**:

After reaching confluence in the 75cm^2 culture flask on the 10th day, the Hep G2 cells were washed two-fold to ensure removal of all traces of medium, following initial aspiration of the last EMEM medium in the flask, using pre-warmed (37^{o}C) sterile 1x phosphate buffered saline (PBS). The cells were subsequently tripsinized, to ensure detachment from the T-75 flask, by carefully adding 2ml of trypsin-EDTA solution (0.02% EDTA and 0.25% porcine trypsin) to the cells, followed by incubation in an incubator at 37^{o}C and 5% CO_2 saturation for 5 minutes. Then, 20ml of fresh EMEM medium was then pipetted into the flask to re-suspend cells. The cell suspension was transferred into 50ml-falcon tubes followed by centrifugation at 250g for 5 minutes. The supernatant was subsequently aspirated, followed by resuspension of the pellet of cells with 5ml EMEM medium in the falcon tubes. 1ml of the cell suspension was then transferred into 20ml pre-warmed sterile EMEM medium supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/ streptomycin in T-75 flasks and kept in an incubator at 37^{o}C and 5% CO_2 saturation. The maximum number of passages of Hep G2 cells maintained in cell bank and used for the experiment was passage 25 to 30, which were securely kept in cryovials and stored in liquid nitrogen until required.

2.2.1.4 **Freezing of Hep G2 cells**

Following the trypsinization and harvest of confluent Hep G2 cells from T-75 flasks as previously described, the resultant suspension of cells (in 5ml EMEM media) was transferred into freezing media [EMEM (70%), DMSO (10%) and FBS (20%)] with 1ml of the subsequent suspension added to cryogenic vials (Sigma, UK). The cryovials were first kept frozen at -20^{o}C and subsequently transferred to -80^{o}C for 24 hours, followed by permanent storage in the liquid nitrogen.

2.2.1.5 **Seeding of Hep G2 cells for B12-lipid metabolism experiments**

Following the initial thawing of cells from the liquid nitrogen and subsequent culturing in T-75 flask until 90-100% confluence on the 10th day, Hep G2 cells were trypsinized and re-suspended in 20ml EMEM medium in 50ml falcon tubes. This was followed by centrifuging the falcon

tubes at 250xg for 5min, decanting the supernatant and re-suspending cell pellets in 5ml fresh sterile EMEM medium, to obtain seeding cells for the experiment. 10µl of 1 in 5 dilution of the cell suspension was loaded in the haemocytometer and counted under x40 objective of the light microscope. To determine the total number of cells in the cell suspension stock (5ml), the number of cells in each quadrant was counted. The average number of cells in the four quadrants was multiplied by the dilution factor (x5) and the volume of suspension medium (5ml) to determine the total number of cells in the suspension. The equivalent volume of suspension medium containing 75 000 (in six-well plates) or 50 000 cells (within 24-well seahorse plates) was diluted in fresh EMEM medium of different concentrations of B12 such as (500nM, 1000pM, 100pM and 25pM). This was further supplemented with 10% FBS, 1% L-glutamine and 1% penicillin / streptomycin and seeded either at a volume of 2ml into wells of sterile six-well plates (Thermo scientific, Loughborough, UK) or 100µl into XF24-seahorse microplates (Agilent Technologies, Stockport, UK) respectively for the assigned assays.

2.2.1.6 **Harvest of Hep G2 cells for B12-lipid metabolism experiments**:

Adherent Hep G2 cells in 2ml EMEM-B12 medium suspension within six-well plates, incubated at 37^{o}C and 5% CO_2 saturation, were routinely replenished with fresh EMEM-B12 medium at 48-hour intervals until 100% confluence was reached on the 10th day. After the last medium change, 1ml of the conditioned medium was transferred into 1.5 ml Eppendorf tubes and stored at -80^{o}C. The Hep G2 cells were washed twice with 1x PBS to remove traces of the medium from the cells. The hepatocyte cell line was then harvested using the following; Qiazol (Trizol) (Thermo Scientific, UK), 1x Phosphate Buffered Saline (PBS) (Sigma, UK) and RIPA lysis buffer (Applied Biosystems, UK) depending on the specific assay to be performed with the cells. In the process, 500µl of lysis solution was carefully added to the cells and placed on shaker for five minutes, followed by scraping of detached cells into 1.5ml Eppendorf tubes. The lysed cell suspensions were stored at -80^{o}C until utilized.

2.2.2 **Gene expression assay**

2.2.2.1 **Total RNA isolation**

Total RNA was successfully isolated from Hep G2 cells in six-well plates by washing the cells twice with ice-cold 1x PBS, followed by harvesting using 500µl Qiazol (Thermoscientific, UK). The lysates in the plates, following initial shaking on a shaker for five minutes, were scraped and transferred into 1.5 ml Eppendorf tubes and stored at -80^{o}C until required. Using the Trizol method of extraction [267], total RNA was obtained by adding 200 µl chloroform to Qiazol

suspension of cells in 1.5 ml Eppendorf tubes. The tubes were subsequently incubated for 2-3 minutes at room temperature, following initial shaking for about 15 seconds. The Eppendorf tubes were centrifuged at 12,000xg speed at 4 ^{o}C temperature for 10 minutes, resulting in phase separation of the suspension into a lower red organic phase, an interphase and a colourless upper aqueous phase (Fig 8). The upper aqueous phase was cautiously transferred into another RNAse-free tube. The RNA present in the aqueous phase was then precipitated by adding 300 µl of 99% isopropyl alcohol (Sigma, UK) and centrifuging at 12,000xg speed, at a temperature of 4^{o}C for 20 minutes. The precipitate (RNA) was subsequently washed by adding 800 µl of 75% ethanol and centrifuging at 12,000xg speed, at 4^{o}C temperature for 20 minutes. The ethanol was carefully aspirated off the pellets and allowed to completely dry. The RNA was re-dissolved in 25 µl nuclease-free water and later utilized for the DNASe step using the DNASe I amplification grade (Sigma, UK).

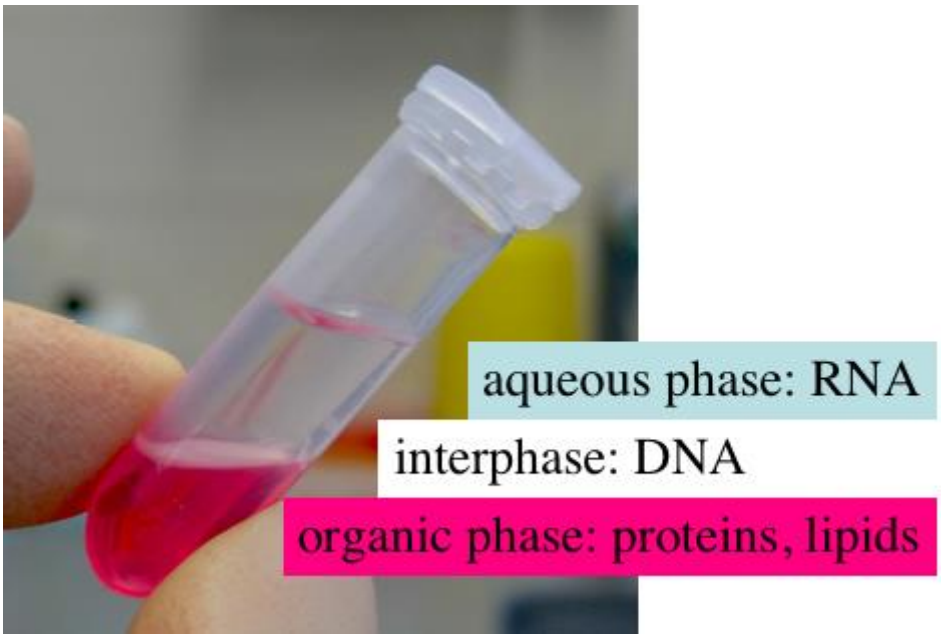

Figure 8: Isolation of RNA using the Trizol method: Separation of sample into phases following the lysis and harvest of cells in 500 µl Qiazol (Thermo scientific, UK), was achieved by adding 200 µl chloroform (Sigma, UK) and centrifuging at 12000xg speed for 10 minutes at 4^{o}C. This was done after the initial incubation of samples for 2-3 minutes at room temperature. The lower red organic phase contained proteins and lipids whereas the white interphase contained DNA, however, the upper clear aqueous phase contained the RNA.

2.2.2.2 **DNASe treatment of RNA**

The aim of the DNASe treatment step was to ensure removal of residual contamination of DNA from RNA samples. In the process, 3 µl of 10x reaction buffer (Sigma, UK) and 3 µl of DNASe I amplification grade (Sigma, UK) were added to 25 µl of RNA suspension in nuclease-free water (Sigma, UK) and incubated at room temperature for 15 minutes, preceded by an initial gentle mixing for 5 seconds. After incubation, 3 µl of the STOP solution (containing 50mM EDTA) (Sigma, UK) was added to ensure inactivation of the DNASe 1 enzyme, calcium (Ca^{2+}) and magnesium (Mg^{2+}). The tubes were incubated at 70^{o}C for 10 minutes, followed by chilling of samples on ice. The DNASe-free RNA was then quantified spectrophometrically (NANOdrop, Labtech, UK) and 300ng was used for cDNA synthesis.

2.2.2.3 **Reverse transcription (cDNA synthesis)**

Principle: Reverse transcribing a single stranded RNA by using the enzyme RNA-dependent DNA polymerase (reverse transcriptase) in the presence of a primer and nucleotides (dNTPs), describes the process involved in the synthesis of cDNA.

In the current study, cDNA was synthesized by using 300ng of DNASe-free RNA in RNASe-free PCR tubes to which 1µl random hexamer primer (Thermo scientific, UK) and DNASe-free water (Sigma, UK) were added and subsequently incubated at 65 ^{o}C for 5 minutes. This was followed by preparing a cocktail consisting of 5x Buffer (Thermo scientific, UK), nucleotides (dNTPs) (Thermo scientific, UK), Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Thermo scientific, UK) and Ribolock RNAse Inhibitor (Thermo scientific, UK). 7.5 µl of the cocktail was then added to the DNASe-free RNA in the PCR tubes, followed by mixing and subsequently placing the tubes in the iCycler (Bio-Rad, UK). Following the manufacturer's protocol for programming the equipment, the assay was conducted by incubating samples at successive temperatures of 25 ^{o}C for about 10 minutes, 42 ^{o}C for 60 minutes and 70 ^{o}C for 10 minutes in the iCycler. Cooling of the synthesized cDNA samples was carried out on ice and further stored at -20 ^{o}C.

2.2.2.4 **Real-time quantitative polymerase chain reaction (RT-qPCR)**

Principle: The principle involves amplification and subsequent quantification of DNA principally at the exponential phase of the reaction, leading to precise estimation of amplicons

which is proportionally equivalent to the original amount of cDNA that is shown by the quantity of fluorescent emission generated.

a. *Taqman qPCR*: In this study, the mRNA expressions in Hep G2 cell line were all analysed using Taqman qPCR. The probe obtained from Taqman operates on the principle of 5'-3' exonuclease activity of the enzyme Taq polymerase, which is involved in slashing of the reporter-quencher-labelled probe, and subsequently hybridized complementarily to its target leading to detection resulting from generation of fluorescence. To run the assay, 24 µl cocktail comprising eukaryotic 18S rRNA endogenous control (Applied Biosystems, UK), universal PCR master mix (Applied Biosystems, UK), and RNASe-free water, was transferred unto 1 µl cDNA in a 96-well PCR plate (Gene Flow, UK).

b. *SYBR Green I qPCR*: mRNA analysis involving mice liver tissues has been well optimized in our laboratory using SYBR Green I PCR and therefore utilized in the current study. Primers for gene expressions were manufactured by Sigma Aldrich, UK. Stock concentration (100µM) of primers were prepared following dissolution of the nucleic acids (dry) in nuclease-free water and kept at -20^{o}C storage. The primers were designed using Ensemble Genome Browser Primers tool (*http://genome.uscs.edu/cgi-bin/hgPcr*). To run the assay, 2µl of cDNA was carefully mixed with 18µl master mix solution in a 96-well plate. Preparation of the master mix was done by cautiously adding 0.1x ROX reference dye, 300nM reverse and forward primers, 1x Jumpstart SYBR Green I mix and nuclease free water to obtain final volume of 20µl.

After carefully mixing the content in 96-well plates by mechanical vortexing, followed by centrifuging at 10,000 rpm for 15 seconds, the PCR assay was performed in about 90 minutes according to the manufacturer's protocol, using a standard RT qPCR analyser (Applied Biosystems, UK). Specificity of the amplification was confirmed with the melting curve analysis. The data was subsequently analysed by using the method of quantitative comparative threshold cycle (qCCT). Also, the expressions of the target mRNA were normalized to the housekeeping gene 18s rRNA / L19. The results were achieved in the form of cycle threshold (C_T) (Fig 9) estimates and utilized for determining the Δ C_T values (Δ C_T = C_T of gene of interest-C_T of r18s/L19). For each sample, measurements were done on a minimum of three occasions. Exclusion of probable bias resulting from averaging was done by transforming the data *via* the power equation $2^{-\Delta\Delta CT}$.

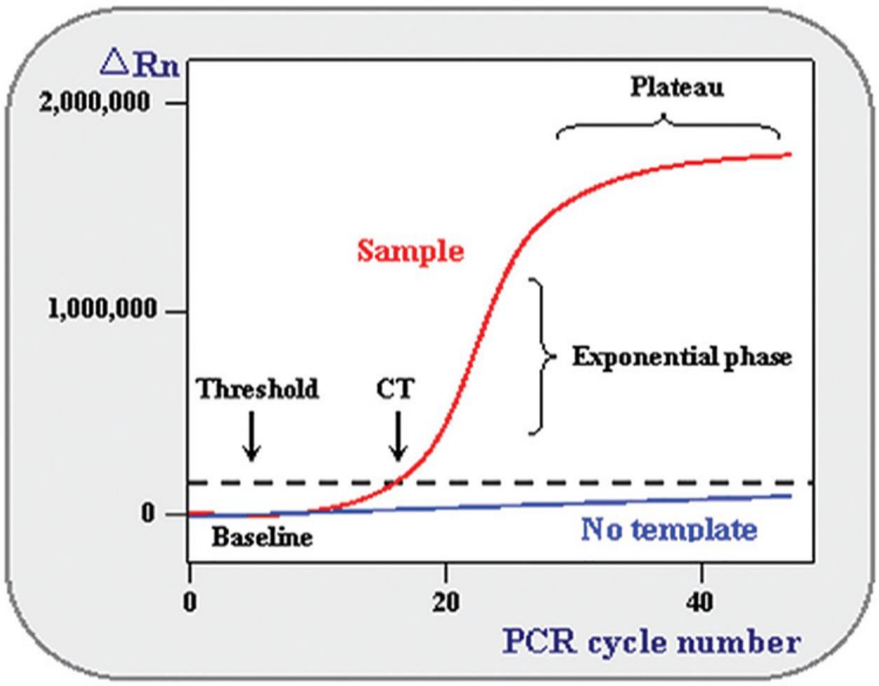

Figure 9: Real time quantitative PCR [268]. ΔRn describes the rise in fluorescent signal detected at individual time point and the values are normally plotted against the number of cycles. The baseline describes cycles of PCR by which accumulation of signals from a reporter fluorescence remains under the detection limits of the equipment. The threshold represents a random fluorescence level usually chosen based on the variability of the baseline. For any sample, detection of a real signal usually higher than the threshold is useful for the definition of the threshold cycle (Ct). Adjustments in the threshold for individual assays could be done to ensure that across all plots, it appears in the exponential amplification region. The threshold cycle (Ct) describes the fractional PCR cycle number whereby the threshold is lower than the reporter fluorescence, and it essentially facilitates the generation of precise and accurate data.

2.2.3 **Estimation of total intracellular TG levels in Hep G2 cells**

Principle: The principle governing the assay includes generation of glycerol and free fatty acids *via* catabolism of TGs by the enzyme lipase. Additional oxidation of the resulting glycerol leads to the development of a product that is made to react with a probe to form a colour and its absorbance measured at 570nm wavelength.

TG was estimated by using a kit from Abcam plc, UK. The frozen stock of 1mM TG standard was thawed in water bath at 80°C – 100°C for 1 minute, gently vortexed for 30 seconds and then diluted to 0.2 mM. The probe was also warmed in water bath at 37°C for 5 minutes to enable thawing of the DMSO solution. TG enzyme mix and lipase were prepared by reconstituting each in 220µl of assay buffer and kept on ice throughout the test. Then the TG standard was diluted with the assay buffer to a total volume of 50µl per well to generate standard concentrations of 0, 2, 4, 6, 8 and 10 nmol/well. 2 µl of lipase was added to each well and

incubated for 20 minutes. A cocktail of reaction mix (50 µl) comprising TG assay buffer, probe and enzyme mix (46:2:2) was added to each well, gently mixed and the absorbance was measured at 570nm wavelength. The data was used to derive a standard curve. Then, the samples were prepared by homogenising cells in PBS and subsequently making up cell suspensions to 50 µl of TG assay buffer per well. Then, 2 µl of lipase was added and incubated for 20 minutes at room temperature. Afterwards, 50 µl of the reaction mix was also added and the final content was gently mixed. The absorbance of the mixture was then measured at 570nm and the concentration of TG was calculated from the standard curve.

2.2.4 **Radioactive flux assessment of TG synthesized *via* utilization of radiolabelled-fatty acid (^{14}C-oleate) by Hep G2 cells**

Principle: The amount of TG synthesized by cells in an interval of time can be quantified by initially exposing cells to radiolabelled fatty acid (14C-oleate) in the presence of L-carnitine.

Preparation of cocktail of BSA and oleate: A cocktail consisting of 497 µl each of 70 mM oleate and 0.25% BSA was prepared in a glass tube. 32µl of the radioactive compound 14C-labelled-0.75mM oleate at 2000 dpm/nmol stored at -20 ^{o}C was transferred in a glass tube and evaporated under nitrogen gas to obtain a dry residue. The radioactive residue in the glass tube was then placed on a shaker at 37^{o}C and the cocktail was gently transferred by drops into the glass tube and left on the shaker for 1hour. Finally, the radiolabelled media was prepared by adding 1mM L-carnitine, 0.75 mM cold glycerol and 0.75 mM ^{14}C-Oleate to the fresh EMEM medium.

Experimental procedure: At 100% confluence, the condition medium in each well of Hep G2 cells was removed and replaced with 1ml of the radiolabelled medium and kept at 37^{o}C incubation for 2 hours (or 5 minutes for background correction control cells). After the incubation, the radiolabelled medium was removed, and the cells were then harvested in 2ml of methanol. The extraction of total lipids from the Hep G2 cells was done using a mixture of chloroform and methanol (2:1 v/v) [269]. The resulting layer of chloroform containing the TG fraction was then transferred into a glass tube and completely made dry under nitrogen gas. The dried content in the glass tube was then reconstituted in 500 µl of chloroform and finally transferred gradually, 15 µl at a time, onto a plate of silica gel 60-coated-TLC. The resultant radiolabelled TG was separated by use of a mobile phase made of formic acid/diethyl ether/hexane (1:30:70, v/v/v). To accurately locate the band of the separated TG from the total lipids, 10 nmol of glyceryltripalmitate (tripalmitin), a TG standard, was run alongside the test

and also included in each sample. Visualisation of the TG bands was aided by use of iodine vapour. The bands were then scraped into scintillation vials and the radioactivity of TG fraction was quantified by the scintillation counter. Finally, the TG concentration was calculated by normalizing with the protein concentration in the chloroform extract by Bradford method as described in section 2.2.6.2

2.2.5 **Estimation of lipid content in Hep G2 cells - Oil Red O (ORO) staining**

Preparation of ORO stain: To obtain a stock of ORO (0.5%) stain, 0.5g of ORO powder (Sigma, UK) was dissolved in 100ml of absolute isopropanol (Sigma, UK). The solution was carefully stirred and placed in water bath at 56^{o}C for 1hour to enhance complete dissolution. After cooling, a working solution of 60% ORO was prepared from the stock, thoroughly stirred and allowed to stand for 10 minutes. The stain was finally filtered through Whatman Number 42 filter paper (Sigma, UK) and kept away from direct exposure to light.

Experimental procedure: At 100% confluence, the culture medium was removed and Hep G2 cells in six-well plates were gently washed twice with PBS. After this, the cells were fixed with 10% formalin at room temperature for an hour, washed with PBS and allowed to dry completely. The cells were then stained for two hours with ORO and washed 3-times until the water contained no noticeable colouration of the dye. The plates were observed under x40 objective of the microscope, followed by capturing of images. After completely drying the wells, an elution assay was then performed by adding 300µl of 99.9% isopropanol to each well and placed on a shaker for about 20 minutes to elute the dye. At this stage, 200µl of isopropanol from the six-wells were transferred into 96 micro-well plate and the absorbance read at 520nm. The absorbance was normalized to the concentration of proteins, estimated by the Bradford method, in the cells of the respective wells.

2.2.6 **Protein characterization study (Western blot analysis)**

2.2.6.1 **Hep G2 cells harvest for western blot analysis**

Hep G2 cells at 100% confluence were lysed using 500 µl of lysis buffer per well of culture plate. To prepare the lysis buffer, a cocktail of protease and phosphatase inhibitors was first prepared by dissolving protease inhibitor, comprising 2 tablets of Roche Complete-Mini protease inhibitor cocktail (Sigma, UK), and phosphatase inhibitors made up of 8mg Sodium Fluoride (Sigma, UK) and 20mg sodium vanadate (Sigma, UK), in 1ml of milli-Q water and stored at -20^{o}C in aliquots. Then, 1x of the RIPA buffer was then prepared from 10x RIPA

stock (Merck Millipore, UK) and mixed with the cocktail of protease / phosphatase inhibitors at 50:1. The cells were subsequently harvested in 500µl of the lysis buffer and transferred into 1.5 ml Eppendorf tubes which were stored at -20^{o}C.

2.2.6.2 **Protein quantification using Bradford reagent**

To quantify proteins, 1:4 dilution of the Bradford reagent (Bio-Rad, UK) and standard BSA of 1mg/ml were first prepared. Then, the standard concentration of BSA (1-7µg/µl) were prepared. To 200 µl of diluted Bradford reagent, standard BSA/ samples (µl) were added to 96-well microplates. The absorbance was obtained spectrophotometrically (Magellan, UK) at 595 nm. The concentration of the protein was then calculated from the standard curve using the absorbance of samples obtained.

2.2.6.3 **Western blot analysis**

Principle: The western blot (BioRad) technique initially separates proteins according to their molecular weights using SDS-poly acrylamide gel electrophoresis (SDS-PAGE) and subsequently electro-transfers the separated proteins unto a nitrocellulose membrane for detection with a primary antibody of a high specificity, followed by secondary antibody labelled with enzyme which is detected on a substrate (Fig 10).

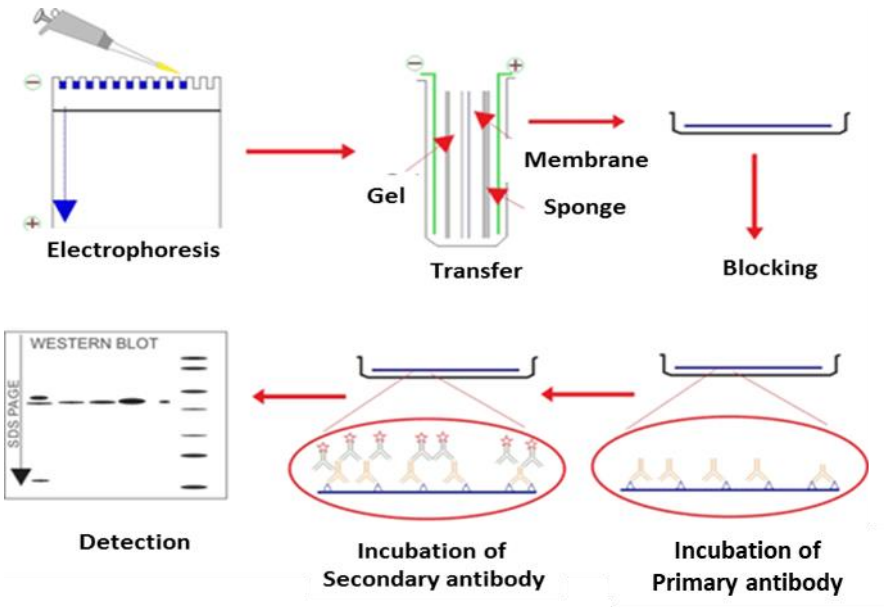

Figure 10: Principle of western blot [270]: The principle of western blot is reliant on the principle of immunochromatography involving the separation of proteins on the basis of their molecular weight into a polyacrylamide gel followed by the electro-transfer unto a nitrocellulose membrane for their detection aided by a substrate together with specific primary and secondary antibodies.

Procedure: For protein expression studies, 10% resolving and 5% stacking gels were prepared and casted, with comb carefully inserted, in the assembled glass plates (BioRad) and allowed to solidify. The samples were prepared by adding 20 µg of protein to 5x dye and milli-Q water to a total of 30ul, followed by vortexing and centrifuging at 10,000 for 15 seconds. Samples were incubated at 95^{o}C for 10 minutes, cooled on ice for 5 minutes and loaded together with 8 µl protein ladder (Sigma, UK) into wells of stacking gel and finally run at 100V for two hours. The gel was carefully removed and assembled in a blotting cassette, together with Whatman filter paper (Sigma, UK), PVDF membrane (Sigma, UK), and sponge, and placed in a tank of transfer buffer as per manufacturer instructions and ran at 100V for 1hour on a magnetic stirrer at room temperature. The membrane was then stained with ponceau S stain (Sigma, UK) and carefully cut at specific regions containing desired protein, followed by blocking and probing with 1:1000 mab conc/µg of primary antibody [(p-AMPKa, New England BioLabs, UK), p-ACC (New England BioLabs, UK), CD320 (Abcam Plc, UK) and TCN2 (Abcam Plc, UK) for 2 hours. The membrane was washed in PBS-T and probed with 1:1000 mab conc/µg of anti-rabbit secondary antibody (Cell signal, UK) for 1 hour, followed by another wash with PBS-T and detection with enhanced chemiluminescence (ECL). Proteins of primary antibodies CD320 and TCN2 were normalized to the housekeeping protein beta-actin. Similarly, pACC and pAMPK were also normalised to total ACC and total-AMPK proteins respectively.

2.2.7 **Analysis of fatty acids in total lipids of hepatic cell pellets**

Fatty acid levels, (µg) normalized per milligram protein of hepatocytes cell line, were obtained after preparing pellets of hepatocyte cell line from cell culture. Hep G2 cells were seeded at 75,000 cells / well in six-well plates under different conditions of B12 [500nM (control), 1000pM, 100pM and 25pM] until 100% confluence was achieved on the 10th day. Confluent cells were harvested, following initial washes 2x in PBS, by adding 300µl of trypsin to cells in each well of six-well plates and incubating at 37 ^{o}C for five minutes. 1000µl of EMEM media was then added to each well to inactivate the trypsin, with the subsequent cell suspension transferred into Eppendorf tubes and centrifuged 2000rpm for 10 minutes at 4 ^{o}C. The supernatant was carefully discarded whereas the pellets were frozen at -80 ^{o}C. Total lipid extraction into chloroform/ methanol (2:1) was carried out after adding 100µl pentadecanoic acid (internal standard) to cell pellets [269, 271]. Drying of the lipid extract was achieved using a flow of nitrogen, followed by synthesis of fatty acid methyl esters (FAME) using 3mol/l

methanolic hydrochloric acid (HCl) (Supelco, Bellafonte, PA) for 45 minutes at a temperature of 85^{o}C [271]. The FAME was then extracted into hexane and subsequently stored at -20^{o}C. Finally, analysis of the FAs, as previously described [272], was done using gas chromatography on a HewlettePackard 6890 from Agilent Technologies, Inc. Palo Alto, CA, together with FID system for detection and SP-2560 capillary column (60m x 0.25mm id x 0.15mm) supplied by Supelco, Sigma Aldrich, St Louis, MO.

2.2.8 **Mitochondrial dysfunction assessment using seahorse extracellular flux assay**

2.2.8.1 **Cell culture for seahorse assay**

Hep G2 cells were first cultured by seeding 75 000 cells in T-25 flasks under four different B12 concentrations [500nM (Control), 1000pM, 100pM and 25pM] of EMEM media supplemented with 10% FBS, 1% L-Glutamine and 1% streptomycin and/ penicillin. The plates were subsequently kept at 37^{o}C incubation with 5% CO_2 saturation. The respective B12-media were changed every 48-hours until 90-100% confluence was achieved on the 10th day in the flasks. On the last day, following the last media change, the cells were washed twice in 1x-PBS, tripsinized, counted and seeded at 50 000 cells /well into the XF-24 seahorse plates.

2.2.8.2 **Cell density optimization and seeding for XF24 seahorse assay**

For the optimum number of Hep G2 cells in 24-well (XF-24) seahorse plates for the experiment, we first optimized using different cells densities such as 10,000, 20,000, 30,000 and 50,000 cells / well. The optimum cell density of 50,000 cells/well showing highest oxygen consumption rate (OCR) was then chosen for the experiment. The cells were first dispensed in 100 µl EMEM-B12 media (supplemented with 10% FBS, 1% L-glutamine, 1% Penicillin and / streptomycin) into seahorse plates and securely incubated at 37^{o}C and 5%-CO_2 saturation for one (1) hour to allow settling of cells on the plate. After this, 150 µl of B12-EMEM medium was further added to each well of seahorse plates, incubated at 37 ^{o}C and 5%-CO_2 saturation overnight (24 hours) and used for the seahorse assay.

2.2.8.3 **Seahorse inhibitors**

Following the optimisation for the optimum concentrations of inhibitors such as oligomycin, carbonyl cyanide-p-tri-fluoro-methoxy-phenylhydrazone (FCCP) and rotenone / antimycin-A to be used for the seahorse assay (Fig 11), we chose optimum 10x concentrations such as 4.0 µM, 7.5 µM and 4.0 µM for oligomycin, FCCP and rotenone/antimycin-A respectively.

The 10x concentration (stock) of the inhibitors were therefore prepared in KHB buffer to obtain final concentrations (1x), of oligomycin (0.4µM), FCCP (0.75µM) and rotenone/ antimycin (0.4µM), after injecting into seahorse XF-24 plates. The inhibitors were loaded into the

allocated ports of the seahorse cartridge which was initially hydrated with 1ml calibrant for 24 hours at 37^{o}C without CO_2 saturation.

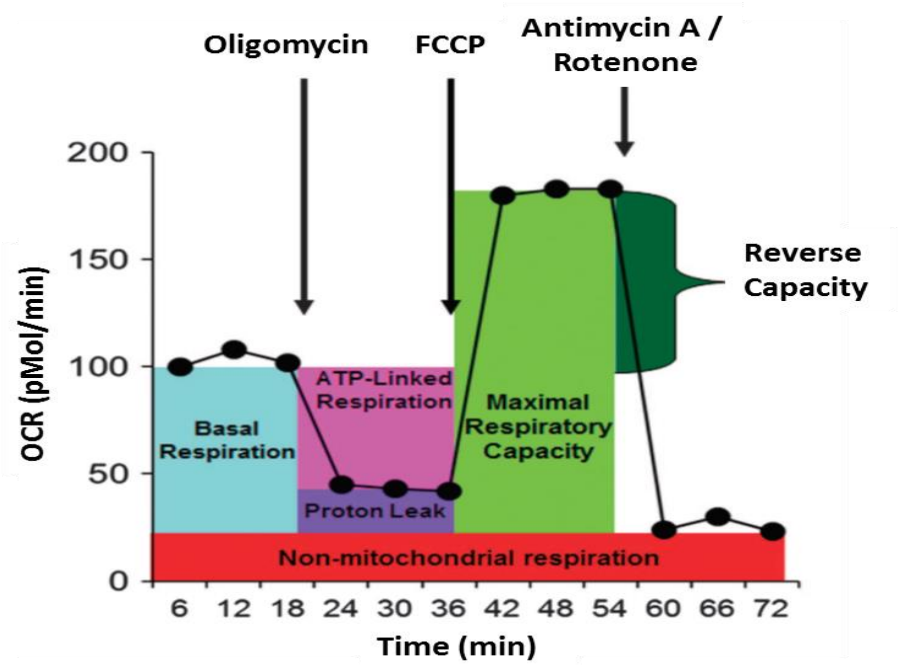

Figure 11: **Seahorse assay [273]:** This involves measuring of oxygen consumption rate (OCR) in cells undergoing respiration prior to and/ or after addition of inhibitors for the derivation of diverse markers of respiration in the mitochondria. OCR measurement is taken repeatedly at 8 mins intervals. An average is obtained from about 3-4 readings to ensure reliability of measurements. First, measurement of a baseline cellular OCR is obtained, and this derives the basal respiration following the subtraction of respiration of non- mitochondrial source. Then, the addition of an inhibitor of complex V or ATP synthase, Oligomycin, is done to produce an OCR which is useful to derive the ATP-linked respiration (Baseline OCR – Oligomycin rate) or respiration of proton leak (Oligomycin rate – non mitochondrial respiration). Next, the addition of FCCP is done to enhance electron transport chain (ETC) to function to its maximal rate following FCCP-induced shutting off the inner membrane gradient. This results in the derivation of the maximal respiratory capacity (FCCP rate – non mitochondrial respiration). Finally, addition of complex III inhibitor, antimycin A, and complex I inhibitor, rotenone, is done to shut down the action of the ETC resulting in the measurement of non-mitochondrial respiration. To calculate the reverse capacity of the mitochondria, basal respiration is subtracted from maximal respiratory capacity.

2.2.8.4 **Seahorse media optimization**

Following the optimisation for the more effective seahorse media for this experiment, [between XF medium and Krebs-Henseleit Buffer (KHB)], we observed a higher spare respiratory capacity in hepatocyte cell line incubated in Krebs-Henseleit Buffer (KHB), therefore, KHB was selected for the experiment. KHB was prepared by using the same protocol described in

section 2.1.7b of chapter 2. The buffer was filter-sterilized, maintained at pH 7.4 and stored at 4 ^{o}C until required.

2.2.8.5 **Sample preparation and seahorse assay.**

After 24-hour incubation at 37^{o}C and 5% CO_2 saturation, the cells were washed in KHB buffer by removing 200 µl EMEM media off the cells in seahorse plates, and subsequently adding 1ml fresh KHB. Then, 950 µl of the KHB in 24-well seahorse plates was removed, and 675 µl of fresh KHB was finally added to the wells of the plate. The plates were then incubated at 37 ^{o}C for one hour without CO_2 saturation. To run the seahorse assay, the cartridge was initially loaded into the seahorse analyser for a calibration and equilibration runs, performed according to the manufacturers' protocol, after which the utility plate of the cartridge was replaced with the seahorse XF24 plate of the hepatocyte cell line. The oxygen consumption rates (OCRs) in the cells were subsequently measured as similarly described in another study [273].

2.2.9 **Animal model**

Studies involving metabolic diseases frequently engage C57BL/6 models due to their capability of developing obesity, intolerance to glucose as well as resistance to insulin following long-term exposure to obesogenic diet, thereby highlighting the essentials of human-related metabolic disorders including MetS and obesity [274].

The study was done in agreement with the UK Animals (Scientific Procedures) Act of 1986 and subject to the UK Home Office License. Appropriate approvals were also obtained from the local ethics review committee at the University of Southampton, the genetic modification and biosafety committee and the animal welfare and ethical review body (AWERB) of the University of Warwick. The C57BL/6 mouse models were all nurtured at the Southampton General Hospital, Division of Biomedical Research, Southampton. In the process, female C57/BL6J mice were sustained under a 12h light/dark cycle (lights on at 07.00h), and at a constant temperature of 22±2°C with food and water available *ad libitum*.

The mice (n=6 per group) were fed either a control diet containing 7.49µg/kg vitamin B12 (RM1 diet, Special Diet Services, UK) or a high vitamin B12 diet containing 30.82µg/kg vitamin B12 (824053 Diet, Special Diet Services, UK) for 8-10 weeks. Following appropriate killing and validation of death by cervical dislocation as similarly described elsewhere [275], the liver, among several other tissues, was taken and the weights assessed, followed by instant freezing in the liquid nitrogen. Blood was obtained *via* cardiac puncture and the plasma was

subsequently acquired by specimen separation in heparinised tubes. B12 concentrations in the plasma of the mice were measured by electrochemiluminescent immunoassay using a Roche Cobas immunoassay analyzer (Roche Diagnostics UK, Burgess Hill, UK).

On this basis, the mice were categorized into two groups: High plasma B12 group, HB (have 6.5-fold higher levels of plasma B12 compared with LB group, n=6) and low plasma B12 group, LB (have low serum levels of B12 compared with HB group, n=6). When required, the liver tissues were homogenised in cryovials with 500µl phosphate buffered saline (PBS), RIPA buffer and Qiazol for intrahepatic B12 measurement, protein isolation for western blots and RNA isolation for RT-qPCR assays respectively. Homogenized samples were stored at -80°C until later use.

2.2.10 **Statistical analysis**

Analysis of all data was performed using the software of Prism 8 (GraphPad, San Diego, USA). All quantitative measurements, where applicable, were obtained in triplicates for standards, controls as well as cases in order to ensure precision of data. Data obtained from the oil red O staining, total intracellular triglycerides, scintillation count of the radioactive flux assay and fatty acid profiling using gas chromatography were normalized with the total protein concentration (mg) in each sample (n) of Hep G2 cells to alleviate possible variations that might misrepresent the data. The final data were then expressed as mean + standard error of mean (SEM). Data for all samples obtained were tested for normality prior to analysis using Kolmogorov-Smirnov and Shapiro-Wilk normality tests to assess if they were distributed normally. For samples that passed the normality assessment, comparisons between cases and their corresponding controls were done using a two-tailed unpaired t-test. Likewise, where test for normality was not passed, comparisons between two sample groups were done using the 2-tailed Mann-Whitney U test. Statistically significant differences were assigned to p-values < 0.05.

Chapter Three - Results

3.0 Uptake and storage of vitamin B12 is modulated by CD320 receptor and TCN2 transporter in hepatocytes in various extracellular B12 concentrations

3.1 Introduction

Vitamin B12 (B12) or cobalamin (Cbl) is synthesized naturally by cobalamin-producing bacteria in the gastrointestinal tract (GIT) of animals [147]. Intestinal absorption of the bulk of B12 is aided by intrinsic factor (IF) binding Cbl, however, only 1% of free B12 is known to be passively absorbed from the GIT [276]. B12 deficiency is associated with various metabolic and neurodevelopment diseases, but its levels are dependent on the B12 receptors and transporters. Specific membrane B12 receptors (CD320) and transport proteins such as haptocorrins (HC) and transcobalamin II (TCN2) facilitate the uptake and delivery of B12 into the tissues [277]. Dietary uptake of B12 occurs following sequential binding of B12 onto HC and IF in the ileal epithelium. Then, IF degrades in the bloodstream allowing B12 to form a B12-TCN2 complex (Fig 12) which is actively available for tissue uptake *via* the transcobalamin receptor (CD320).

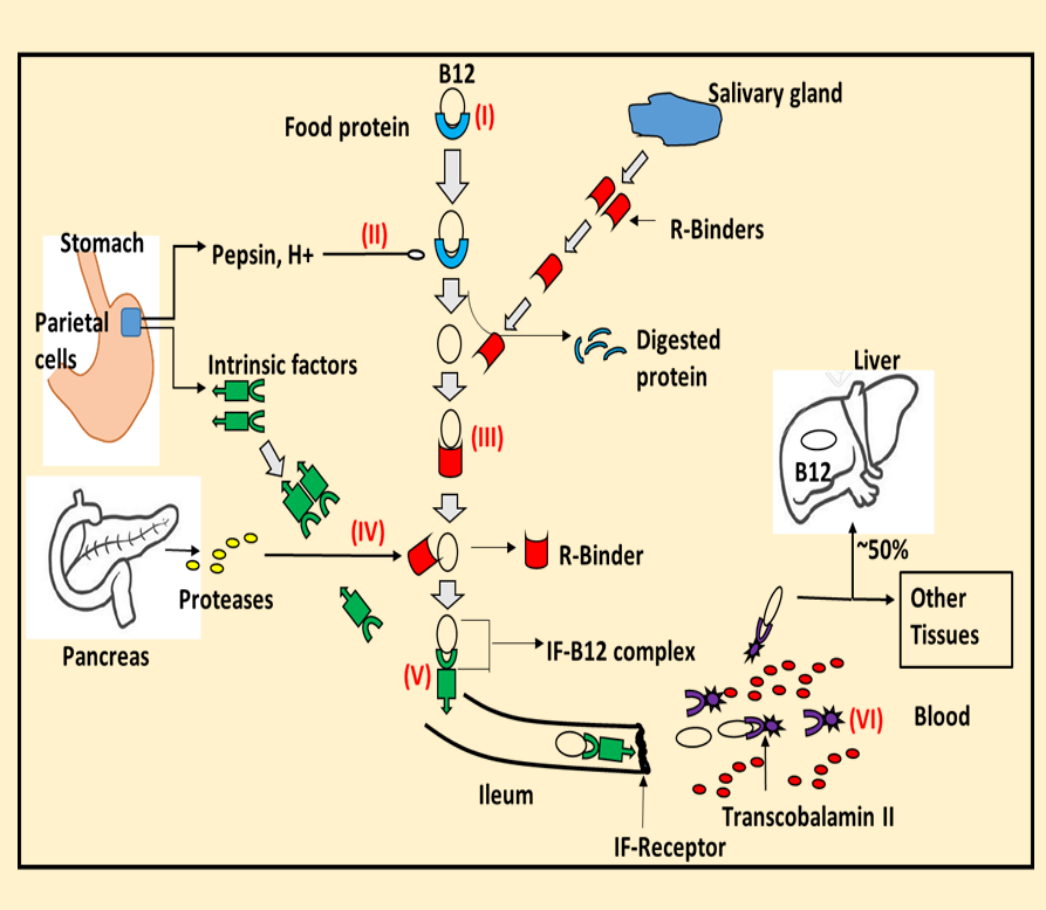

Figure 12: Intestinal absorption and transport of B12 in the circulation:

(I) Following ingestion, B12 is usually complexed with food protein prior to entering the stomach.

(II) Protein undergoes breakdown releasing free B12 in the acidic medium of the stomach following the action of pepsin secreted by the gastric parietal cells.

(III) Free B12 is then bound to R-binders (haptocorrins) following their secretion and release into the stomach by the salivary glands.

(IV) B12 is again liberated into the duodenum by pancreatic proteases which cause degradation of the R-binders.

(V) The gastric parietal cells generate and release intrinsic factor (IF) into the duodenum that binds free B12 forming the B12-IF complex. Specific IF receptors known as cubam take up the B12-IF complex and release B12 into circulation.

(VI) B12 is then bound to transcobalamin II (holo-TC) in the circulation and transported to all tissues of the body expressing specific transcobalamin receptors (CD320), with the liver taking about 50% of the total circulating B12.

TCN2 is an essential protein for the delivery of B12 from blood to various tissues. Its potential role in the transport of B12 from maternal to foetus was shown in pregnant adolescents, where higher placental TCN2 abundance was associated with higher cord blood B12 concentrations [278]. CD320 is required for the uptake of TCN2 bound B12. Studies have shown that CD320 knockout mice have associated B12 deficiency and demonstrate behavioural deficits, macrocytic anaemia, infertility and hypomethylation of DNA in brain [279]. However, the current knowledge on the absorption and tissue distribution of B12 have been reported only in few animal studies [280-285]. Tissue-specific concentrations of B12 according to a study in Wistar rats were: lungs (10 pmol/g tissue), spleen (25 pmol/g tissue), brain (33 pmol/g tissue), heart (72 pmol/g tissue), liver (74 pmol/g tissue) and kidney (1350 pmol/g tissue) [286]. A study in B12 deficient rats has shown that the liver uptake of hydroxyl Cbl was more than twice that of cyano Cbl, while the opposite was observed in other tissues such as kidney, brain and spleen [281]. Similarly, animals fed with imbalanced methyl containing nutrients (low B12, high folate, and high methionine) during pregnancy showed a redistribution of B12 in the maternal tissue from the kidneys to the liver and in foetal compartment (uterus, placenta, foetuses) [287].

Although it is known that the liver is the primary source of TCN2 and storage organ for B12 [288], the mechanism of B12 transporter and receptor modulation as well as hepatic levels of B12 in relation to circulatory B12 levels are unexplored. No data exists on the role of B12 transporter (TCN2) and receptor (CD320) and their association with intracellular levels of B12 in hepatocytes. Therefore, the present study was designed to; (a) assess and understand the

relationship between different circulating B12 levels, their corresponding intracellular hepatic concentrations and the levels of CD320 and TCN using Hep G2 cell line and C57BL/6 mice models. (b) Identify the appropriate concentrations of B12 required in the circulation for optimum hepatic uptake. (c) Studies show that B12 transport into the placenta is increased during pregnancy regardless of reduced circulating levels, therefore, we aimed to understand the discrepancy of B12 transport in diverse circulating levels.

3.2 Methods

3.2.1 **Cell culture:**

The cell culture was performed with slight modifications, as described in another study [289] and previously described in sections 2.2.1.5 - 2.2.1.6. In this experiment, Hep G2 cells were seeded in ten (10) different concentrations of B12 medium: 500nM (Control), 400nM, 200nM, 100nM, 50nM, 20nM, 10nM, 1nM, 100pM and 25pM B12 in six-well plates. At 100% confluence the cells were harvested for intracellular B12, RNA and protein assessments, whereas the conditioned medium (1ml) was taken into Eppendorf tubes and stored at -20^{o}C for extracellular B12 quantification.

3.2.2 **Animal model**

The liver tissues and plasma from HB (n=6) and LB (n=6) mice used in this study were obtained using the same method described in section 2.2.9.

3.2.3 **RNA isolation, cDNA synthesis and quantitative real time-PCR (qRT-PCR)**

Total RNA and cDNA synthesis for gene expression assays in Hep G2 cells and liver tissues (mice) were done following the protocol as previously described in sections 2.2.2.1-2.2.2.4 and similarly performed in another study [290]. In the qRT-PCR assays, as described in section 2.2.2.4, the housekeeping genes 18s rRNA (Applied Biosystems, Paisley, UK) and L19 (Sigma, UK) were used to normalize RNA expressions in Hep G2 and liver tissues respectively.

3.2.4 **Western blot analysis**

Hep G2 cells in plates were harvested using the lysis buffer, radioimmunoprecipitation assay (RIPA), with dissolved phosphatase and protease inhibitors and stored at −80°C until required [290]. Similarly, liver tissues of the mice were also suspended in RIPA buffer, homogenised

and stored at -80°C. Protein samples of both Hep G2 and liver tissues were subsequently quantified using the Bradford assay. 30µg of the protein was used for the western blot analysis as described in section 2.2.6.3. Primary antibodies targeting CD320 (molecular weight: 29 kDa) and TCN2 (molecular weight: 48 kDa) were normalized to beta-actin (molecular weight: 42 kDa).

3.2.5 **Measurement of intracellular B12 levels**

Intracellular concentrations of B12 in Hep G2 cell lysates and liver tissues, prepared by homogenising cells / tissues in PBS, were determined by electrochemiluminescent immunoassay using a Roche Cobas immunoassay analyzer (Roche Diagnostics UK, Burgess Hill, UK).

3.2.6 **Measurement of B12 concentrations in EMEM media and plasma**

Before the Hep G2 cell culture, stock concentration of B12 (10mM) was carefully prepared from B12 powder (Sigma Aldrich, UK), filter-sterilized and supplemented in the EMEM media to obtain various final B12 concentrations such as 500nM, 400nM, 200nM, 100nM, 50nM, 20nM, 10nM, 1nM, 100pM and 25pM. Moreover, to confirm whether the exact concentrations of B12 supplemented in the media were similar to the concentrations present in the media, 1000µl of the different B12 fresh media were taken into Eppendorf tubes and stored at -20^{o}C until required. Similarly, 1000µl of the Foetal Bovine Serum (FBS) (Labtech international LTD, UK), used in the cell culture, was transferred into Eppendorf tubes and stored at -20^{o}C. The fresh medium was initially custom-made (without B12 supplementation) and was subsequently supplemented with 10% FBS in addition to 1% penicillin/streptomycin and 1% L-glutamine. Therefore, for B12 concentrations within Hep G2 cells, their corresponding levels in the media were measured using electrochemiluminescent immunoassay with Roche Cobas immunoassay analyzer (Roche Diagnostics UK, Burgess Hill, UK). Likewise, B12 levels in the plasma of mice were also measured the same way.

The B12 concentration present in the FBS stock was found to be 234pM, therefore, supplementation of the fresh medium with 10% FBS contributed low levels of B12 (23.4pM) to the medium. Other probable sources of B12 in the medium are unknown, however, sources of proteins and/or amino acids used in the medium (powder) preparation, by the manufacturer, might be accountable for additional low levels of B12. In the fresh medium containing 10%

FBS, without B12 supplementation (0pM), B12 concentration of about 48pM was detected. Likewise, in the media supplemented with low B12 concentrations such as 25pM, about 69pM B12 concentration was measured, whereas 100pM supplementation accounted for about 136pM B12 measurement (Fig 13). This data showed that the clinical definition of B12 deficiency (<150pM) or low B12 (150-230pM) was applicable to the low B12 conditions used in the current study.

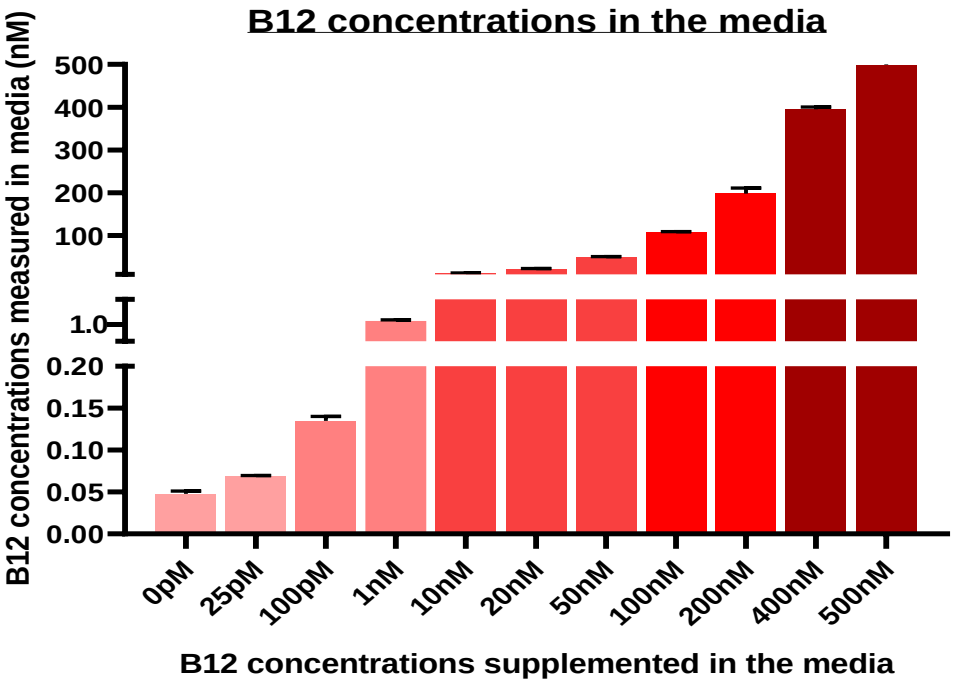

Figure 13: B12 concentrations in fresh and conditioned media: B12 levels supplemented in the EMEM medium, containing 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine, were measured in the EMEM media using the electrochemiluminescent immunoassay with Roche Cobas immunoassay analyzer (Roche Diagnostics UK, Burgess Hill, UK).

3.3 Results

3.3.1 **Hepatic uptake of B12**

3.3.1.1 **B12 uptake in Hep G2 cell line**:

The lower B12 concentrations (25-100pM) in the extracellular medium resulted in 2.84 - 2.11-fold increase in the intracellular levels of B12 in the hepatocyte cell line. However, an increase in the extracellular concentration to 1nM B12 resulted in only 0.43-fold storage in the hepatocyte cell line. Likewise, further increased circulating concentrations: 10nM, 20nM,

50nM, 100nM, 200nM, 400nM and 500nM B12 resulted in progressive decreases (0.05, 0.04, 0.03, 0.02, 0.02, 0.01 and 0.02-fold) in Hep G2 cell line uptake of B12, respectively (Fig 14).

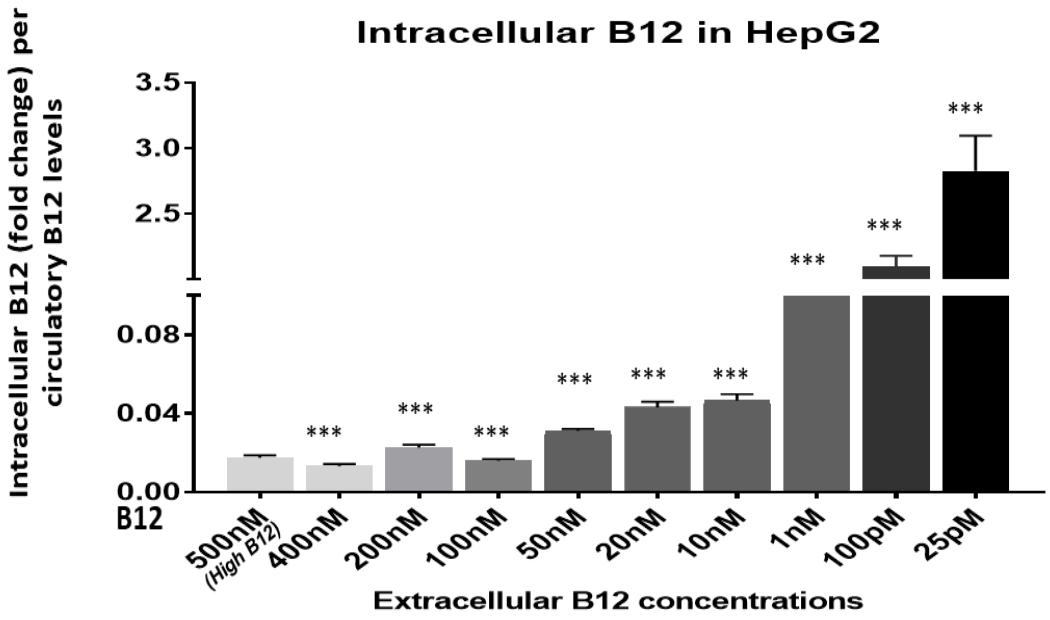

Figure 14: I**ntracellular fold uptake of B12 in hepatocyte cell line**: Hep G2 cell line was cultured in ten (10) different concentrations of B12 [500nM (high B12), 400nM, 200nM, 100nM, 50nM, 20nM, 10nM, 1nM, 100pM and 25pM] in EMEM media supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. These were incubated at 37°C with 5% CO_2 saturation until reaching 100% confluence on the 10th day after seeding the cells in six-well plates with 48hours-interval medium change. One ml of conditioned medium was taken and the hepatocytes, following 2x washing in PBS, were lysed in 250 µl 1x PBS with the B12 of both medium and cell lysate determined by electrochemiluminescent immunoassay using a Roche Cobas immunoassay analyzer (Roche Diagnostics UK, Burgess Hill, UK). All the data are graphed as mean ± SEM (n=6). * indicates significance compared with 500nM (high B12-control); * p< 0.05, ** p< 0.01, *** p< 0.001

3.3.1.2 **B12 uptake in mice liver**:

To evaluate the tissue-specific effect of uptake of B12, we assessed the intracellular levels of B12 in liver tissues from mice in relation to B12 levels in plasma. The average B12 concentration in the group of mice with low levels of B12 in plasma (LB (n=6)) was 0.82nM and in the group with high levels of B12 in plasma (HB (n=6)) was 5.36nM. The average B12 concentration in the HB group was therefore 6.5-fold higher than the concentration in the LB group of mice. Like the *in-vitro* model, we observed that the intrahepatic B12 uptake in the low B12 group (LB) was significantly higher compared with the high B12 group (HB) (Fig 15).

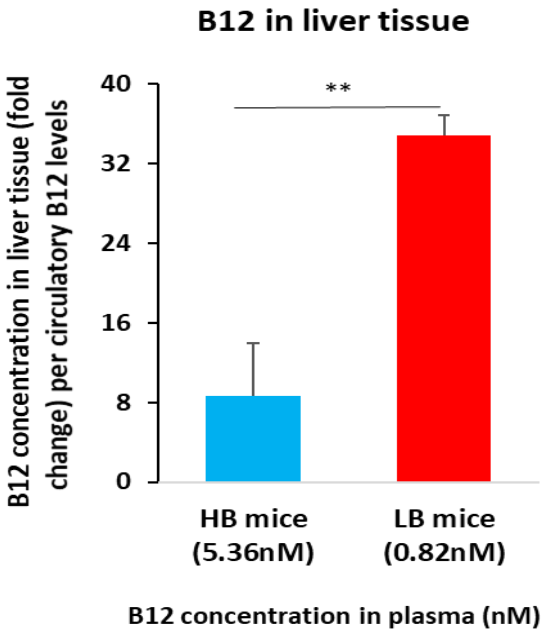

Figure 15: I**ntrahepatic B12 fold uptake in mice liver**: The mice (n=6 per group) were fed either a control diet containing 7.49µg/kg vitamin B12 (RM1 diet, Special Diet Services, UK) or a high vitamin B12 diet containing 30.82µg/kg vitamin B12 (824053 Diet, Special Diet Services, UK) for 8-10 weeks. Following the measurement of B12 in the plasma, the mice were categorized into two groups: High plasma B12 group, HB (have 6.5-fold higher levels of plasma B12 compared with LB group, n=6) and low plasma B12 group, LB (have low serum levels of B12 compared with HB group, n=6). Liver tissues of both HB (5.36nM) and LB (0.82nM) mice were homogenized in 500µl of PBS and the B12 of lysates were measured. All the data are graphed as mean ± SEM (n=6). * indicates significance compared with HB mice (control); * p< 0.05, ** p< 0.01, *** p< 0.001

3.3.2 **Transcobalamin receptor CD320**

3.3.2.1 **Hep G2 cell line**

We assessed the protein and mRNA expression of CD320 in hepatocyte cell line under ten different B12 medium concentrations. Low extracellular B12 concentrations of 1nM, 100pM and 25pM resulted in significantly increased expression of the CD320 receptors in hepatocytes compared with control (500nM). Increasing B12 medium concentrations: 10nM, 20nM, 50nM, 100nM, 200nM and 400nM resulted in decreased transcobalamin receptor (CD320) gene expression and were not significantly different from control (500nM) (Fig 16).

Similarly, we also observed upregulation in protein levels of CD320 in decreasing B12 concentrations of the medium: 20nM, 10nM, 1nM, 1nM and 25pM compared with control (500nM). The highest CD320 protein expression was observed in hepatocyte cell line cultured in the lowest B12 medium concentration of 25pM. However, hepatocyte cell line in the

increasing B12 medium concentrations: 50nM, 100nM, 200nM and 400nM, showed no significant change compared with control (Fig 17).

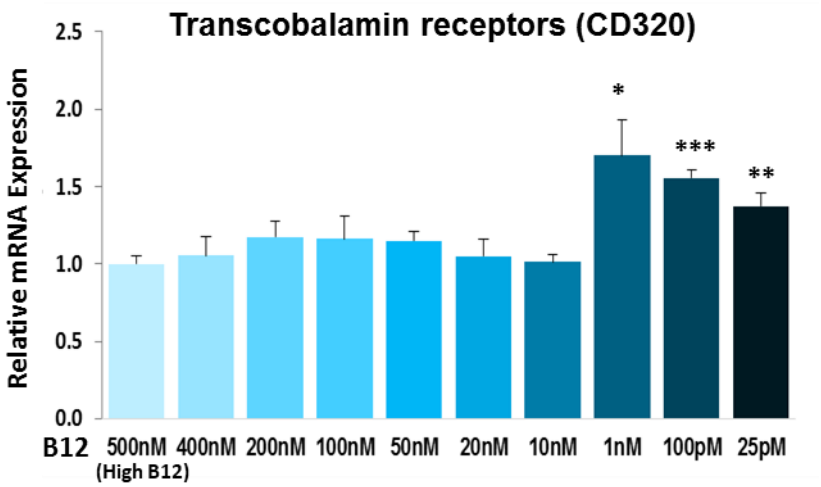

Figure 16: **Transcobalamin receptors (CD320) expression in Hep G2**: The mRNA expressions of transcobalamin receptors (CD320) in hepatocyte cell line of ten (10) different B12 conditions [500nM (control), 400nM, 200nM, 100nM, 50nM, 20nM, 10nM, 1nM, 100pM and 25pM] normalized to 18S rRNA endogenous control (Applied Biosystems, UK) were analysed using RT-qPCR. All the data are graphed as mean ± SEM (n=6). * indicates significance compared with 500nM (high B12-control); * p< 0.05, ** p< 0.01, *** p< 0.001

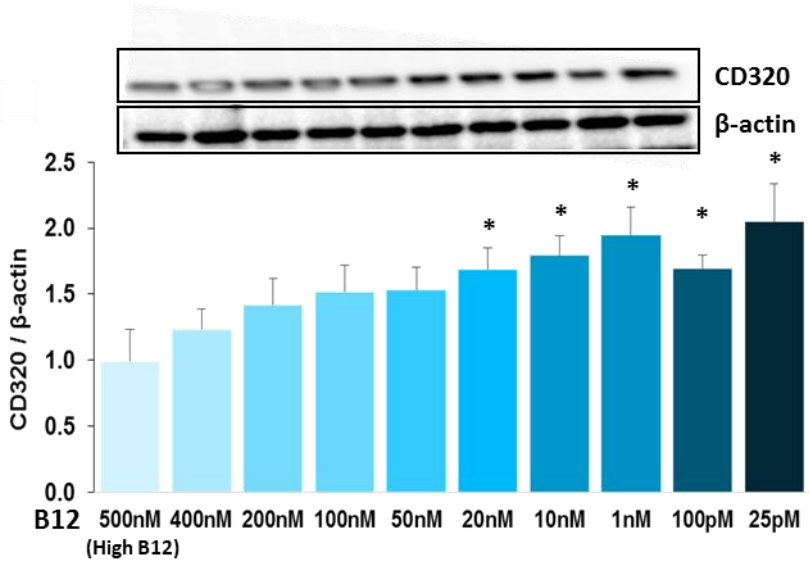

Figure 17: **Level of transcobalamin receptor (CD320) proteins in Hep G2**: CD320 proteins in hepatocyte cell line of different B12 concentrations [500nM (control), 400nM, 200nM, 100nM, 50nM, 20nM, 10nM, 1nM, 100pM and 25pM] were extracted and analysed using western blotting. All the data are graphed as mean ± SEM (n=6). * indicates significance compared with 500nM (high B12-control); * p< 0.05, ** p< 0.01, *** p< 0.001

3.3.2.2 **Mice liver**

Then, we assessed the mRNA expression and protein levels of CD320 in the liver tissue of mice with high (HB) and low (LB) levels of B12. There was no significant difference in the mRNA expression of CD320 between the LB and HB mice (Fig 18A). However, we observed that liver tissues from the LB mice had significantly increased level of CD320 protein compared with HB group of mice (Fig 18B).

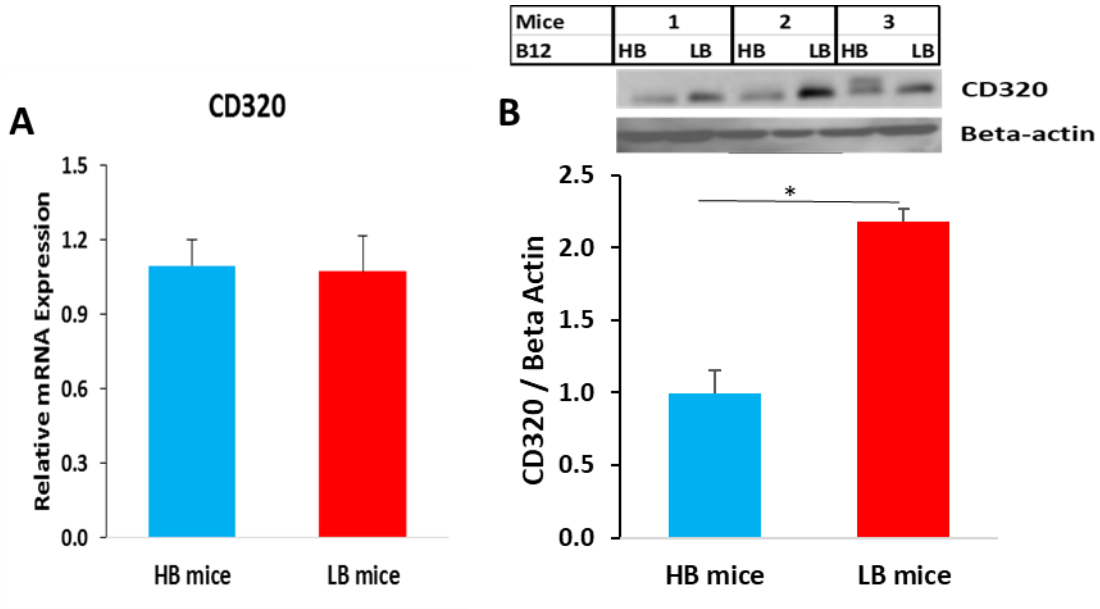

Figure 18: **Transcobalamin receptors (CD320) in mice liver:** (**A**) The CD320 mRNA expression in liver tissues of HB and LB mice normalized to L19 endogenous control and (**B**) CD320 protein levels in liver tissues of HB and LB mice following homogenisation and extraction of RNA and protein from liver tissues and analysed with RT-qPCR (Applied Biosystems, UK) and western blotting respectively. All the data are graphed as mean ± SEM (n=6). * indicates significance compared with HB mice (control); * p< 0.05, ** p< 0.01, *** p< 0.001

3.3.3 **B12 transporter transcobalamin II (TCN2)**

3.3.3.1 **Hep G2 cells**

To further evaluate the transporter involved in B12 uptake, we assessed the protein and gene expressions of B12 transporter transcobalamin II (TCN2) in hepatocytes cultured in ten different B12 medium concentrations. In B12 deficient conditions 100pM and 25pM, the mRNA expression of hepatic TCN2 was significantly higher. However, we observed that mRNA expression of TCN2 was decreased in hepatocyte cell line cultured in increasing medium concentrations of B12: 1nM, 10nM, 20nM, 50nM, 100nM, 200nM and 400nM,

showing no significant difference compared with control (500nM). The highest TCN2 mRNA expression was observed in hepatocyte cell line cultured in the least concentration of B12 medium (25pM) compared with control (500nM) (Fig 19).

Similarly, Hep G2 cells cultured in low medium concentrations of B12 resulted in high expression of protein levels of TCN2 (50nM, 20nM, 10nM, 1nM, 100pM and 25pM) compared with control (500nM). However, high B12 media concentrations such as 100nM, 200nM and 400nM showed low levels of TCN2 protein and were not significantly different from control (500nM) (Fig 20).

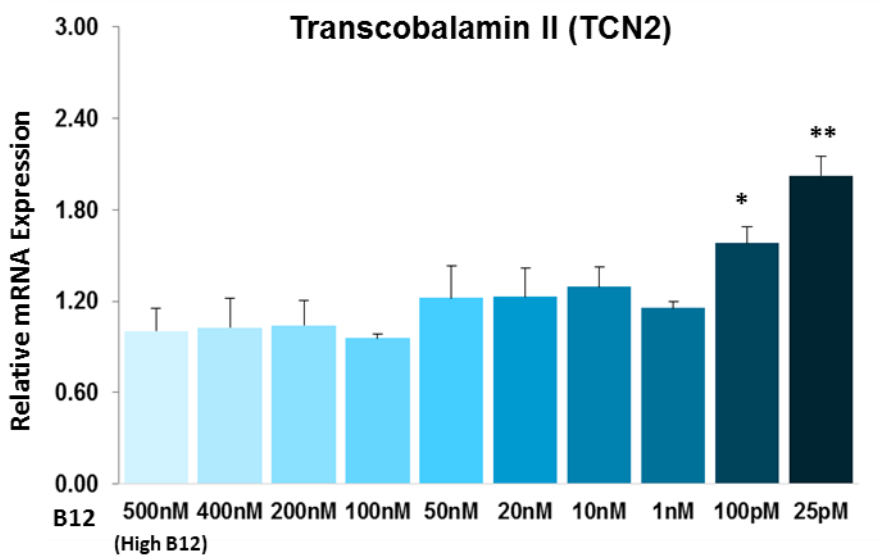

Figure 19: G**ene expression of transcobalamin II (TCN2) in Hep G2:** mRNA expressions of transcobalamin II (TCN2) in hepatocyte cell line of ten (10) different B12 medium concentrations such as [500nM (control), 400nM, 200nM, 100nM, 50nM, 20nM, 10nM, 1nM, 100pM and 25pM] were analysed using RT-qPCR (Applied Biosystems, UK) and normalized to 18S rRNA endogenous control (Applied Biosystems, UK). All the data are graphed as mean ± SEM (n=6). * indicates significance compared with 500nM (high B12-control); * p< 0.05, ** p< 0.01, *** p< 0.001

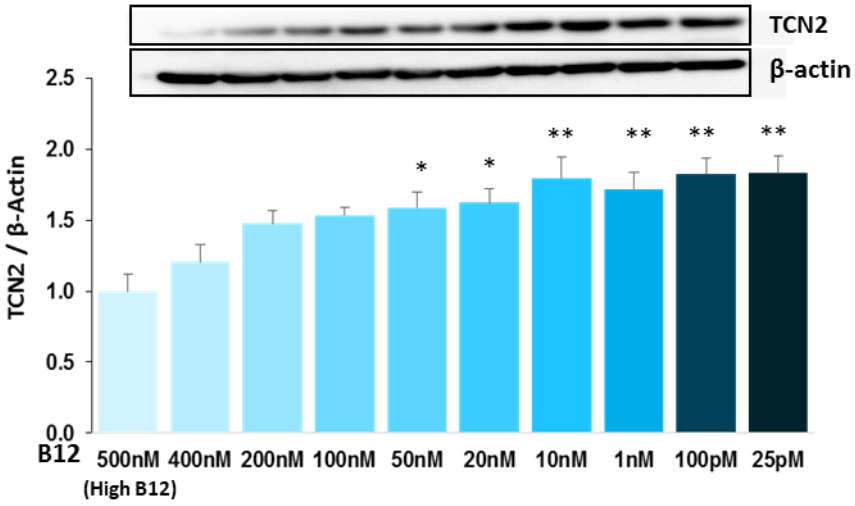

Figure 20: P**rotein levels of B12 transporter transcobalamin II (TCN2) in Hep G2**: B12 transporter transcobalamin II (TCN2) protein levels in hepatocyte cell line of ten (10) different B12 medium concentrations such as 500nM (control), 400nM, 200nM, 100nM, 50nM, 20nM, 10nM, 1nM, 100pM and 25pM were analysed using western blotting. All the data are graphed as mean ± SEM (n=6). * indicates significance compared with 500nM (high B12-control); * p< 0.05, ** p< 0.01, *** p< 0.001

3.3.3.2 **Mice liver**

Like Hep G2 cells, we observed that both mRNA expression and protein levels of TCN2 were significantly higher in the liver tissue with low levels of B12 (LB) compared to HB group. We found that liver tissue expressed about 75% increase in the mRNA levels (Fig 21A) and 50% increase in the protein levels (Fig 21B) of TCN2 in LB mice compared with HB mice.

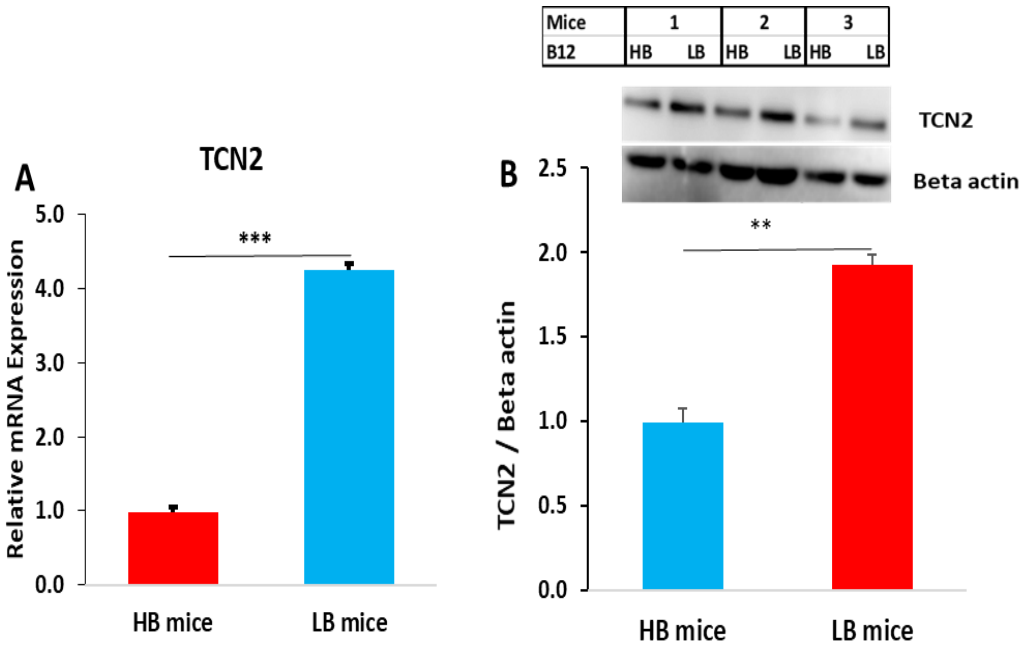

Figure 21: B12 transporter TCN2 in mice liver: (**A**) The TCN2 mRNA expression in liver tissues of HB and LB mice normalized to L19 endogenous control and (**B**) levels of B12 transporter transcobalamin II (TCN2) protein in HB and LB groups of mice following homogenisation, extraction and analysis of RNA and proteins using RT-qPCR (Applied Biosystems, UK) and western blotting respectively from liver tissues. All the data are graphed as mean ± SEM (n=6). * indicates significance compared with HB mice (control); * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$

3.4 Discussion

In this study, Hep G2 cells in increasing extracellular B12 medium concentrations resulted in progressive decreased intracellular B12 uptake. Both protein and gene expressions of B12 receptor CD320 and transporter TCN2 decreased progressively in increasing extracellular B12 medium concentrations. Similarly, liver tissues of the HB mice demonstrated decreased B12 uptake and lower expressions of CD320 and TCN2 compared with LB mice.

In clinical practice, both higher and lower concentrations of circulating B12 are common. For instance, B12 deficiency may be due to vegetarianism, prolonged metformin treatment in T2DM patients and during pregnancy [291, 292]. However, high plasma levels may result from either supplementation with a traditional B12 supplement or lack of affinity of B12 receptor and transporter for B12 [282]. The transcellular movement of B12 across membranes of cells involves mechanisms such as transcytosis of holo-TC (B12-TC) *via* CD320 receptors and exocytosis of free B12 (Cbl) according to a recent *in-vitro* study in bovine aortic

endothelial cells [293]. In the current study, we assessed the uptake of B12 in Hep G2 cells cultured in increasing concentrations of ten different B12 media. When the circulatory level in low B12 medium was 25pM, the intracellular level was 178pM (2.8-fold) but when circulatory level in High B12 medium was 500nM (20,000-fold higher than 25pM), the intracellular level was only 8nM (0.02-fold). Similarly, in the animal model, we observed that when the circulatory level in LB was 0.82nM, the intracellular level was 27nM (i.e. 33.8 fold higher) but when the circulatory level in HB was 5.36nM (6.5 fold higher than 0.82nM), the intracellular level was only 46nM (8.6 fold). This was also supported by a recent study in rats that a single dose supplementation with 4-fold higher B12 concentration in plasma was unable to deliver any further B12 to the tissues [282]. Our findings are confirmed by a recent study in rat models which showed that low circulating B12 resulted in elevated endogenous B12 concentration in different tissues such as spleen, brain, heart and liver (higher tissue/plasma B12 ratio) [284]. In the cobalamin-deficient rats, Scott et al found that the proportion of the administered [57Co] Cbl present in the liver was twice than in normal rats [294]. Similarly, in an earlier study in folate (B9) deficient rats, low folate resulted in the elevation in the uptake of folate in the brush border membrane vesicles [295]. Our data hence suggests that in low circulating B12, intracellular uptake as observed in this study could indicate a probable active transport of B12 [296] from the circulation leading to intracellular accumulation within the hepatocyte cell line, a possible mechanism to compensate physiologically for depleted intracellular hepatic stores. In high B12 environment, the intracellular level of B12 observed in both our *in-vitro* (8nM) and *in-vivo* model (46nM) demonstrated that these levels are sufficient for the cells to perform their action, therefore, the high amount of B12 supplemented to cells or the levels prevailing high in circulation are not required for further cellular uptake. Since the liver tissue plays a paramount role in the maintenance of B12 homeostasis among other tissues such as gut and kidney [297], this may account for the adjustment in hepatic B12 uptake amidst variant extracellular conditions.

Furthermore, in the current study, we observed increased protein and gene expressions of B12 receptor (CD320) in Hep G2 particularly in low circulating B12 concentrations. However, low B12 medium concentrations resulted in higher expression of CD320 in the hepatocyte cell line. In liver of HB mice, decreased protein level of CD320) was observed compared with the LB group. Our findings supports an earlier study in hepatocytes which showed that modulations in the cellular uptake of B12 was the result of alterations in the number

of cellular receptors but not affinity [298]. Clinically, B12 deficiency may be observed during pregnancy due to increased demand of B12 for foetus [299] but a recent study showed progressive increase in serum levels of soluble CD320 receptors up to 35 weeks of gestation [300]. High serum sCD320 receptors in pregnancy might be derived from the placenta where higher expression of the receptor has been shown [301]. Similarly, a recent study has shown that serum levels of the B12 co-receptor [amnionless (AMN)], which plays a key role in anchoring the transport complex of B12 to epithelial cell membranes [297], were increased in aged humans presenting with B12 deficiency [297]. In the same study, a negative association was observed between serum AMN and B12 levels in rats [297]. The authors also observed increased expression of AMN in the kidneys of the B12-deficient-aged subjects, suggesting that the high serum AMN levels originated from kidney accounting for high renal uptake [297]. Although high B12 uptake and accumulation are common in the liver and kidneys [296, 302, 303], there might be a threshold beyond which cells might limit further uptake in excessive supply. Probable modulation of receptors or the availability of an efflux system which actively expels excessive B12 from cells, has been proposed to account for regulation of intracellular concentration of B12 [304, 305].

The B12 transporter - TCN2 is principally produced by hepatocytes and less commonly intestines, endothelia and monocytes [306-308]. The *de novo* biosynthesis of TCN2 has been demonstrated in liver perfusates as well as cultured liver parenchymal cells using models involving rats [309, 310], supporting our observation of TCN2 expression in Hep G2 cells and liver tissues from mice. Although TCN2 accounts for about 22-37% binding and transport of plasma B12 [311], it is the key transporter of up to 99% of active B12 (holo-TC) into tissues [312]. Therefore, modulations in both gene and protein expressions of TCN2 in the liver reflects its crucial role involved in the regulation of B12 uptake and metabolism in different circulating B12 conditions. In the current study, we also observed higher TCN2 levels in hepatocytes under decreasing concentrations of extracellular B12 media. However, decreased levels of TCN2 were observed in the hepatocyte cell line under higher medium concentrations of B12. Similarly, the liver tissues of HB mice showed significantly decreased TCN2 levels compared with LB mice. In pregnancy, the TCN2 levels required for B12 transport are produced by the liver and placenta of the mother [300]. In spite of the reduction in serum B12 levels observed in pregnancy, the placenta was shown to have an increased expression and release of TCN2 into the circulation, accounting for increased cord blood [278] and foetal concentrations of B12 [300]. Similarly, following B12 supplementation in a clinical study, the level of total serum

transcobalamin (TC) was observed to be higher in low B12 patients [313]. In support to these evidences, our data indicates that the receptor and transporter levels only uptake the B12 levels to a certain level showing a threshold at cellular membrane entry and suggests the cellular uptake is limited even though high levels of B12 are in circulation.

3.5 Conclusion

In summary, our study highlights that when extracellular B12 levels were low, the intracellular B12 levels were 2-3fold higher whereas the opposite was observed in higher extracellular B12 levels. This was supported by the increased gene and protein expression of receptor/ transporter in both hepatocyte cell line and liver tissues in lower extracellular B12 levels. This shows that at low B12 concentration, the active transport of B12 in these tissues is higher and might be due to the higher expression of B12 receptors/transporters in the membrane of hepatocytes. However, at high B12 concentrations, neither intracellular B12 levels nor the receptor and transporter expression increase according to the corresponding rise in B12 in circulation. Moreover, some recent studies indicate that elevated circulatory B12 levels are associated with cancer, liver diseases, blood disorders and solid neoplasms where cell proliferation is evident. Hence, we suggest the optimal physiological levels of B12 are required rather than overloading with supplements. This provides us with the impetus to study further whether the tissue-specific effect of low B12 in liver dysregulates the hepatic metabolism.

4.0 Vitamin B12 deficiency alters lipid synthesis triggering accumulation in hepatocyte cell line

4.1 Introduction

The impact of higher adiposity on metabolic health is vastly explored in recent times due to rapid increase in the global incidence of obesity [314, 315]. Individuals above 18 years of age were reported to be 39% overweight, 13% obese and 48% NAFLD [316-318]. Obesity, characterized by excessive storage of fat in both adipose and hepatic tissues, is been associated with dyslipidaemia, insulin resistance and non-alcoholic fatty liver disease (NAFLD) [319]. Hepatic *de novo* lipogenesis (DNL) is a major pathway of lipid biosynthesis that is actively influential on fat balance in the body. Similarly, hepatic fatty acid oxidation (FAO) is crucial in suppressing lipolysis of adipose fats as well as improving liver physiology [320, 321]. However, increased free fatty acids (FFA) from these hypertrophic tissues have been associated with insulin resistance (IR), inflammation and GDM [322, 323].

Environmental factors including nutrition (macro and micronutrients) [324] may affect metabolism of lipids (DNL and FAO) and regulate the expression of lipogenic and FAO genes [254]. Deficiency in methyl donors such as vitamin B12 (B12) and folate is associated with obesity [325], liver steatosis [326] and increased risk of metabolic syndrome [327]. Low B12 in pregnancy was associated with elevated BMI, IR and GDM in mothers [328-330]. This was subsequently associated with higher risk of offspring adiposity, IR and NAFLD incidence in later life [331, 332]. Clinical studies showed that maternal low B12 was associated with higher levels of lipids in both maternal and cord blood [333]. Human adipocyte models showed that the nuclear transcription factor and master regulator of lipogenesis, sterol regulatory element binding protein (SREBP1c) and regulator of cholesterologenesis, low density lipoprotein receptor (LDLR), were dysregulated leading to dyslipidaemia by low B12 [193]. The same study has also elucidated the epigenetic mechanisms of B12 deficiency *via* regulation of DNA methylation [193] and microRNAs leading to higher adiposity and dyslipidaemia [334]. B12 restricted rat models have demonstrated in offspring, higher maternal adiposity [335], dyslipidaemia [335], upregulation of enzymes in lipogenesis [336] and lipid oxidation [337, 338]. Though dysregulation of lipid metabolism in human adipose tissue has also been extensively explored in relation to B12 deficiency [193, 334], the contribution of adipose tissue to lipid metabolism to the entire body is considerably lower compared to the liver [339-341]. Therefore, if parallel effects of B12 are evident within hepatocytes, this may enlighten the observation of low B12 induced dyslipidaemia in humans.

DNL is the key lipid synthesis pathway within hepatocytes that account for generation of lipids for hepatic storage and secretion into circulation [342]. Hepatic DNL utilizes substrates from metabolism of glucose or glycolysis, suggesting that increased carbohydrate-rich diet could account for higher hepatic DNL rate due to substrate overload [343]. The products of DNL, principally FAs, which may be utilized for TG and cholesterol biosynthesis (Fig 22-25) may end up accumulating within hepatocytes resulting in harmful effects. Low B12 has been shown to be associated with elevation in key enzymes and or genes involved in regulating key DNL pathways in human adipocytes [193]. Therefore, moderation of the level of lipids in human is crucial, achieved through careful modulation in the DNL and FAO pathways (Fig 6).

Excessive circulating levels of free fatty acids (FFAs) is a key contributor to dyslipidaemia as well as adverse effects associated with obesity. Increased level of FFAs is shown to be associated with the development of IR in the liver as well as muscles [344]. In animal models, apart from the increased TG and cholesterol levels [345], deficiency of B12 demonstrated unfavourable alterations in the levels of long chain polyunsaturated FAs (LC-PUFAs) in the plasma and liver of subjects across three generations [345]. Recent evidences suggest that FAs in circulation may be implicated in metabolic dysregulation. Abnormal circulating FA profile was demonstrated in GDM [322], postmenopausal obese and overweight women [346], MetS [347], T2DM and CVD [348]. Despite the varied origins and diverse roles of these FAs, studies are lacking on these individual FAs with respect to micronutrient deficiency in particular cell type. Therefore, actual measurement of these individual FAs will provide the understanding of their distinct pathophysiologic roles in relation to B12 deficiency.

Therefore, we hypothesised that low B12 levels in hepatocytes might dysregulate lipid metabolism. We therefore aimed to investigate (1) the effects of B12 deficiency on lipogenesis in human hepatocyte cell line (Hep G2) and (2) the effects of B12 deficiency on the FA profile in Hep G2.

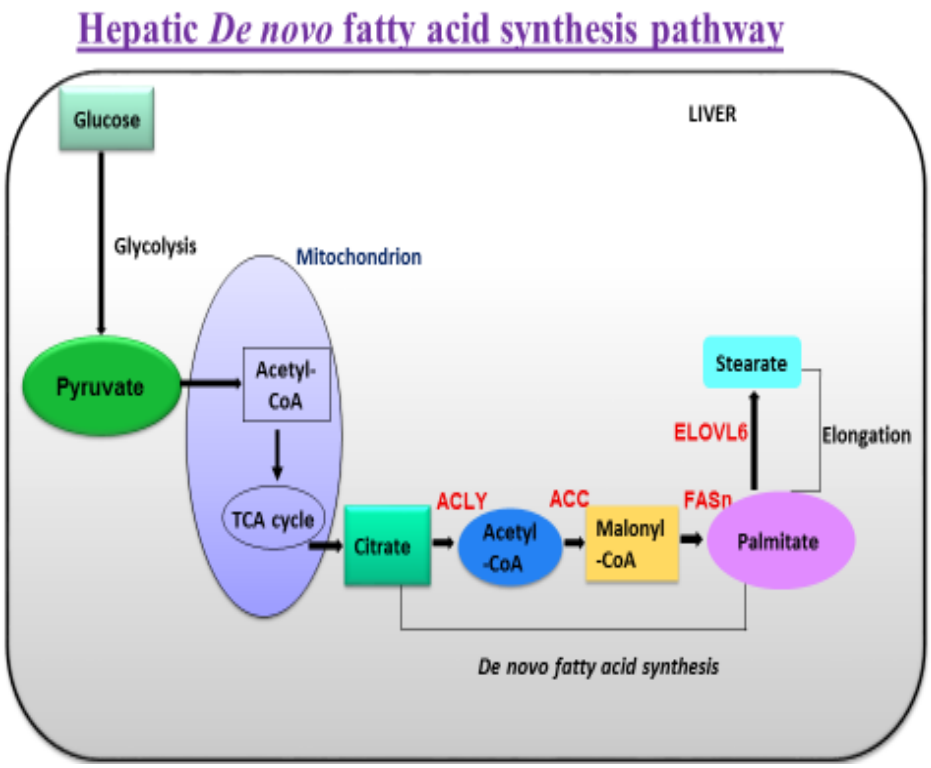

Figure 22: **Hepatic *de novo* FA synthesis pathway**

Hepatic lipogenesis constitutes FA, TG and cholesterol biosynthesis. *De novo* FA synthesis begins with the utilization of citrate, a product from the tricarboxylic acid (TCA) cycle in the mitochondria using pyruvate from glycolysis, which is further converted to acetyl co-enzyme A in the cytosol using the enzyme ATP citrate lyase (ACLY). Acetyl CoA is then carboxylated to malonyl CoA, a potent inhibitor of fatty acid oxidation by limiting carnitine palmitoyl transferase 1-alpha (CPT1α), by acetyl CoA carboxylase (ACC) and further converted by fatty acid synthase (FASN) into palmitate. Palmitate (16:0) is, finally, elongated by the enzyme elongation of a very long chain fatty acid 6 (ELOVL6) into stearate (18:0).

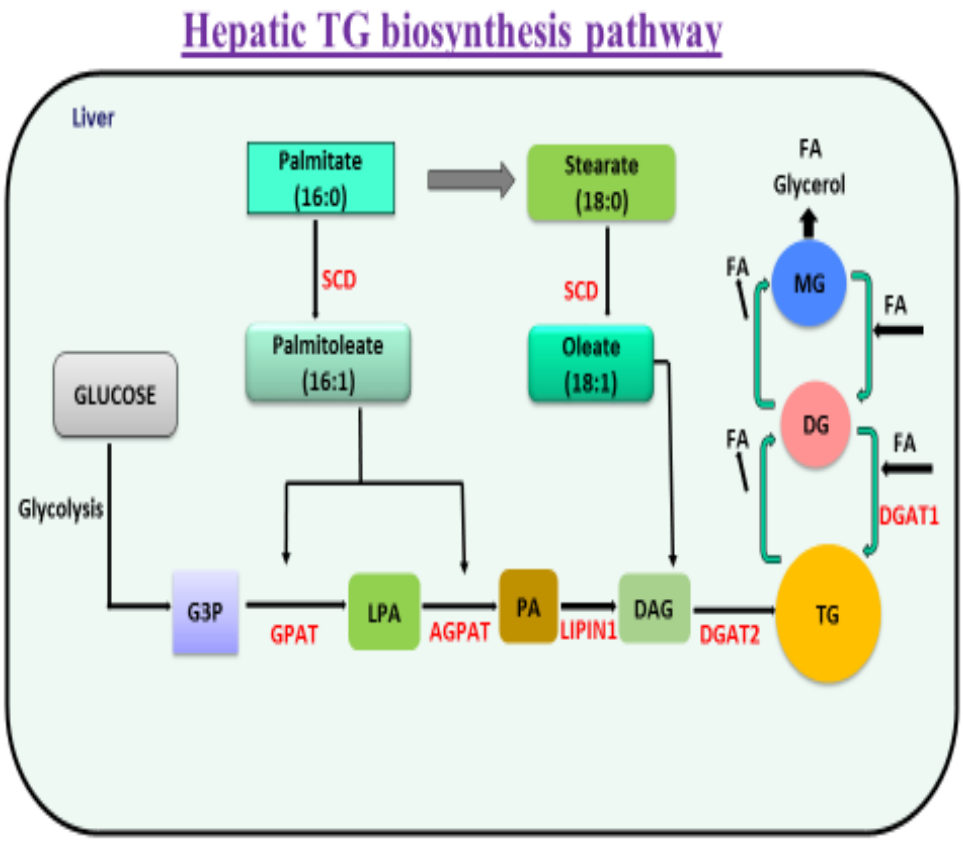

Figure 23: TG biosynthesis pathway. In the TG synthesis pathway, the saturated FA: palmitate and stearate, are desaturated by stearoyl CoA desaturase (SCD) into palmitoleate (16:1) and oleate (18:1) respectively, with either of these fatty acids incorporated into glycolysis-derived glycerol-3-phosphate (G3P) substrate, using the enzymes glycerol-3-phosphate acyl transferase (GPAT), acyl glycerol-3-phosphate acyl transferase (AGPAT), phosphatidic acid phospatase 1 (Lipin1), diacylglycerol acyl transferase 1 and 2 (DGAT 1&2), to derive lysophosphatidic acid (LPA), phosphatidic acid (PA), diacylglycerol (DAG) and triacylglycerol (TG) respectively.

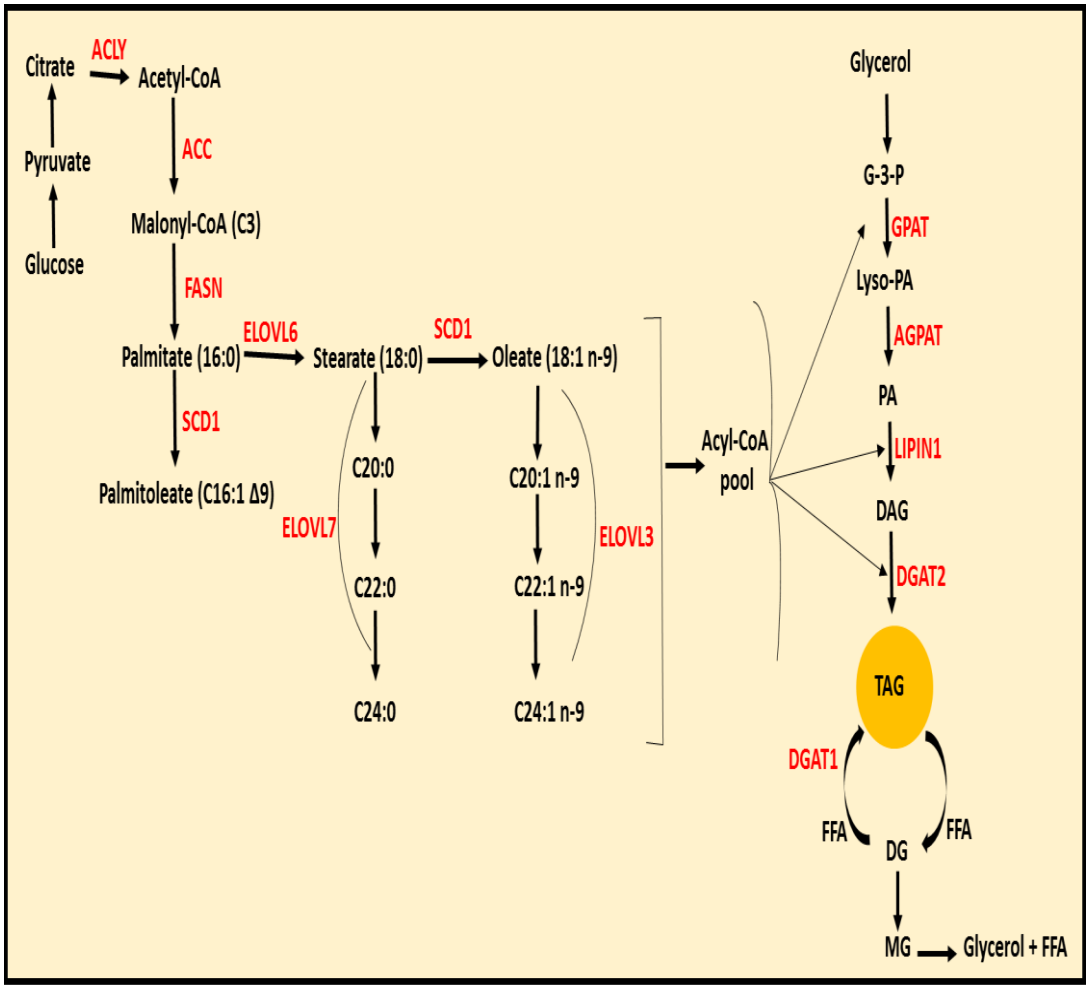

Figure 24: *De novo* biosynthesis of diverse FAs. *De novo* synthesis of FA begins with the generation of short to medium, long and very long chains. FAs may be saturated, thus presenting with no double bonds in the carbon chains, e.g.: palmitate (16:0), stearate (16:0), arachidic acid (C20:0), behenic acid (C22:0) and lignoceric acid (C24:0). Unsaturated FAs may either be monounsaturated, presenting with a single double bond in carbon chains, e.g.: oleate (18:1 n-9), gondoic acid (C20:1 n-9), erucic acid (C22:1 n-9) and nervonic acid (C24:1 n-9), or polyunsaturated with more than a one double bond and are commonly referred to as essential FAs. A pool of acyl-CoAs from these fatty acids are utilized for esterification into a glycerol-3-phosphate substrate for triglyceride biosynthesis.

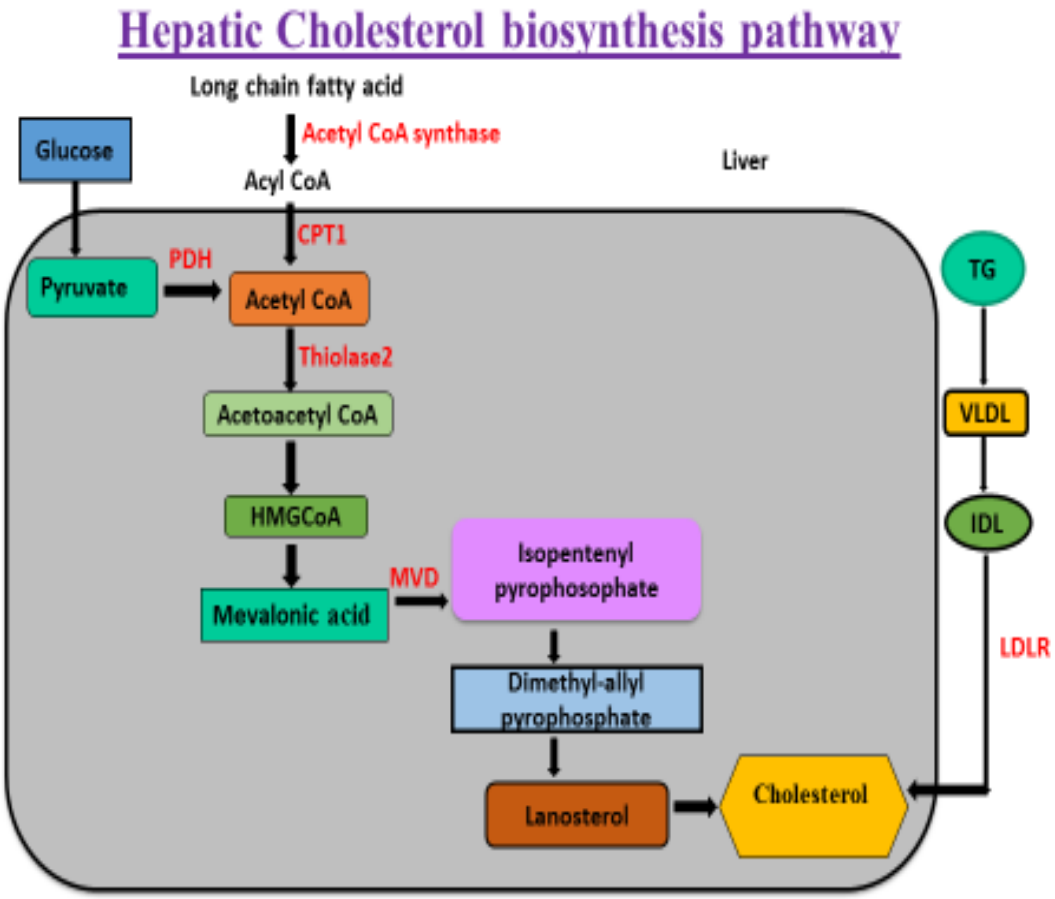

Figure 25: The Cholesterol synthesis pathway: The cholesterol biosynthesis pathway begins with acetyl CoA which is a product of the beta oxidation of a fatty acid and or pyruvate incorporation in the TCA cycle in the mitochondria regulated by rate limiting enzymes CPT1α and pyruvate dehydrogenase (PDH), respectively. Acetoacetyl CoA formation from acetyl CoA substrates under the influence of the thiolase enzyme, facilitates their metabolism into mevalonic acid through the 3-hydroxy-3-methylglucatyl-CoA intermediate, which is synthesized by the 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS) and, subsequently, reduced by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR). Mevalonate is further dehydrogenated to isopentenyl pyrophosphate by the enzyme mevalonic acid dehydrogenase (MVD) and, subsequently, subjected to series of modulations to derive lanosterol and cholesterol. Cholesterol may also be derived from triglyceride stores within very low-density lipoprotein (VLDL) by enzymatically modifying them into intermediate-density lipoproteins (IDS) and cholesterol by nuclear transcription factor low density lipoprotein receptor (LDLR)

4.2 **Methods**

4.2.1 **Cell culture**

Hep G2 cell culture was the same as previously described (sections 2.2.1.5 – 2.2.1.6), however, it was performed under four (4) different concentrations of B12: 500nM (Control), 1000pM, 100pM and 25pM. The cells were either harvested for isolation of RNA, total intracellular TG estimation and FA profiling, or maintained within plates for oil red o (ORO) staining and radioactive flux assays.

4.2.2 **Oil Red O staining and elution assay**

The oil red o (ORO) staining followed by elution assay of lipids within the Hep G2 cells, at 100% confluence, was the same as previously described in section 2.2.5.

4.2.3 **RNA isolation, cDNA synthesis and gene expression**

Total RNA isolation, cDNA synthesis and gene expression assays were the same as previously described in sections 2.2.2.1- 2.2.2.4, for the four conditions of B12 (500nM, 1000pM, 100pM and 25pM). The qRT-PCR involved the use of 18s rRNA (Applied Biosystems, Paisley, UK, 4319413E) for normalizing expressions of RNA.

4.2.4 **Total intracellular TG estimation**

An estimation of total intracellular TG in hepatocytes under four (4) conditions of B12 (500nM, 1000pM, 100pM and 25pM) was performed using the commercial TG quantification kit (ab65336) from Abcam plc, Cambridge, UK following the same protocol as described in section 2.2.3.

4.2.5 **Radioactive flux assay for TG biosynthesis**

Hep G2 cells at 100% confluence, after the last medium change for four different B12 conditions: 500nM, 1000pM, 100pM and 25pM within six-well plates, were incubated in EMEM medium containing radiolabelled oleates (^{14}C-Oleate) at 37^{o}C for two hours. The cells were harvested and the radiolabelled TG within the hepatocytes were extracted and assessed following the same protocol as previously described in section 2.2.4.

4.2.6 **Analysis of FA in total lipids of hepatic cell pellets**

The profile of fatty acids (FA) in the lipids of Hep G2 cells in different conditions of B12 [500nM (control), 1000pM, 100pM and 25pM] was analysed as previously described in section 2.2.7.

4.3 **Results**

4.3.1 Lipogenesis

4.3.1.1 **Effect of B12 on lipid droplets formation in hepatocytes**

To obtain the fundamental evidence of a B12 effect on hepatic lipid synthesis (lipogenesis), we observed lipid droplets in hepatocyte cell line using x40 objective of a light microscope, under different conditions of B12 [500nM (control), 1000pM, 100pM and 25pM] following the initial fixation and staining of cells with ORO. First, we observed more densely stained lipids in the cell-culture plates of hepatocytes under low B12 compared with the control which had Hep G2 cells that were only faintly coloured (Fig 26B). Observing images captured under the x40 objective, hepatocyte cell line had a high number of intensely stained lipid droplets under low B12 compared with the control B12 which had only few lightly stained lipids (Fig 26A). The above data were confirmed by our quantitative assessment, using the elution assay standardized by milligram protein concentration of hepatocyte cell line in respective wells of various B12 conditions, which indicated that significantly higher amounts of lipids were eluted from cells of low B12 compared with control (Fig 26C). These data gave preliminary evidence that low B12 significantly increased lipid levels in hepatocyte cell line compared with control, hence motivating the need for further investigations into the diverse pathways of lipid metabolism trying to account for this effect.

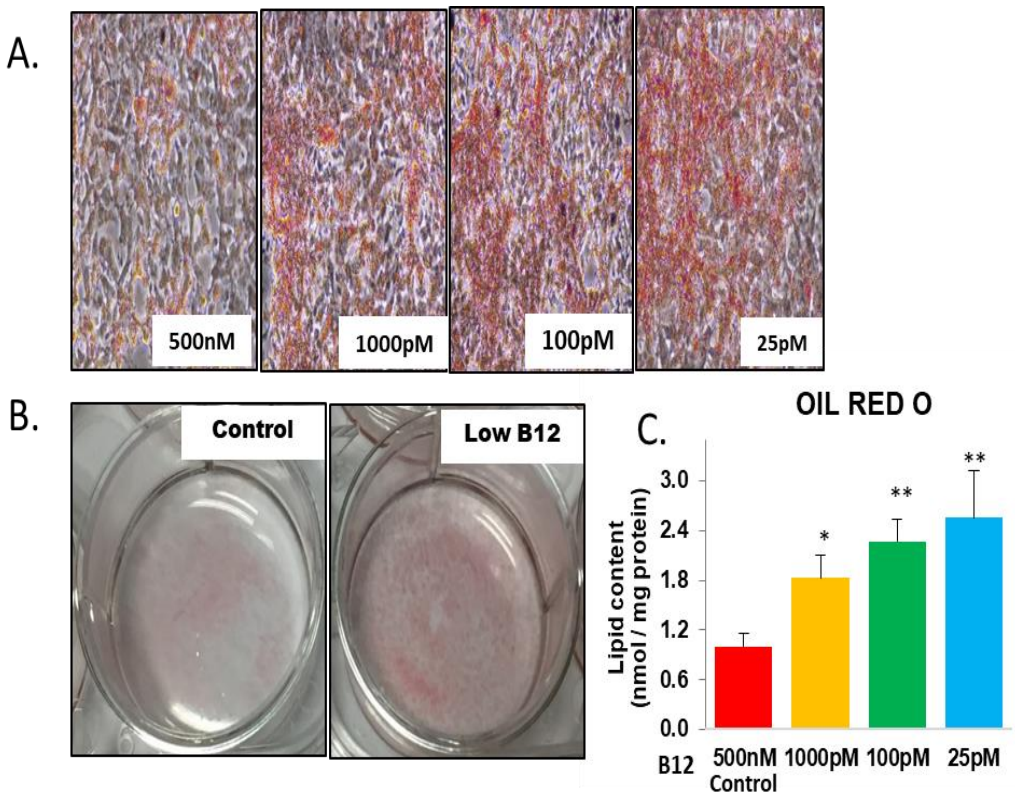

Figure 26: Oil Red O staining of lipid droplets in hepatocytes of different B12 conditions

Hep G2 cells were cultured in a custom-made eagle's minimal essential medium (EMEM) using EMEM powder (catalogue number 41500) from Thermo-Scientific, which contained 1000 mg/dL concentration of D-glucose. Lipids *de novo* synthesized in Hep G2 cells from metabolism (glycolysis) of the excessive glucose were visualized and quantified. Unaided visualization of cell culture plates of Hep G2 cells, following fixation and staining with formalin and ORO respectively, indicating more deeply stained lipids in the low B12 hepatocyte cell line compared with control (B). Images of Hep G2 captured under x40 objective showing an increasing intensity of ORO colouration of lipid droplets with decreasing concentrations of B12 [500nM (Control), 1000pM, 100pM and 25pM] (A). Spectrophotometric estimation of lipids eluted from hepatocyte cell line of different B12 [500nM (Control), 1000pM, 100pM and 25pM] conditions standardized per milligram protein showed higher amount of lipids in low B12 (25pM) compared with control (500nM) (C). The data are representative of mean ± SEM (n=6). * indicates significance compared with 500nM (control): * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$

4.3.1.2 **Effect of B12 on total intracellular TG levels in hepatocyte cell line**

To obtain more specific evidence on the effect of low B12 on hepatic lipogenesis, we assessed the total intracellular TG levels synthesized by hepatocyte cell line under various conditions of B12 normalized per milligram protein of cells. Low B12 hepatocyte cell line had significantly higher levels of total intracellular TG compared with control (Fig 27), thus confirming the earlier evidence obtained in ORO staining and elution assays.

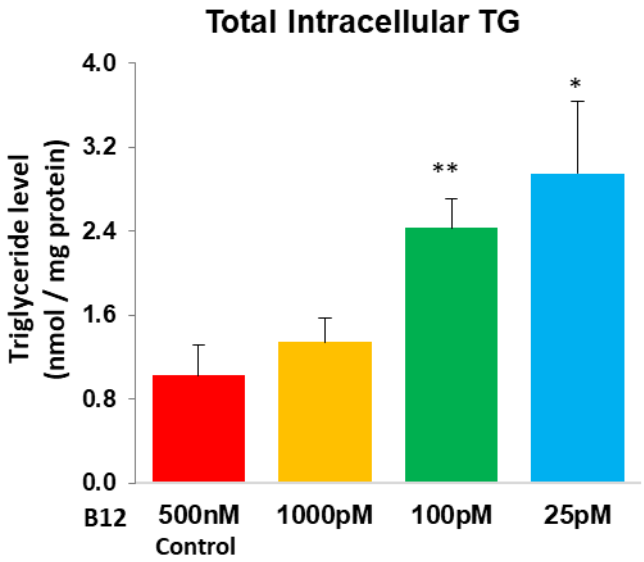

Figure 27: Effect of B12 deficiency on hepatic total intracellular triglyceride levels

Total intracellular levels of triglycerides were quantified in hepatocyte cell line under different B12 (500nM, 1000pM, 100pM and 25pM) conditions using the TG kit (ab65336) from Abcam Plc and normalized per milligram protein of Hep G2 under each B12 condition. The data are representative of mean ± SEM (n=6). * indicates significance compared with 500nM (control): * p< 0.05, ** p< 0.01, *** p< 0.001

4.3.1.3 **Effect of B12 on genes regulating FA synthesis**

Next, we assessed effect of B12 on gene expression of sterol regulatory element-binding protein (SREBF), which is a transcription factor and master regulator of the biosynthesis pathways of FAs, TG and cholesterol. Then we also assessed B12 effect on downstream genes regulating hepatic *de novo* FA synthesis (Fig 22 and Fig 24). Hepatic DNL uses substrates from diet or adipocyte lipolysis. Through a series of coordinated enzymatic reactions, the substrates are metabolized to pyruvate, which enters mitochondria to be used for energy production. When energy stores are adequate, citrate is transported out to cytoplasm and is converted to acetyl-CoA by ATP citrate lyase (ACLY). Acetyl-CoA carboxylase (ACC) converts acetyl-CoA to malonyl-CoA. Fatty acid synthase (FASN), the key rate-limiting enzyme in *de novo* FA synthesis, converts malonyl-CoA into palmitate, which is further elongated to stearate or longer FAs by the action of elongation of very long-chain fatty acids (ELOVL6). We observed that gene expression of SREBF1 and the genes in *de novo* FA synthesis such as ATP citrate lyase (ACLY), Acetyl-CoA carboxylase (ACC), Fatty acid synthase (FASN) and elongation of very long-chain fatty acids (ELOVL6) were increased in low B12 hepatocyte cell line compared with control, suggesting that hepatocyte cell line in low B12 could synthesize higher levels of *de novo* FAs (Fig 28).

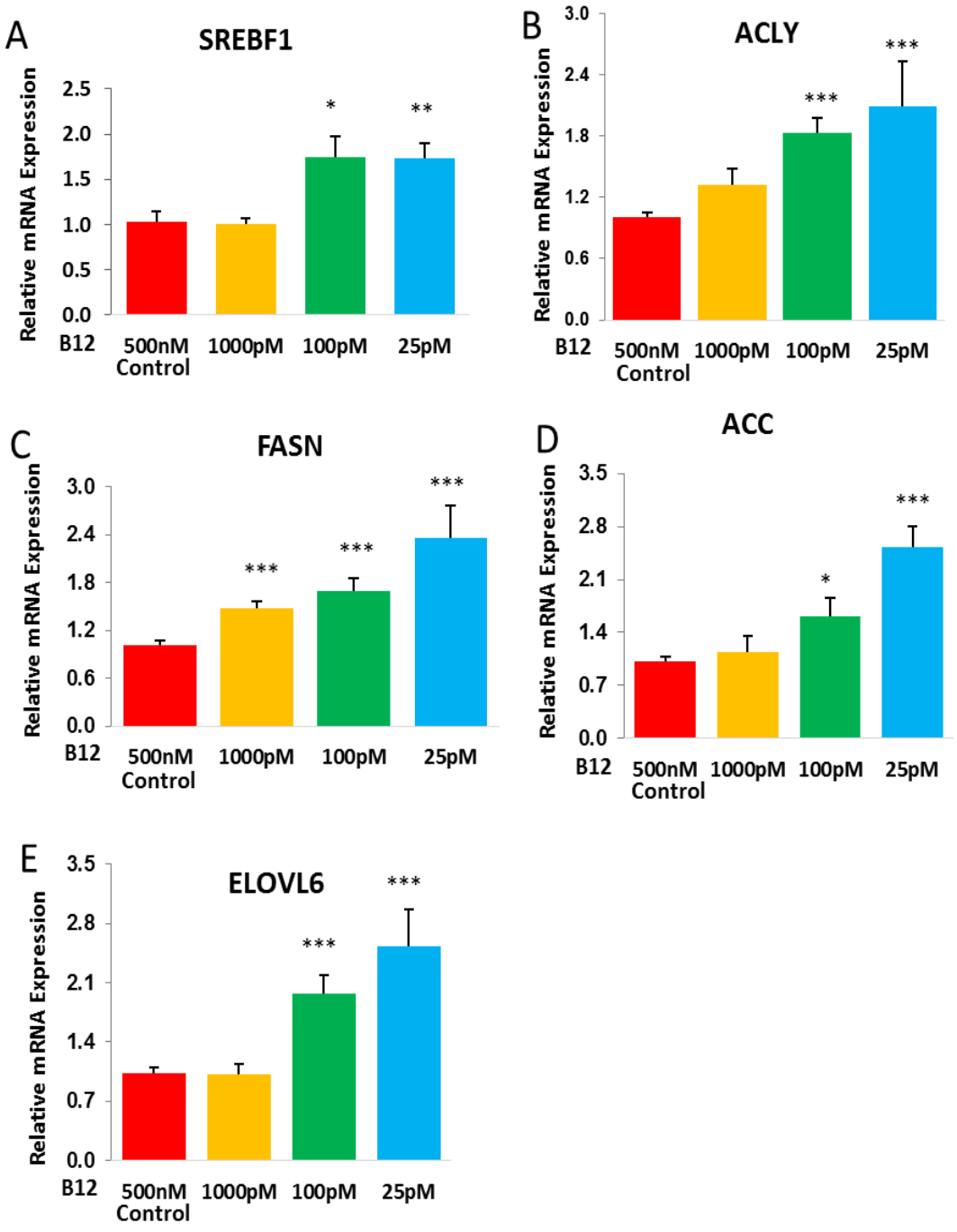

Figure 28: Low B12 increases mRNA expression of enzymes regulating hepatic *de novo* FA synthesis

The mRNA expression of enzymes regulating *de novo* FA synthesis [SREBF1 (A) ACLY (B) ACC (C) FASN (D)] as well as elongation [ELOVL6 (E)] normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data are representative of mean ± SEM (n=6). * indicates significance compared with 500nM (control): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

4.3.1.4 **Effect of B12 on genes regulating TG biosynthesis**

TG biosynthesis occurs by sequential esterification of FAs into glycerol-3-phosphate (G3P) and conversion of saturated FAs to monounsaturated FAs by Stearoyl-CoA desaturase 1 (SCD1). Glycerol-3-phosphate acyltransferase (GPAT) adds acyl-CoA to glycerol-3-phosphate (G3P) to form lysophosphatidicacid (LPA). Then 1-acylglycerol-3-phosphateacyltransferase (AGPAT) adds a second acyl-CoA to produce phosphatidic acid (PA), which is then dephosphorylated by Phosphatidate phosphatase (LPIN1) to form 1, 2-diacylglycerol (DAG). Finally, Diacylglycerol acyltransferase (DGAT) converts diacylglycerols into triglycerides (TG), which may be stored in the liver or assembled into VLDL and exported to circulate in the blood (Fig 23).

Since we initially observed higher total intracellular TGs in low B12 hepatocyte cell line, we further investigated the expression of genes involved in the TG biosynthesis pathway. The expression of genes such as Stearoyl-CoA desaturase 1 (SCD1), Glycerol-3-phosphate acyltransferase (GPAM), 1-acylglycerol-3-phosphateacyltransferase (AGPAT), phosphatidate phosphatase (LPIN1) and Diacylglycerol acyltransferase 2 (DGAT2) crucial in *de novo* TG biosynthesis including desaturation, monoacyl, diacyl and triacylglycerol synthesis, were increased in low B12 hepatocyte cell line compared with control. Similarly, gene expression of DGAT1 involved in TG recycling was increased in hepatocytes under low B12 compared with control. Therefore, these findings demonstrate that both *de novo* synthesis and regeneration of TGs were increased in hepatocyte cell line under low B12 compared with control (Fig 29).

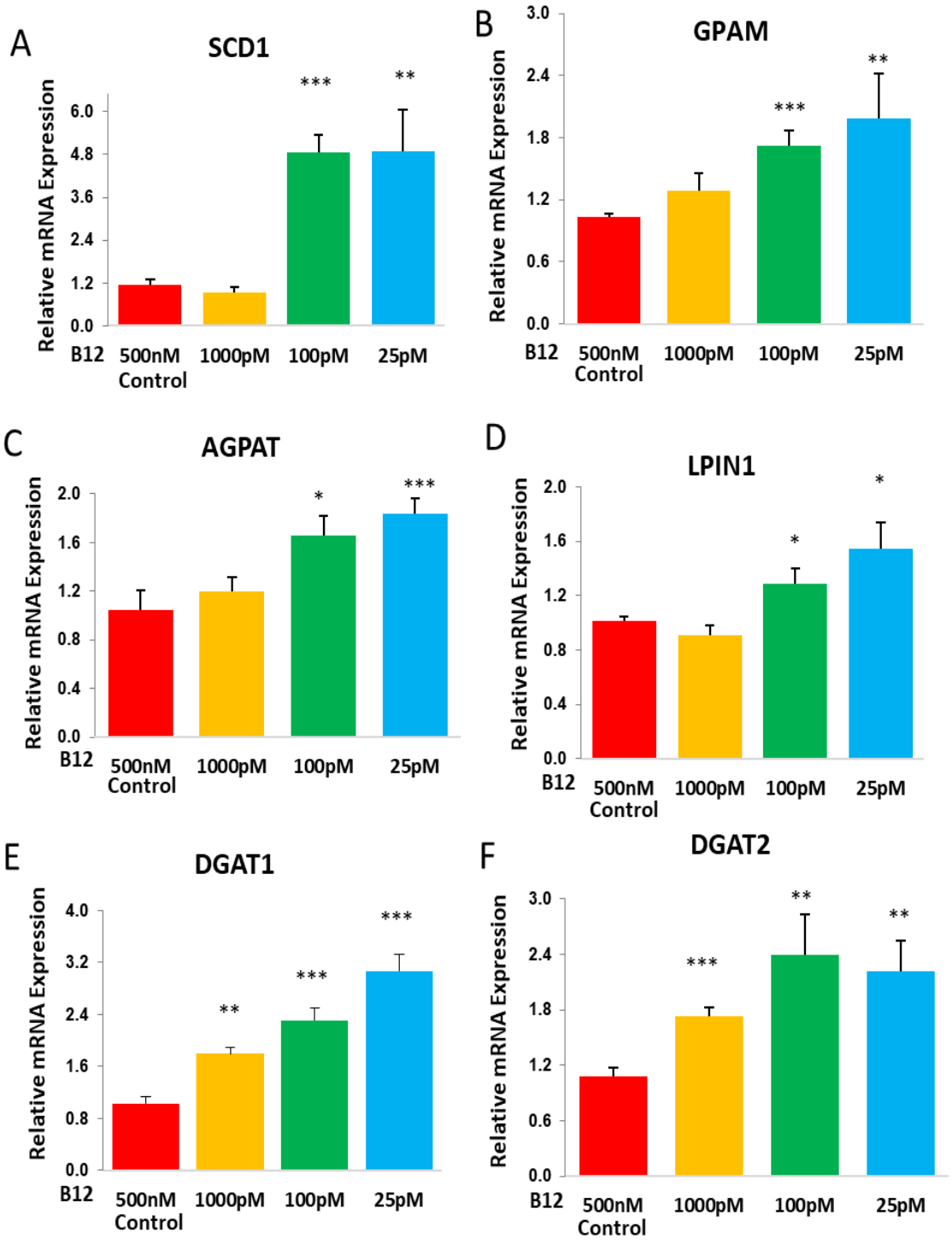

Figure 29: Low B12 increases mRNA expression of genes regulating TG biosynthesis

The mRNA expression of enzymes involved in the regulation of *de novo* TG biosynthesis such as [SCD (A), GPAM (B), AGPAT (C), LIPIN (D) and DGAT1 (F)] and or TG regeneration from residues of hydrolysed lipids by the enzyme DGAT1 in hepatocyte cell line normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data are representative of mean ± SEM (n=6). * indicates significance compared with 500nM (control): * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$

4.3.1.5 **Effect of B12 on TG synthesis utilizing radiolabelled FA**

To further confirm our observation of high total intracellular TGs and dysregulated genes involved in *de novo* TG synthesis and regeneration in hepatocytes under low B12 condition, we performed a real-time flux assay [349]. We incubated the hepatocytes with radiolabelled FA (^{14}C-Oleate) and FA transporter (L-carnitine) and assessed dysregulation of enzymes involved in TG synthesis pathway by investigating the amount of FA incorporation in the TG biosynthesis for a stipulated period. Interestingly, we observed high measure of radioactivity (disintegration per minute, DPM) by scintillation count in low B12 hepatocyte cell line following initial extraction and isolation of radiolabelled-TGs using glyceryl tripalmitate as standard. This provided a direct indication that increased number of FAs were incorporated and synthesised in hepatocytes treated with low B12 compared with control (Fig 30).

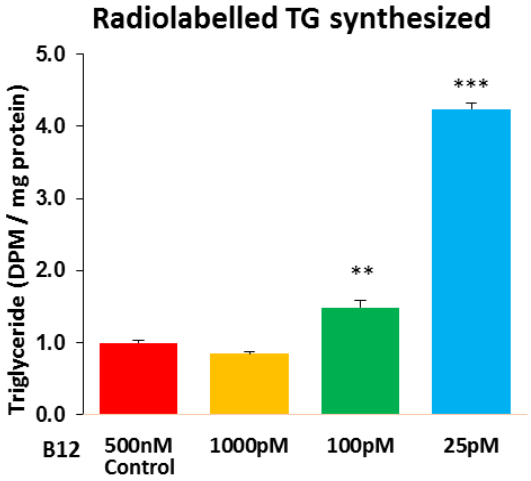

Figure 30: Low B12 increased biosynthesis of radiolabelled-TG in hepatocytes exposed to ^{14}C-oleate and L-carnitine: Flux assay was performed after seeding 75,000 Hep G2 cells in six-well plates under different conditions of B12 [500nM (control), 1000pM, 100pM and 25pM] until reaching confluence at the 10th day of the cell culture. After the last medium change, the cells were incubated with ^{14}C-Oleate (0.75mM concentration at 2000 dpm/nmol) and L-carnitine (1mM) to facilitate incorporation of ^{14}C-Oleate into hepatocytes for two (2) hours in EMEM, against five minutes incubation for background normalizing control at 37^{o}C and 5% CO_2 saturation. Hep G2 cells were harvested in 2ml methanol and the synthesized radiolabelled-TG was extracted, following an initial total lipid isolation with the liquid-liquid (chloroform-methanol, 2:1 v/v) extraction method, using a mobile phase such as hexane/diethyl ether/formic acid (v/v/v, 70/30/1) on a thin layer chromatography (TLC) plate with 10 nmol glyceryltripalmitate (tripalmitin) as a standard. TG bands on TLC plates were transferred into vials of 5ml scintillation fluid for scintillation counter assessment of radioactivity (for 5 minutes) of the synthesized TG fraction, normalized per milligram (mg) protein concentration by the Bradford method. The data are representative of mean ± SEM (n=6). * indicates significance compared with 500nM (control): * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$

4.3.1.6 **Effect of B12 on genes regulating cholesterol synthesis**

Dysregulation in FA and TG metabolism could affect cholesterol biosynthesis as the latter utilizes acetyl-CoA and VLDL which are derivatives of FA's (endogenous or exogenous) and TGs in the mitochondria and cytosol, respectively (Fig 24). Therefore, assessment of gene expression in the hepatic cholesterol biosynthesis pathway was performed to examine possible dysregulation resulting from B12 conditions. We observed an increased gene expression of the nuclear transcription factor LDLR (master regulator of cholesterol synthesis) in low B12 compared with control. Likewise, the expressions of key cholesterol genes such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS1) and Isopentenyl-Diphosphate delta Isomerase 1 (IDI1) were also increased in low B12 compared with control (Fig 31). This evidence therefore suggests that hepatocyte cell line in low B12 had higher levels of cholesterol compared with control.

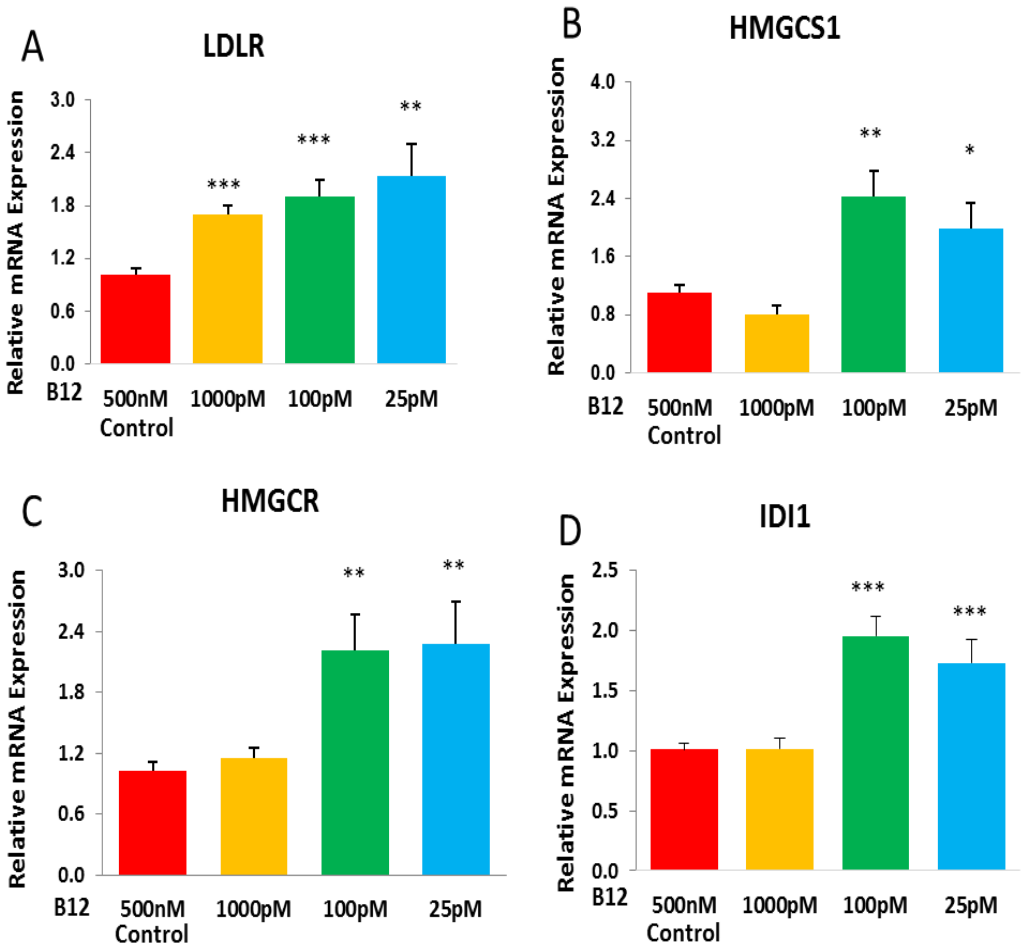

Figure 31: Low B12 upregulates genes in the cholesterol synthesis pathway

The mRNA expression of enzymes involved in the regulation of cholesterol biosynthesis such as LDLR (A) HMGCS1 (B), HMGCR (C) and IDI1 (D) in hepatocyte cell line normalized to 18S rRNA endogenous control

(Applied Biosystems, UK). The data are representative of mean ± SEM (n=6). * indicates significance compared with 500nM (control): * p< 0.05, ** p< 0.01, *** p< 0.001

4.3.2 Fatty acid (FA) profiling in hepatocytes

4.3.2.1 **Total FA**

We showed evidence of low B12-induced upregulation of genes involved in pathways leading to *de novo* synthesis, elongation and desaturation of certain FA. We further performed FA profiling in hepatocyte cell line under different conditions of B12 to assess how levels of different FA were affected in the hepatocyte cell line. We observed that the highest group of FAs synthesized in the hepatocyte cell line was saturated FA (SFA - 43%), then monounsaturated FA (MUFA - 32%), polyunsaturated n-6 FA (PUFA n-6 - 12%), polyunsaturated FA–n-3 (PUFA n-3 - 10%), long chain polyunsaturated n-9 FA (LC-PUFA n-9 - 0.9%), and trans FA (0.5%) [(Fig 32 (control)]. Interestingly, our data showed that the level of total FAs synthesized in hepatocytes in low B12 was 38% higher compared with control (Fig 33A).

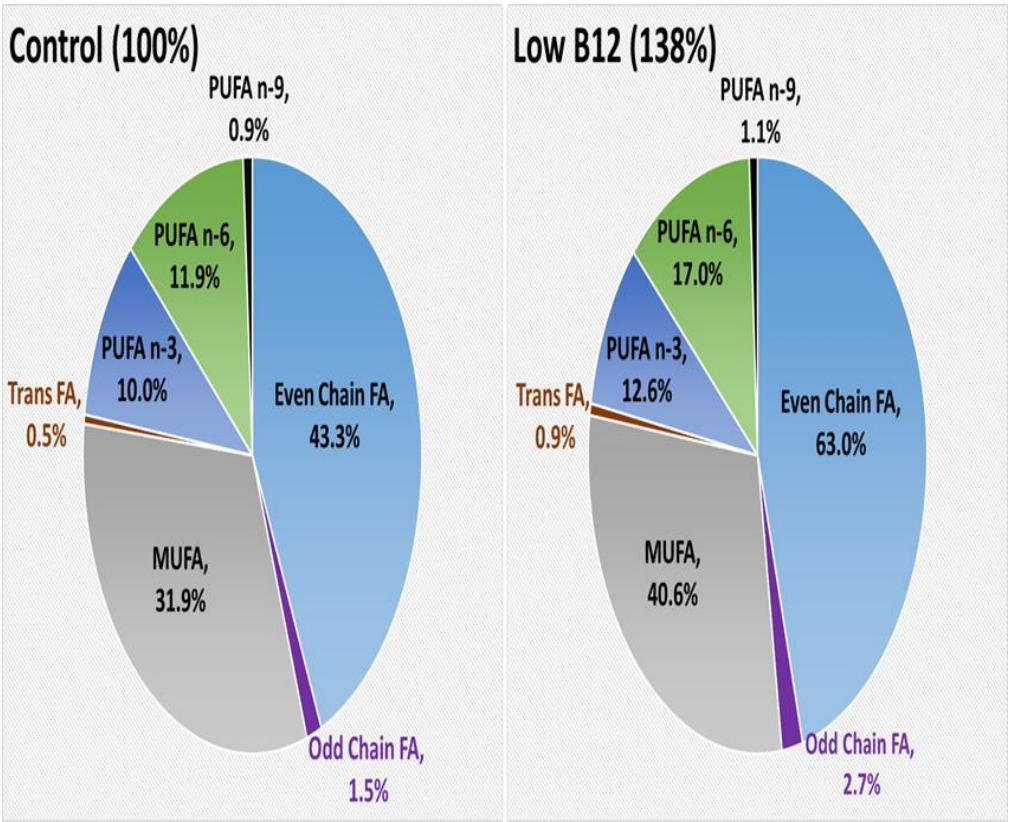

Figure 32: Profile of total FA groups in hepatocytes: The composition (%) of various FA groups in hepatic total FA levels: Even chain FA, Odd chain FA, MUFA, Trans FA, PUFAn-3, PUFAn-6 and PUFA n-9 in hepatocyte cell line under low B12 compared with control.

4.3.2.2 **Grouped fatty acids**

Saturated FAs (SFA)

Next, we assessed the level of diverse groups of FAs in hepatocyte cell line under different B12 conditions. We observed that the predominant FA group, the SFA, was significantly higher in low B12 compared with control (Fig 33.B).

Monounsaturated FAs (MUFAs)

We observed that MUFA levels in low B12 hepatocyte cell line were significantly higher compared with control (Fig 33. C). Since the FA profile was predominated by SFAs and MUFAs, higher levels of both groups observed in low B12 accounted for the overall increase in the total fatty acids (TFAs) under low B12 hepatocyte cell line compared with control.

Polyunsaturated fatty acids (PUFAs)

In PUFAs-total, there was no significant difference observed between low B12 and control however, sub-groups of PUFA such as PUFA n-6, LC-PUFA n-6, PUFA n-6/n-3 and Trans-FA were significantly higher in low B12 hepatocyte cell line than control (Fig 33. E, H, J). Moreover, in groups such as PUFA n-3 and LC-PUFA n-3, no significant difference was observed between low B12 and control (Fig 33, F, G).

4.3.2.3 **Individual FA profile in hepatocytes**

Even chain FAs

We observed that all even chain FA's such as lauric acid (C12), myristic acid (C14), palmitic acid (C16) and stearic acid (C18) comprising 43% of the total FA in the control were significantly increased to 63% in low B12 hepatocyte cell line (Fig 32).

Odd chain FAs

Similarly, odd chain FAs such as margaric acid (C17), heneicosylic acid (C21) and tricosylic acid (C23) comprising 1.5% of the total FA in the control were significantly increased to 2.7% in low B12 hepatocyte cell line (Fig 32).

MUFAs

Among the MUFAs, oleic acid (C18:1n-9) and cis-vaccenic acid (C18:1n-7) were present predominantly than other MUFAs such as hexadecanoic acid (C16:1n-9), palmitoleic acid (C16:1n-7) and gondoic acid (C20:1n-9). These MUFA's comprising 31.9% of the total FA in

the control were significantly increased to 40.6% in low B12 hepatocyte cell line (Fig 32). However, the only very long chain MUFA, gondoic acid was significantly decreased in low B12 hepatocyte cell line compared with control (Table 2).

Trans FAs

Among the trans FAs accounting for only 0.5% of total FAs in control, higher levels of palmitelaidic acid (C16:1t) and elaidic acid (C18:1t) were observed in low B12 hepatocyte cell line (which accounted for 0.9%). There was no significant difference in linoelaidic acid (all trans-9, 12) (C18:2tt) and linoelaidic acid (trans-12) C18:2ct (Table 2)

PUFAs

PUFA n-3 comprising 10% of the total FA in the control were significantly increased to 12.6% in low B12 hepatocyte cell ine, PUFA n-6 from 12% to 17% and PUFA n-9 from 0.9% to 1.1 (Fig 32). PUFAs n-3 (such as eicosapentaenoic acid (EPA) (C20:5 n-3), docosapentaenoic acid (DPA)(C22:5 n-3) and docosahexaenoic acid (DHA) (C22:6 n-3)), PUFAs n-6 (such as linoleic acid (C18:2 n-6), dihomo-γ-linolenic acid (C20:3n-6), arachidonic acid (AA) (C20:4 n-6) and docosapentaenoic acid (C22:5 n-6)) and PUFA n-9 such as mead acid (C20:3 n-9) were significantly higher in hepatocyte cell line with low B12. However, no significant difference was observed in some PUFAs n-3 (such as α-linolenic acid (C18:3n-3) and eicosatrienoic acid (C20:3n-3)) and PUFAs n-6 (such as adrenic acid (C22:4n-6)) (Table 2).

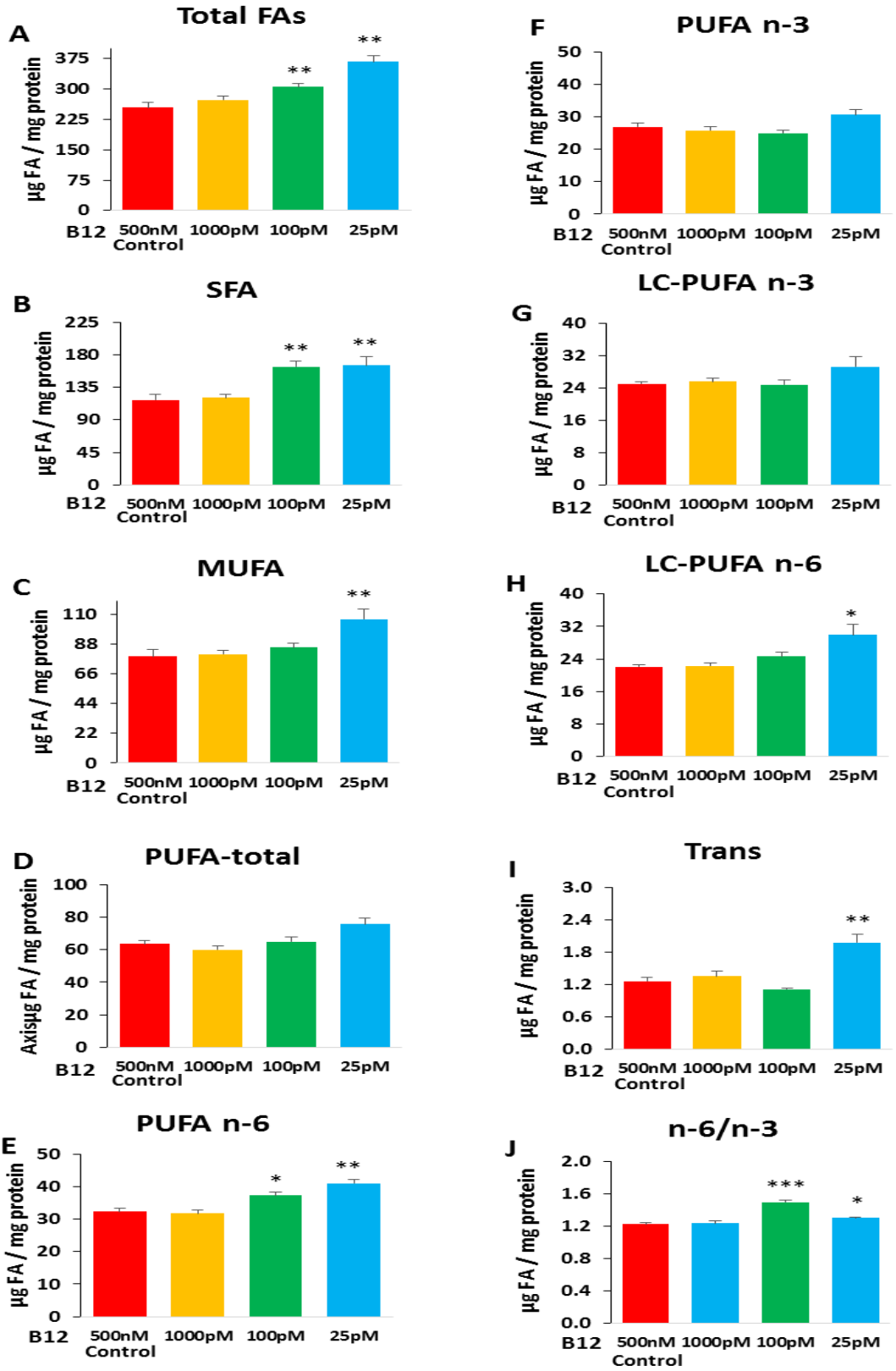

Figure 33: **Profile of various groups of fatty acids in hepatocytes under different conditions of B12**

The fatty acid profile of Hep G2 cells under different B12 conditions [500nM (Control), 1000pM, 100pM and 25pM] obtained in total lipids extracted from cell pellets of the hepatocyte cell line and analysed using gas chromatography in connection with SP-2560 capillary column (60m x 0.25mm id x 0.15mm) supplied by Supelco,

Sigma Aldrich, St Louis, MO and FID system for detection. Fatty acid levels (µg) normalized per milligram protein showing various fatty acid groups such as total fatty acid (A), saturated fatty acids (SFA) (B), monounsaturated fatty acids (MUFA) (C), total polyunsaturated fatty acids (PUFA-total) (D), polyunsaturated n-6 fatty acids (PUFAn-6) (E), polyunsaturated n-3 fatty acids (PUFAn-3) (F), long chain polyunsaturated n-3 fatty acids (G), long chain polyunsaturated n-6 fatty acids (LC-PUFAn-6) (H), Trans fatty acids (I) and polyunsaturated fatty acids n-6/n-3 ratio (J). The data are representative of mean ± SEM (n=6). * indicates significance compared with 500nM (control): * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$

Fatty acids µg FA/mg protein	Vitamin B12 500nM (Control) (n=6)	1000pM (n=6)	100pM (n=6)	25pM (n=6)
Even chain				
C12	**0.00** ± 0.0	**0.00** ± 0.0	**0.93** ± 0.3***	**0.61** ± 0.1***
C14	**3.73** ± 0.7	**3.86** ± 0.3	**4.82** ± 0.4**	**5.71** ± 1.1**
C16	**75.47** ±12.3	**77.65** ± 7.8	**102.85** ± 11.3**	**111.72** ± 15.8**
C18	**34.76** ± 5.9	**35.07** ± 3.7	**47.53** ± 5.0**	**47.74** ± 9.0*
Odd Chain				
C17	**1.97** ± 0.4	**2.01** ± 0.2	**3.05** ± 0.5**	**3.43** ± 1.2*
C21	**0.66** ± 0.1	**0.70** ± 0.2	**0.81** ± 0.2	**1.45** ± 0.4**
C23	**1.20** ± 0.2	**1.29** ± 0.1	**2.38** ± 0.3***	**2.34** ± 0.7**
MFA				
C16:1 n-9	**3.47** ± 0.6	**3.70** ± 0.5	**3.9** ± 0.5	**5.00** ± 1.0**
C16:1 n-7	**7.95** ± 0.7	**8.36** ± 1.2	**8.39** ± 0.8	**10.27** ± 1.7*
C18:1 n-9	**47.66** ± 2.3	**47.21** ± 5.3	**47.20** ± 5.0	**61.04** ± 10.6*
C18:1 n-7	**23.01** ± 1.3	**23.66** ± 2.9	**23.54** ± 2.2	**29.94** ± 5.2*
C20:1 n-9	**1.80** ± 0.4	**2.20** ± 1.0	**0.55** ± 0.6**	**0.53** ± 0.1***
Trans FA				
C16:1t	**0.33** ± 0.1	**0.47** ± 0.1	**0.6** ± 0.0***	**0.99** ± 0.4**
C18:1t	**0.32** ± 0.0	**0.38** ± 0.1	**0.00** ± 0.0***	**0.54** ± 0.1**
C18:2tt	**0.00** ± 0.0	**0.00** ± 0.0	**0.00** ± 0.0	**0.17** ± 0.3
C18:2ct	**0.66** ± 0.1	**0.53** ± 0.1	**0.49** ± 0.01	**0.63** ± 0.1
PUFA n-6				
C18:2 n-6	**10.31** ± 1.1	**10.47** ± 0.9	**12.75** ± 1.5**	**14.4** ± 2.4**
C20:3n-6	**3.03** ± 0.5	**3.17** ± 0.4	**3.24** ± 0.4	**4.27** ± 1.1*
C20:4 n-6	**17.72** ± 2.6	**18.07** ± 2.0	**21.17** ± 2.7*	**25.09** ± 5.1**
C22:4 n-6	**0.00** ± 0.0	**0.00** ± 0.0	**0.00** ± 0.0	**0.25** ± 0.3
C22:5 n-6	**0.29** ± 0.0	**0.29** ± 0.0	**0.49** ± 0.04**	**0.58** ± 0.2*
PUFA n-9				
C20:3 n-9	**2.32** ± 0.3	**2.41** ± 0.3	**2.88** ± 0.4*	**2.86** ± 0.4*
PUFA n-3				
C18:3 n-3	**2.32** ± 0.1	**2.16** ± 0.1	**1.90** ± 0.2	**2.93** ± 0.5
C20:3 n-3	**0.43** ± 0.1	**0.46** ± 0.0	**0.43** ± 0.0	**0.67** ± 0.4
C20:5 n-3	**3.66** ± 0.6	**3.98** ± 0.5	**3.92** ± 0.5	**5.04** ± 1.1*
C22:5 n-3	**2.5** ± 0.5	**2.97** ± 0.9	**2.97** ± 0.5	**3.45** ± 0.7*
C22:6 n-3	**17.41** ± 1.3	**17.83** ± 1.8	**17.87** ± 1.7	**21.00** ± 2.9*

Table 2: **Profile of individual fatty acids in hepatocytes under different B12 conditions**

Fatty acid levels (µg) normalized per milligram protein of hepatocyte cell line under different B12 conditions [500nM (control), 1000pM, 100pM and 25pM]. The data are representative of mean ± SEM (n=6). * indicates significance compared with 500nM (control): * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$

4.4 **Discussion**

Here we show that B12 deficiency in hepatocyte cell line influences increased gene expression of lipogenesis and decreased lipid oxidation, resulting in increased intracellular TG and accumulation of more lipid droplets. In addition, we also observed subclasses of FAs such as SFAs, MUFAs, PUFA n-6, LC-PUFA n-6 and trans-FAs were also significantly higher in low B12 hepatocyte cell ine. Hence our findings indicate that increased fatty acid synthesis in low B12 condition and subsequent impairment of fatty acid oxidation in catabolising FAs in hepatocyte cell ine might likely lead FAs to accumulate in hepatocytes in low B12 resulting in dyslipidaemia.

Hepatocytes demonstrate an elevated turnover of FAs, which is immediately converted to TG and are stored in lipid droplets [350]. In the current study, Hep G2 cells were supplied with excess glucose concentration (1000 mg/dL), beyond the normal circulating levels (63.0-99.0 mg/dL) [351], in the media. This was to facilitate the utilization of the excess products from glycolysis (pyruvate) for *de novo* lipid synthesis. The lipids synthesized were stored in the hepatocyte cell line as lipid droplets. We observed that lipid droplets were higher in Hep G2 cells in low B12 and subsequent increase in total intracellular TGs, radiolabelled-FA uptake for TG synthesis in low B12 compared with control. This evidence was similar to the observation of elevated accumulation of lipid droplets, increased levels of TG [334] and cholesterol in adipocytes differentiated in deficient B12 condition [334]. Early studies have hypothesized that lipid droplets (LDs) are much sensitive to conditions such as nutritional stress capable of inducing unique alterations in the lipidome of LDs present in mice hepatocytes [350]. Lipid droplets resident in hepatocytes may demonstrate variations in numbers and sizes which are reflective of the precise state of the pathological and or physiological condition of an individual [350]. Accumulation of LDs underlies and defines steatosis of the liver [352], therefore implying that our observation of low B12-induced hepatic accumulation of LDs may be associated with the pathogenesis of hepatic steatosis. We also observed that total intracellular TGs and radiolabelled-FA uptake for TG synthesis in hepatocyte cell line were increased in low B12. Clinical evidences have showed that serum B12 negatively correlates with triglyceride [196, 333, 353] and VLDL levels in Indian subjects [354, 355]. In an animal study, severe B12 deficiency was associated with higher triglyceride levels compared with control which resulted in adverse pregnancy outcome [356]. These findings therefore confirm that in hepatocytes B12 deficiency causes increased accumulation of LDs.

Furthermore, we observed that gene expression of the master regulator of lipogenesis (SREBF1) and cholesterol (LDLR), and downstream genes regulating synthesis of FAs (ACLY, ACC, FASN, ELOVL6), TGs (SCD, GPAM, AGPAT, LPIN1, DGAT1 and DGAT2) and cholesterol (HMGCR, HMGCS1 and IDI1) was increased in low B12 hepatocyte cell line. In a previous study, hepatic transcription factors: SREBP1c, liver X receptor (LXRα) and retinoid X receptor (RXRα) in Wistar rat offspring, were differentially regulated by B12 restriction [357]. SREBP1c was high but not significant in B12 deficient rats compared with control [357]. However, significantly higher expression of SREBF1 and LDLR were observed in human adipocytes with low B12 levels [193] and increased level of SREBP-1c in the fatty livers of ob /ob mice, [358, 359]. Similar studies have also shown increased expression of genes involved in biosynthesis of FA, TG and cholesterol in animals [336] and human adipocytes [189]. Three separate clinical studies involving women at (a) child-bearing period but non-pregnant (b) early pregnancy and (c) at the time of delivery showed associations between low B12 and higher LDL cholesterol, total cholesterol and ratio of cholesterol-to-HDL [193]. It was further revealed in these studies that, low B12-induced elevation in cholesterolgenesis and lipogenesis may result from epigenetic modulations *via* hypomethylation of SREBF and LDLR [360] and micro RNAs targeting PPARg and IR [189], respectively. Generally, DNL in humans is reported to be responsible for about 26% of total TG levels in the liver and contributes to the incidence of hepatic steatosis and NAFLD [361]. Therefore, our findings may suggest that increase in hepatic DNL resulting from low B12 may be associated with the risk of developing NAFLD.

A particularly interesting finding in the present study was that FA profile was altered in hepatocyte cell line with low B12. Total FA, subclasses of FAs (SFAs, MUFAs, PUFA n-6, LC-PUFA n-6 and trans-FAs) and individual FAs (saturated even chains (C12, C14, C16 and C18), odd chains (C17, C21 and C23) and MUFA (C18:1n-9, n-7and C16:1n-9, n-7) were higher in hepatocyte cell line in low B12 compared with control. A prospective and longitudinal study showed that circulating even chain SFA's in early pregnancy (10week gestation) was associated with glucose metabolism, cardio-metabolic markers and subsequent GDM risk [362]. Another study in pregnant women at delivery showed that grouped FAs (SFA, MUFA and PUFA) were elevated in GDM women and showed a graded trend between the severity of maternal hyperglycaemia and serum FA composition [363]. Similarly, Chen *et al,* have showed that SFA - myristic acid was inversely associated with IR and inflammation [364]. Several studies have also shown evidence of association of SFAs [365], MUFAs [366] and trans-FAs [367, 368] with subclinical inflammation, increased risk of T2DM and CVD. Adolescent

females presenting with slightly lower levels of active B12 (holo-TC) than males, had significantly higher plasma levels of SFA, MUFA and trans-fatty acids and showed association with B12 [367]. The same study found a positive correlation between serum B12 and n-3 PUFAs, and a negative association with n-6 PUFAs and PUFA n-6/n-3 ratio [367]. Similar relationship in liver tissues of rat offspring was shown between B12 and PUFAs n-3 (omega-3) and PUFA n-6 (Omega-6) [345]. Likewise, we observed higher levels of individual n-3 and n-6 fatty acids, total PUFA n-6 (PUFAn-6 and LC-PUFAn-6) and PUFA n-6/n-3 ratio in low B12 hepatocyte cell line. Higher n-6 /n-3 ratio is associated with dysregulation in hepatic metabolism of lipids [369] and the risk of developing NAFLD [370]. Finally, higher levels of individual FAs such as myristic acid (C14:0), palmitate (C16:0), palmitoleate (C16:1), stearate (C18:0), oleate (C18:1), as shown in this study, were supported by an animal study involving liver tissues of B12-deficient rat [371]. In support to this, placental tissues and plasma from GDM women and NAFLD patients [372, 373] showed similar observation. Our data in agreement with these evidences indicate that B12 deficiency may alter the FA composition and may lead to dyslipidaemia and increased IR. Furthermore, a recent study in adolescents showed that an anti-obesity program comprising calorie restricted diet, increased physical activity, psychological therapy and nutritional education on adolescents modified plasma FA, reduced adiposity and improved cardiometabolic markers [374]. This indicates that similar programs with relevance to B12 supplementation should be encouraged in young women and women planning for pregnancy where B12 deficiency is common to reduce the burden of metabolic disorders including obesity, GDM, T2DM and CVD.

4.5 **Conclusion**:

In summary, our study provided novel evidence that B12 deficiency in hepatocyte cell line accumulated more lipids, intracellular TG and increased uptake of FA under the influence of increased FA synthesis. The nuclear transcription factors, SREBF1 and LDLR, that regulate the genes involved in the biosynthesis of FA, TG and cholesterol pathways were also significantly increased in hepatocyte cell line due to B12 deficiency. Interestingly, we found that there was alteration in the FA profile especially the abundant subclasses of SFA and MUFA. Our evidence may therefore suggest that low B12 dysregulates lipid synthesis in hepatocytes and may predispose to NAFLD and dyslipidaemia. Future studies elucidating possible epigenetic mechanisms and clinical studies endorsing its relevance to B12 deficiency are warranted.

5.0 Vitamin B12 deficiency reduces oxidation of fatty acids and compromises mitochondrial functional integrity

5.1 Introduction

Obesity increases the risk of developing metabolic disorders including IR, CVD and T2DM, therefore, compromising the health of adults and children globally [320]. Failure of excessive fat storage in the adipose tissues accounts for inappropriate build-up in other tissues including the liver [375]. Within hepatocytes, the entry of medium and short-chain FAs (MCFAs and SCFAs) into the mitochondria for oxidation may occur freely without activation [376]. However, enzymes including the carnitine palmitoyltransferase (CPT) system which comprises CPT1, CPT2 and carnitine acylcarnitine translocase (CACT) may be required to facilitate the entry of long-chain FAs (LCFAs) into the mitochondria [377, 378] for beta oxidation (Fig 34). Moreover, CPT1 (the rate limiting enzyme) may be inhibited due to changes in levels of methyl malonyl-CoA [378] regulated by vitamin B12 (B12) [379].

It is hypothesized that promotion of fatty acid oxidation (FAO) is a probable mechanism to alleviate the risk of developing resistance to insulin [375, 380]. Also, growing evidence supports the hypothesis that white adipose tissue (WAT) oxidation of fats contributes significantly towards the maintenance of metabolic homeostasis [381-383]. FAO is further shown to be inversely associated with *de novo* lipogenesis [376]. However, the rate of FAO in adipocytes is relatively minimal compared with other tissues such as liver and muscles [384, 385]. Some clinical studies have shown evidence of association between low B12 and higher obesity/adiposity in pregnancy among the British [314] and Indian [386] populations. Similar evidence was also shown in pre-clinical studies involving animal [336] and cell line [360] models. Moreover, the potential role of B12 in limiting tissue accumulation of long chain FAs in humans through CPT1 regulation in the FAO pathway has been reported [371, 387]. In a study involving rat model, impairment in the oxidation of FAs resulting from deficiency in methyl donors (B12 and folate) was demonstrated in liver tissues [327]. Clinically, systemic deficiency of carnitine during pregnancy was shown to be associated with CVDs [388]. Also, increasing evidence suggests that dysfunction in the mitochondria of the liver is associated with lipid accumulation underlying the incidence of non-alcoholic fatty liver disease (NAFLD) [389].

We initially showed evidence of increased lipogenesis resulting from B12 deficiency in human adipose tissues [360] and currently in hepatocytes, including increased levels of total,

saturated and monounsaturated FAs (TFA, SFA and MUFA). Higher levels of long FAs including palmitate (C16:0), stearate (C18:0) and oleate (18:1) were also observed in hepatocytes as a result of low B12.

Since mitochondrial oxidation of FAs is crucial in limiting the accumulation of lipids in the liver, we therefore assessed the effect of B12 deficiency on the FAO pathway as well as mitochondrial functional potency in the utilization of long chain FA for ATP metabolism under various conditions of B12.

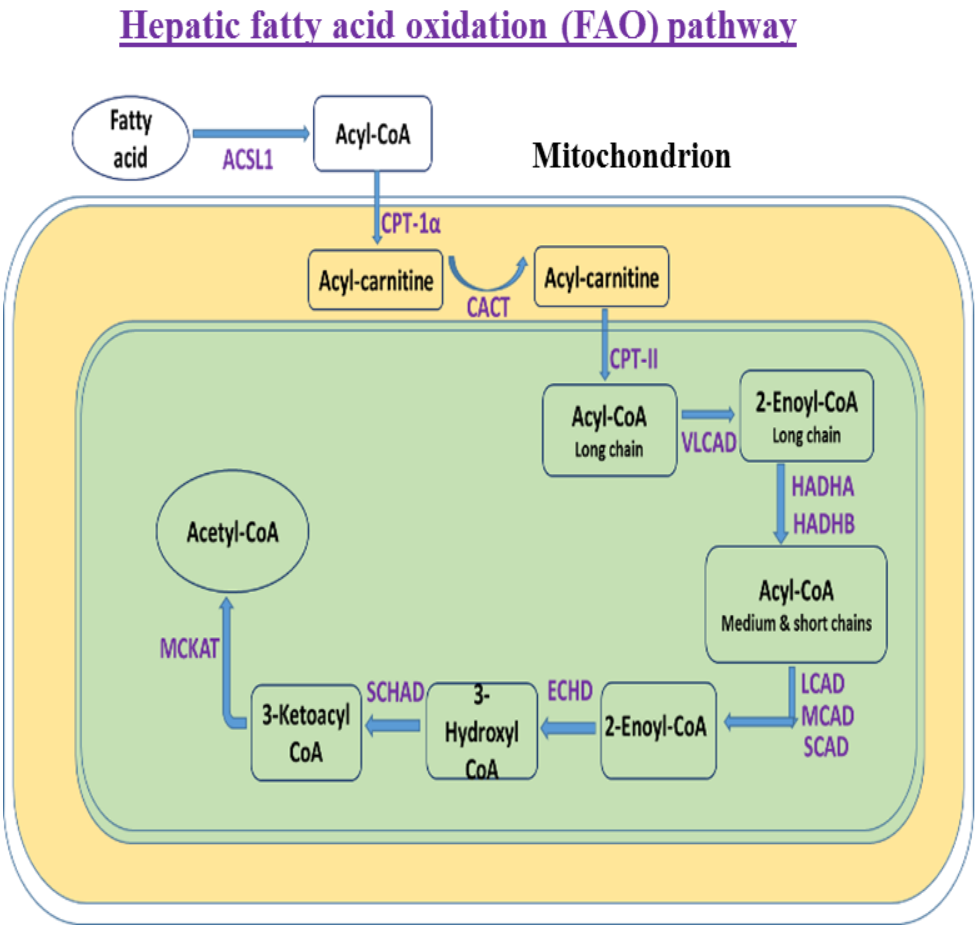

Figure 34: Fatty acid oxidation pathway: Long chain FAs derived from lipolysis of TGs and *de novo* FA synthesis may be utilized in the beta oxidation of FA pathway in the mitochondria to maintain levels of energy (ATP) for cellular metabolism. In the process, long chain fatty acyl CoA is synthesized from FA by the enzyme fatty acyl CoA synthase (ACSL1) and transported from the cytosol across the outer and inner mitochondrial membranes with the help of carnitine palmitoyl transferase 1α (CPT1α) and CPT II respectively through acyl carnitine intermediate modulated by acyl carnitine acyl translocase (CACT). Long chain fatty acyl CoA is sequentially shortened by cleavage of two-carbon units until generation of only two-carbon compounds, acetyl CoAs, by various enzymes long chain Acyl–CoA dehydrogenase (LCAD), medium chain Acyl–CoA

dehydrogenase (MCAD), short chain Acyl–CoA dehydrogenase (SCAD) and long-chain 3-hydroxyacyl-CoA dehydrogenase alpha (HADHA). Acetyl CoA is finally utilized in the Krebs or TCA cycle for ATP generation

5.2 **Methods**

5.2.1 **Beta-oxidation of fatty acid assessment**:

5.2.1.1 **Cell culture for gene expression assay**: The culture of Hep G2 cells in four different concentrations of B12: 500nM (Control), 1000pM, 100pM and 25pM, was done as described in sections 2.2.1.5 – 2.2.1.6.

5.2.1.2 **Gene expression assay**:

Total RNA isolation, cDNA synthesis and gene expression assays were same as previously described in sections 2.2.2.1-2.2.2.4, for the four conditions of B12 (500nM, 1000pM, 100pM and 25pM).

5.2.2 **Mitochondrial dysfunction assessment using seahorse XF24 extracellular flux assay**

Hep G2 cell culture for seahorse assay was done as described in section 2.2.8.

However, optimization for the desired final (1x) concentrations of the respiratory inhibitors oligomycin, FCCP and rotenone / antimycin was done by testing a range of different inhibitor concentrations: Oligomycin (0.2µM, 0.4µM, 0.6µM, 0.8µM and 1.0µM), FCCP (0.1µM, 0.3µM, 0.5µM, 1.0µM, 3.0µM and 5.0µM) and rotenone /antimycin (0.1µM, 0.2µM, 0.4µM, 0.6µM, 0.8µM and 1.0µM) (Fig 35).

Likewise, optimization for the cell density was done by seeding Hep G2 cells at different densities comprising 10 000 (10K), 20 000 (20K), 30 000 (30K) and 50 000 cells (50K) / well of seahorse XF24 plate. In the presence of respiratory inhibitors (oligomycin, FCCP and rotenone/antimycin), the highest maximal respiratory capacity, estimated by the oxygen consumption rate (OCR) in hepatocyte cell line, was observed in the 50K cell density with the lowest seen in the 10K cells (Fig 36).

We also optimized for the more efficient substrate medium, between Krebs-Henseleit Buffer (KHB) and seahorse XF buffer, accounting for higher spare respiratory capacity (SRC) in hepatocytes (Fig 37). Therefore, following optimization of assays, 50 000 cell density per well of seahorse XF24 plate, KHB medium and inhibitor final (1x) concentrations of 0.40 µM oligomycin, 0.75 µM FCCP and 0.40 µM rotenone/antimycin were used for the experiment.

Assessment of mitochondrial functional competence in condition of B12 was done using same protocol as described in section 2.2.8 of chapter 2.

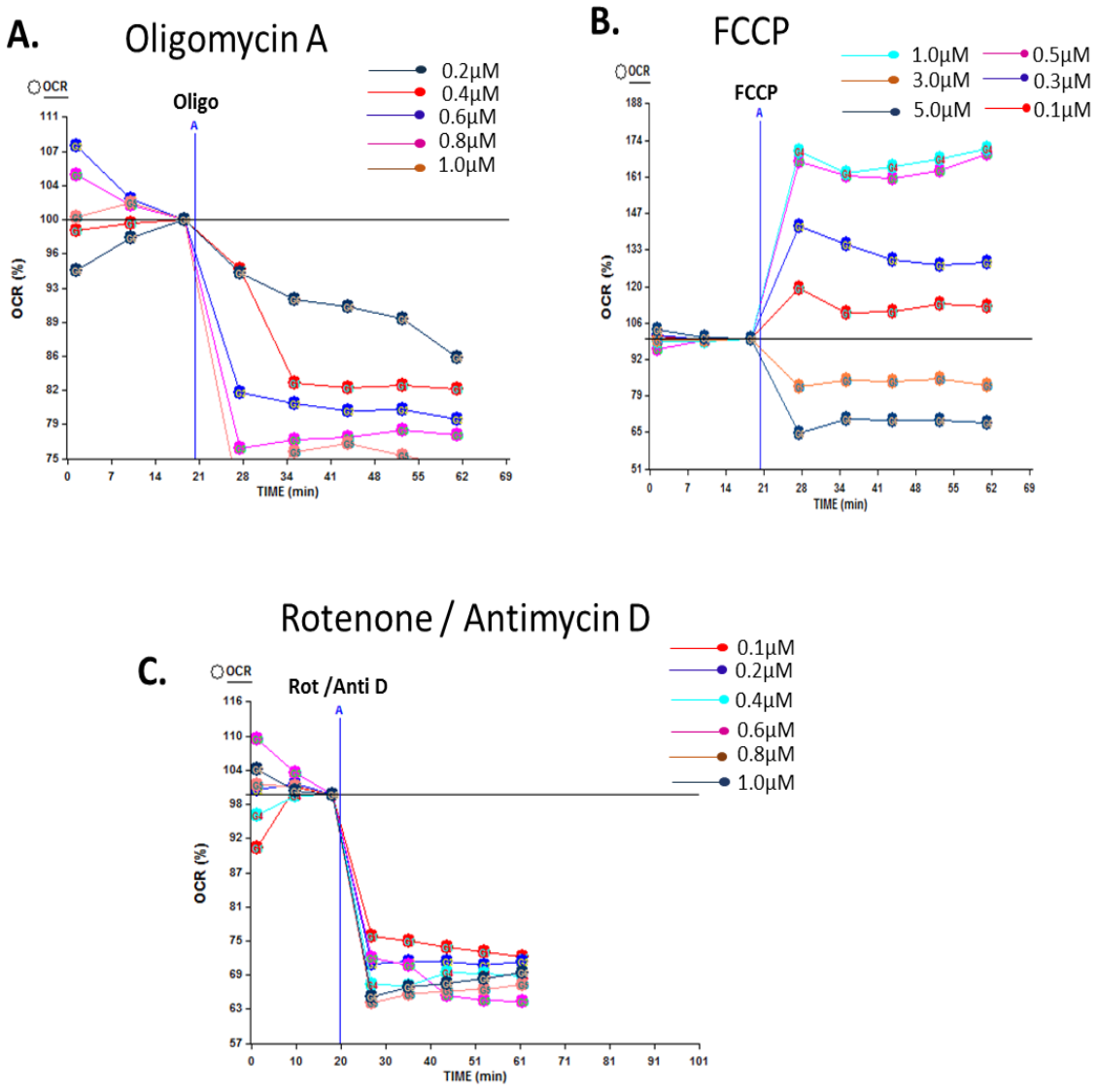

Figure 35: **Optimisation for concentrations of inhibitors for seahorse assay**: Oxygen consumption rate (OCR) of hepatocyte cell line (50K cells/well) obtained following injection of different concentrations of inhibitors such as oligomycin (A), FCCP (B) and rotenone / antimycin D (C) after basal respiration measurements.

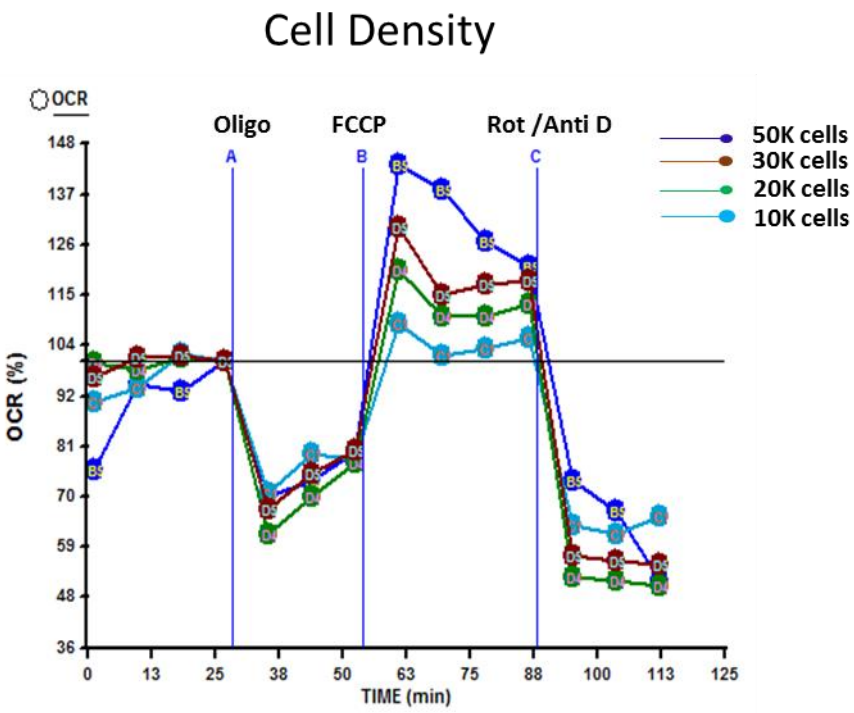

Figure 36: Optimisation of Hep G2 cell density for seahorse flux assay: Oxygen consumption rate (OCR) for different densities of Hep G2 (10K, 20K, 30K and 50K cells) following the sequential injection of different inhibitors Oligomycin, FCCP and rotenone/ antimycin after an initial basal respiration measurement.

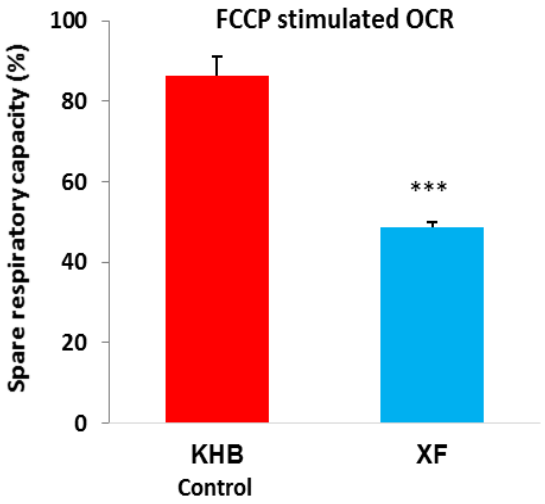

Figure 37: **Optimisation for the desired seahorse media**: Spare respiratory capacity (%) of hepatocyte cell line incubated in two different seahorse buffers such as KHB and XF-buffer. The data is representative of mean ± SEM (n=6), and * indicates significance compared with control; * p< 0.05, ** p< 0.01, *** p< 0.001

5.3 RESULTS

5.3.1 Effect of B12 on the expression of genes regulating the FAO pathway

Free long chain-FAs are activated and transported across the mitochondrial membranes by the enzymes: acyl-CoA synthetase (ACSL), carnitine palmitoyl transferase 1 (CPT1), carnitine acyl carnitine translocase (CACT) and carnitine palmitoyl transferase 2 (CPT2). Subsequently, consecutive processes involving dehydrogenation, hydration, oxidation and thiolytic cleavage to finally generate acetyl-CoA (two-carbon units), are crucial in the beta-oxidation (FAO) pathway under the regulation of the enzymes such as acyl-CoA dehydrogenase long chain (ACADL), acyl-CoA dehydrogenase medium chain (ACADM), acyl-CoA dehydrogenase short chain (ACADS) and hydroxyacyl-CoA dehydrogenase trifunctional multi enzyme complex subunits beta and alpha (HADHB and HADHA).

B12 deficiency is associated with elevation in the levels of methyl malonic acid (MMA) resulting from the inefficient conversion of methyl malonyl-CoA to succinyl CoA during propionate metabolism by the enzyme methyl malonic CoA mutase (MCM) in the mitochondria. MMA is however known to be a potent inhibitor of CPT1 [390], the rate limiting enzyme in the FAO pathway. Since the lipid metabolism is co-ordinated by an intricate balance of FA synthesis and fatty acid oxidation (FAO), we assessed the effect of B12 deficiency on CPT1α and the downstream genes involved in FAO in the mitochondria.

We observed that, the genes regulating oxidation of FAs: carnitine palmitoyl transferase 1α (CPT1α), carnitine acyl carnitine translocase (CACT), acyl-CoA synthase (ACSL1), acyl-CoA dehydrogenase long chain (ACADL), acyl-CoA dehydrogenase medium chain (ACADM), acyl-CoA dehydrogenase short chain (ACADS) and hydroxyacyl-CoA dehydrogenase trifunctional multi enzyme complex subunits beta and alpha (HADHB and HADHA), were significantly decreased in low B12 hepatocyte cell line compared with control. This suggests that FAO may be significantly impaired in hepatocyte cell line in low B12 compared with control (Fig 38).

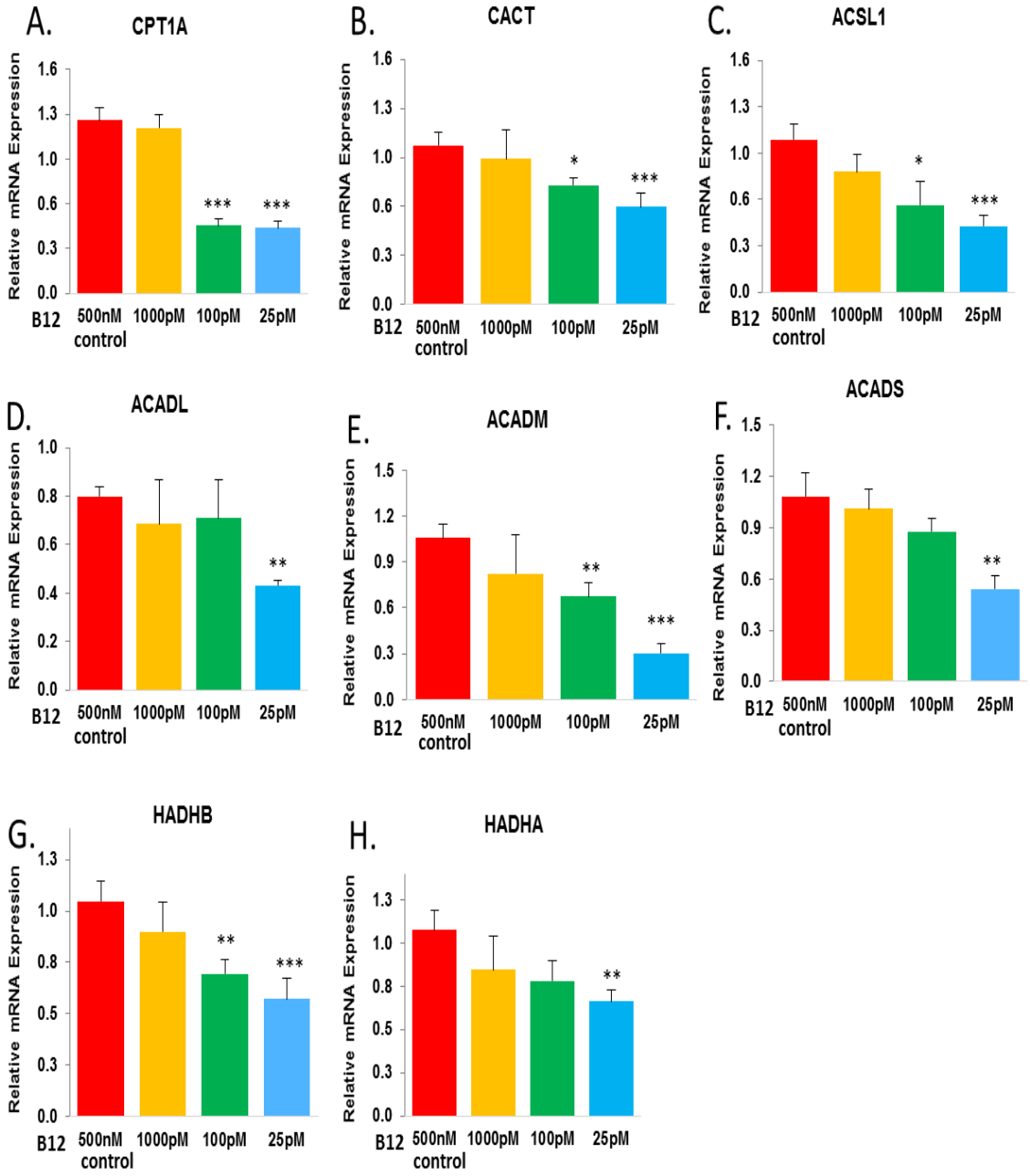

Figure 38: Low B12 downregulates gene expression of enzymes involved in fatty acid oxidation (FAO) in hepatocytes : The mRNA expression of enzymes involved in the regulation of FAO such as CPT1a (A), CACT (B), ACSL1 (C), ACADL (D), ACADM (E), ACADS (F), HADHB (G) and HADHA (H) in hepatocyte cell line normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data are representative of mean ± SEM (n=6). * indicates significance compared with 500nM (control): * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$

5.3.2 **Effect of B12 on mitochondria functional integrity**

5.3.2.1 **Efficiency of mitochondria in utilizing a rich-substrate supply**

The electron transport chain (ETC) is partly dependent on the beta-oxidation of FA in the mitochondria for supply of electrons required for the synthesis of ATP [391]. We initially observed a decrease in the genes facilitating FAO in the mitochondria, that may account for a compromised beta-oxidation in low B12 hepatocyte cell line. We, therefore, further assessed the effect of B12 on mitochondrial respiration by measuring the OCR in hepatocyte cell line as an assessment of mitochondrial functional integrity in various conditions of B12.

In the presence of respiratory inhibitors such as oligomycin (inhibits ATP synthase or complex V), FCCP (protonophore uncoupler which shuts the inner membrane gradient) and rotenone (inhibits complex I) / antimycin A (inhibits complex III of the ETC), we measured the maximal respiratory capacity by the OCR in hepatocyte cell line (in a rich-substrate medium) and found that the OCR was decreased in low B12 compared with control (Fig 39B). Then, we estimated the spare respiratory capacity (SRC), a measure of the capacity of electron transport chain and substrate supply to respond to elevation in energy demand. We observed that the SRC was also decreased significantly in low B12 hepatocyte cell line compared with control (Fig 39A). This suggests that the efficiency of the mitochondria in utilising a rich-substrate for energy metabolism was compromised in low B12.

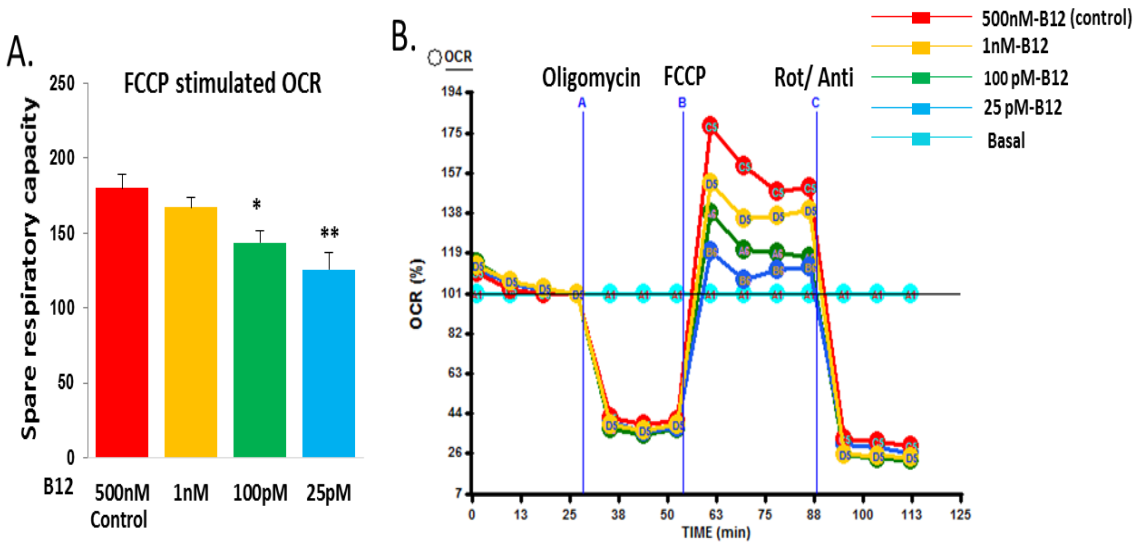

Figure 39: Low B12 compromises mitochondrial fumctional integrity of hepatocyte cell line in a rich-substrate medium: The spare respiratory capacity (**A**) and oxygen consumption rate (OCR) (**B**) of hepatocytes in a rich-substrate KHB medium containing glucose (2.5mM), pyruvate (1mM), amino acid (L-Glutamine) (2mM)

and BSA (0.1%) at pH 7.4 and 37°C temperature, in various conditions of B12 [500nM (control), 1nM (1000pM), 100pM and 25pM]. The data are representative of mean ± SEM (n=6). * indicates significance compared with 500nM (control): * p< 0.05, ** p< 0.01, *** p< 0.001

5.3.2.2 **Efficiency of mitochondria in utilizing limited-substrate (palmitate) supply**

Then, to examine how the low B12 hepatocyte cell line function with the endogenous supply of high extracellular levels of palmitate and other limited substrate, we incubated hepatocyte cell line in a limited-substrate KHB medium, which is poorly enriched with other supplements, except palmitate, compared with the rich-substrate KHB medium, containing only 0.5mM L-carnitine and 1.25mM glucose for one hour. After the basal respiration (OCR) was measured in the hepatocyte cell line, the cells were then exposed to 200µM palmitate or 33.3µM BSA (basal control) in the substrate medium to assess how Hep G2 cells efficiently uptake palmitate for ATP metabolism. We observed that in low B12 condition, the spare respiratory capacity (SRC) of the hepatocyte cell line upon exposure to (a) palmitate (Fig 40A) and (b) palmitate and respiratory inhibitors (Fig 40B), was significantly lower compared with control. This observation further suggests that the capacity of the mitochondria to catabolise long chain fatty acid (palmitate) for energy metabolism was impaired in low B12, therefore, implying that FAs were likely to accumulate in the hepatocyte cell line treated with low B12, leading to dyslipidaemia.

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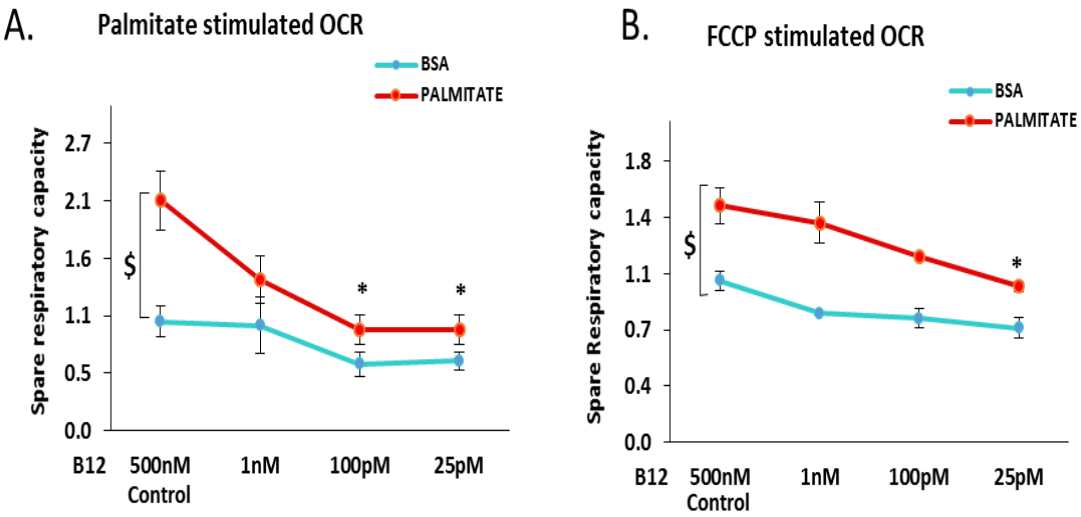


Figure 40: **Low B12 impaired FA (palmitate) uptake leading to decline in spare respiratory capacity in hepatocyte cell line:** The spare respiratory capacity of hepatacytes in response to; (A) uptake of palmitate compared with BSA (basal control) and (B) uptake of palmitae in the presence of inhibitors such as oligomycin A, FCCP and rotenone / antimycin A in a substrate-limited KHB medium containing only 0.5mM L-carnitine and

1.25mM glucose for one hour at pH 7.4 and 37°C temperature without CO_2 saturation. The data is representative of mean ± SEM (n=6), * indicates significance compared with 500nM-palmitate control; and $ indicates significance compared with 500nM-BSA control; * p< 0.05, ** p< 0.01, *** p< 0.001; $ p <0.05, $$ p < 0.01, $$$ p < 0.001

5.4 Discussion

In the current study, we observed that gene expression of the rate limiting enzyme (CPT1α) as well as key enzymes (CACT, ASCL1, ACADL, ACADM, ACADS, HADHA and HADHB) involved in the FAO pathway was significantly decreased in low B12 hepatocytes cell line compared with control. Likewise, the mitochondrial functional potency in low B12 hepatocyte cell line was compromised, resulting in the impairment of long chain FA (palmitate) utilization for ATP metabolism.

Low B12 impaired the beta-oxidation of FAs by decreasing expression of the rate limiting enzyme carnitine palmitoyl transferase 1-alpha (CPT1α) and downstream enzymes crucial in the FAO pathway. Similarly, an animal model study showed evidence of decrease in the hepatic FAO due to methyl donor (B12 and folate) deficiency [327]. The authors observed a deficit of carnitine in the liver of subjects compared with control [327]. However, FAO improvement *via* CPT1α upregulation following supplementation with methyl donors (B12) in mice was shown in another study [392]. Reduction in the expression of CPT1β and CPT2 due to low B12 was also observed in a similar study involving human adipocytes [393].

Catabolism of FAs to prevent their accumulation in hepatocytes is achieved *via* beta oxidation in the mitochondria [394]. Normally, the transport of acyl-CoA across the mitochondrial membrane represents the rate limiting step. FAs presenting with an overall number of carbon atoms from one to six are generally short-chain fatty acids (SCFAs), whereas those with seven to twelve carbon atoms are medium-chain fatty acids (MCFAs) [395]. SCFAs and MCFAs are basically independent of carnitine-facilitated uptake and do not require prior activation into acyl-CoAs before transported across the mitochondria membranes for catabolism [395]. However, long chain fatty acids (LCFAs) such as palmitate (C16) and stearate (C18) [396], the most abundant of total FAs in animal tissues [397], require the role of carnitine to enhance their oxidation. Therefore, reduction in the levels of CPT1α in low B12 hepatocyte cell line might affect the efficient transport of LCFAs across mitochondrial membrane for beta-oxidation.

Similarly, we observed that other key enzymes (ACLS1, CACT, ACADL, ACADM, ACADS, HADHB and HADHB) crucial in the FAO pathway were also decreased in low B12 compared with control. Similar evidence has been shown in human adipocyte model where low

B12 triggered adipocyte dysfunction by promoting TG biosynthesis as well as inhibiting FAO [393]. In that study, the expressions of key FAO genes including ACSL1, Acyl-CoA-dehydrogenase group (ACDL, ACADS and ACADM) and HADHB were all shown to be decreased in adipocytes as a result of low B12 [393]. Similar evidence of impaired FAO was also shown in myocardial cells resulting from low B12 and folate [398].

Generally, an inverse relationship between *de novo* synthesis of LCFAs and the rate of beta oxidation in the mitochondria has been reported [376]. It therefore confirms our earlier observation of higher levels of LCFAs synthesized in hepatocyte cell line under low B12. Since LCFAs are also associated with the development of hepatic steatosis [370], it suggests that compromised beta-oxidation due to low B12 and may account for long chain-FA accumulation in hepatocytes leading to the development of NASH or NAFLD [399]. However, the precise mechanism of low B12 downregulation of the mitochondrial FAO downstream enzymes requires further investigation, nevertheless, epigenetic modulation may be the most probable mechanism [400, 401].

Finally, we observed that the mitochondrial functional integrity, assessed by OCR and SRC, was impaired in low B12 compared with control. This observation was evident in the hepatocyte cell line in both rich and limited (palmitate)-substrate supply in low B12. Similarly, a recent study has shown that functional deficiency of B12 is associated with dysfunctional lysosomal metabolism (Gaucher disease) [402], which in turn is accompanied by mitochondrial dysfunction [402, 403]. Moreover, acute fatty liver of pregnancy (AFLP) is shown to be triggered by impaired hepatic mitochondrial function [404] which is further suggested to underlie several pathological disorders in pregnancy [405].

Beta-oxidation of FAs (FAO) and oxidative phosphorylation involving the electron transport chain (ETC) complexes in the mitochondria are not entirely independent pathways of cellular energetics [406]. Deficiencies identified in the FAO in patients are associated with detection of biochemical and/or clinical evidence suggestive of a subsequent abnormality in the oxidative phosphorylation pathway, or *vice versa* [406]. Also, a potential reduction of key FAO enzymes due to B12 deficiency may affect the supply of end-products (acetyl-CoA) of beta- oxidation to TCA (Krebs) cycle. Altogether, dysregulation in both beta-oxidation and TCA cycle may collectively affect the overall electrons (NADH and $FADH_2$) supply to the electron transport chain (ETC) of the mitochondria for efficient ATP metabolism [406]. This may explain our observation of compromised mitochondrial respiration in hepatocyte cell line in low B12, therefore, suggesting that LCFAs are likely to accumulate in hepatocytes. In conclusion, our

evidence in low B12 supports the hypothesis that impairment of FAO and mitochondrial respiration increases the incidence of NAFLD [405].

5.5 **Conclusion**

In conclusion, this study provides novel evidence of the role of B12 deficiency in hepatic FAO as well as mitochondrial respiration (oxidative phosphorylation) by utilization of LCFA for energy metabolism. The study shows that B12 deficiency may limit the entry of LCFAs into the mitochondria due to decreased CPT1α expression. Low B12 further decreased beta-oxidation by decreasing expression of the key downstream enzymes involved in the FAO pathway. Likewise, low B12 compromised the mitochondrial functional integrity by decreasing the maximal respiratory capacity, assessed by OCR, and the SRC in hepatocyte cell line compared with control. This may therefore explain the observation of lipid accumulation in hepatocyte cell line in low B12 accounting for elevation in the risk of NAFLD and incidence of T2DM.

6.0 The desired lipid lowering effect of metformin in human hepatocytes may be decreased in B12 deficient state

6.1 Introduction

The emergence of non-alcoholic fatty liver disease (NAFLD) is increasingly reported with the concomitant rise in cases of obesity. The prevalence of NAFLD is 25% in the general population of Asia [407] and Western countries [408]. Whereas prevalence rate in T2DM [409, 410] and obese population [409, 411] is much higher at 70% in the West, in lean or non-obese Asians [407], it is lower at 8-19%. NAFLD is known to be an independent risk factor associated with the development of T2DM and cardiovascular disease (CVD) [412]. Evolving evidence show that micronutrients may demonstrate a crucial role in the progression of NAFLD [331] and the association of NAFLD with certain vitamins has further been suggested by different studies [331]. In NAFLD patients presenting with higher levels of circulating alanine aminotransferase (ALT), serum B12 concentrations were shown to be significantly lower [332]. Supplementation with diverse vitamins, according to different studies, present as a hopeful alternative in NAFLD management [331]. Likewise, metformin offers some lipid lowering effect along with other potential drugs such as glucagon-like peptide 1 (GLP-1) [413], although their efficacy are still being tested.

In the management of T2DM, and in reduction [414] of fat accumulation in the liver [415-419] in other co-morbidities, metformin is used as the first line therapy. It is also increasingly accepted as an alternative therapy to insulin in GDM. Recently, the MiG (Metformin in Gestational Diabetes) trial reported similar pregnancy outcomes for metformin versus insulin treatment, however, supplemental insulin was required in 46% of women on metformin [420]. Studies in animals [421] and humans [422] have shown that metformin treatment had no effect on intra-hepatic TG content [415]. This suggests failure of metformin therapy in a subgroup of these patients, however, the exact underlying mechanism accounting for this remains unclear. Several clinical studies have shown that prolonged treatment with metformin is linked to deficiency of B12 [423-428]. Though metformin has a lipid lowering effect, some studies show metformin reduces B12 levels, and B12 deficiency induces lipid dysregulation. These observations thus provide us a clue that B12 might interfere with the action of metformin (Fig 41). Therefore, we hypothesize that B12 deficiency might impair the lipid lowering effect of metformin. Hence, we aimed to investigate the relation between deficiency of B12, metformin and adverse lipids and the underlying mechanisms. In the present

study, therefore, we performed to determine the effect of metformin on lipid synthesis, oxidation and mitochondrial function in hepatocytes treated with low B12.

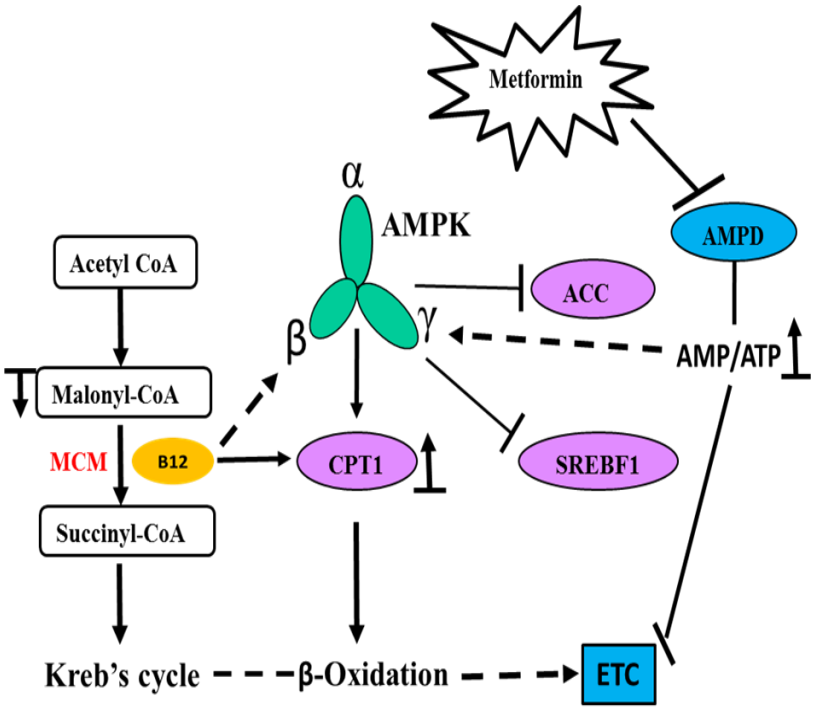


Figure 41: **Mechanism underlying metformin and B12 actions resulting in lipid lowering effect**: Metformin plays a key role in the activation of the adenosine monophosphate activated protein kinase (AMPK) by encouraging higher AMP/ATP ratios achieved *via* suppression of ATP production resulting from inhibition of complex 1 of the electron transport chain (ETC) as well as blocking of AMP deaminase (AMPD) which is capable of degrading AMPs present. Higher levels of AMPs achieved *via* metformin treatment subsequently results in the activation of AMPK by binding to its gamma subunit. Activated AMPK inhibits lipogenesis by directly phosphorylating and inactivating rate limiting enzymes such as acetyl CoA carboxylase (ACC) and sterol regulatory element binding protein (SREBF) involved in lipid biosynthesis. AMPK again ensures upregulation of CPT1, enhancing beta-oxidation of FAs to prevent their accumulation. Concurrently, B12 (adenosyl cobalamin) acting as a cofactor to methylmalonyl-CoA mutase (MCM) engaged in the propionate metabolism in the mitochondria plays a key role in alleviating the build-up of methyl malonic acid (MMA) by facilitating the generation of succinyl-CoA from methyl malonyl-CoA, thereby providing succinyl-CoA substrates to the Kreb's cycle leading to generation of AMP, ADP and ATP molecules required for AMPK activation. Similarly, B12 *via* inhibition of MMA build-up facilitates activation of CPT1 which decreases accumulation of FAs by enhancing their breakdown *via* the beta-oxidation.

6.2 **Methods**

6.2.1 **Cell culture**

Hep G2 cell culture was done as described in sections 2.2.1.5-2.2.1.6, in control B12 (500nM) and low B12 (25pM) conditions. After the last media change at the 10^{th} day of incubation, the cells (cases) were treated with 1mM metformin (Sigma, UK) in a serum-free EMEM media for 24-hours or maintained in serum-free media without metformin. The cells were harvested for isolation of RNA, proteins and total intracellular TG estimation. Some cells were also maintained in six-plates for radioactive flux assays.

6.2.2 **RNA isolation, cDNA synthesis and gene expression**

Total RNA isolation, cDNA synthesis and gene expression assays were same as previously described in sections 2.2.2.1-2.2.2.4 for cases (1mM metformin treated hepatocytes) and controls (without metformin treatment) under conditions of low B12 (25pM) and control B12 (500nM).

6.2.3 **Western blot analysis**

Protein characterization studies were done as previously described in section 2.2.6. Primary antibodies (cell signalling) targeting pAMPKα (molecular weight: 62 kDa) and pACC (molecular weight: 280 kDa) were normalised to total AMPK (molecular weight: 62 kDa) and total ACC (molecular weight 280kDa) proteins respectively.

6.2.4 **Total intracellular TG estimation**

Total intracellular TG in hepatocytes was quantified as previously described in section 2.2.3 of chapter 2. In cases involving Hep G2 cells treated with 1mM metformin as well as control (without metformin treatment) in low (25pM) and control (500nM) B12 conditions, TGs were quantified using the commercial TG quantification kit (ab65336) from Abcam Plc, Cambridge, UK, following the manufacturer's protocol.

6.2.5 **Radioactive flux assay for TG biosynthesis**

Upon reaching 100% confluence in six-well plates following the last media change for different conditions of B12: 500nM (control) and 25pM, Hep G2 cells were treated with 1mM metformin in serum-free media for 24-hours in cases, whereas controls were maintained in serum-free

EMEM medium at 37^{o}C and 5% CO_2 saturation. The radioactive flux assay was then performed as previously described in section 2.2.4 of chapter 2.

6.2.6 **Mitochondrial dysfunction assessment using seahorse assay**

Hep G2 cell culture was done as described in section 2.2.8. Additionally, the cells in control B12 (500nM) and low B12 (25pM) conditions were trypsinized and seeded at 50 000 per well of the seahorse XF 24 plate and allowed to settle overnight at 37 ^{o}C and 5% CO_2 saturation. This was followed by replacement of the B12-EMEM medium with a serum-free medium for 24-hours incubation, followed by subsequent treatment with 1mM metformin in a serum-free medium for 24-hours. The samples, seahorse substrate medium and respiratory inhibitors (oligomycin, FCCP and rotenone /antimycin) were then prepared and used for the seahorse assay as previously described in section 2.2.8.

Following the estimation of OCR in a rich-KHB substrate medium, we further incubated the Hep G2 cells in a limited-KHB substrate medium containing only 0.5mM L-carnitine and 1.25mM glucose for one hour. After measuring the basal respiration (OCR) in hepatocytes, the cells were then exposed to 200µM palmitate or 33.3µM BSA (basal control) in the substrate medium to assess how the hepatocyte cell line efficiently uptake palmitate for ATP synthesis.

6.3 **Results**

6.3.1 **Effect of metformin on total intracellular TG level in hepatocyte cell line**

Metformin is shown to significantly reduce plasma levels of TG and cholesterol [429]. We therefore quantified the total intracellular TG in metformin treated hepatocyte cell line to assess whether metformin would achieve a significant lowering effect in different B12 conditions. In low B12 hepatocyte cell line (without metformin), the total intracellular TG level was significantly higher compared with control. As predicted, metformin treatment in control cells showed significant reduction (20.7%) of TG levels but treatment with metformin in low B12 cells did not achieve significant reduction (6.2%). This finding suggests that there is higher TG level in low B12 hepatocyte cell line following metformin treatment and thereby confirms our hypothesis that B12 deficiency in metformin treatment might lessen its lipid lowering effect (Fig 42).

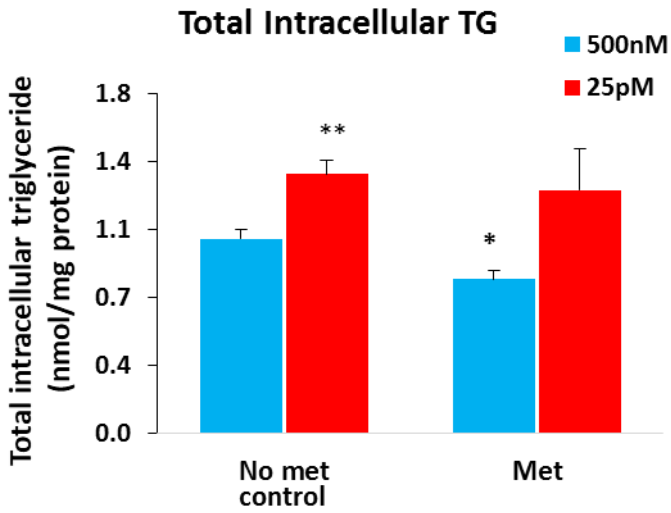


Figure 42: Effect of metformin on total intracellular TG levels in hepatocytes

Total intracellular levels of TGs were quantified in metformin (Met) and non-metformin treated (No met) / basal hepatocyte cell line in different B12 conditions: 25pM (low B12) and 500nM (control), using the TG kit (ab65336, Abcam plc, UK) and normalized per milligram protein of hepatocytes under each B12 condition. The data is representative of mean ± SEM (n=6). * represents significance compared with 500nM-No-met control; and $ represents significance compared with 25pM-No-met control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

6.3.2 **Effect of metformin on FA (radiolabelled) uptake for TG synthesis**

Following the observation that metformin treatment in low B12 did not reduce total intracellular TG levels, we further assessed to see how this affects the uptake of FA in hepatocyte cell line for TG synthesis. We therefore performed the radioactive flux assay to assess the amount of radiolabelled-FA utilized in the hepatocytes cell line for TG synthesis after a period, following the extraction and isolation of TG in metformin treated hepatocyte cell line.

We observed that, the level of TG synthesized following the uptake of radiolabelled-FA was significantly higher in hepatocyte cell line in low B12 (without metformin) compared with control. However, in metformin treated hepatocyte cell line, the level of TG synthesized was significantly reduced in control hepatocyte cell line (58%) but not in low B12 hepatocyte cell line (13%) (Fig 43). This evidence may also suggest that the esterification of FA for TG synthesis in low B12 hepatocyte cell line might not be significantly reduced by metformin treatment.

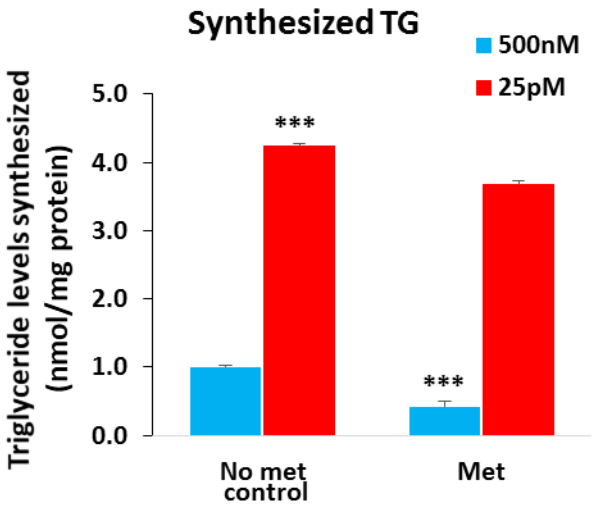


Figure 43: TG synthesized, following radio-labelled-FA uptake in metformin treated hepatocyte cell line, in different B12 conditions: Radioactive flux assay was performed after seeding 75,000 Hep G2 cells in six-well plates under different conditions of B12 [500nM (control) and 25pM (low B12)] until reaching about 100% confluence at 10th day of the cell culture. The cells were then treated with 1mM metformin in a serum-free media for 24-hours after which hepatocytes were incubated with ^{14}C-Oleate (0.75mM concentration at 2000 dpm/nmol) and L-carnitine (1mM) to facilitate incorporation of ^{14}C-Oleate into hepatocytes for 2-hours in EMEM, against 5-minutes incubation for background normalizing control, at 37^{o}C and 5% CO_2 saturation. Hep G2 cells were harvested in 2ml methanol and the synthesized radiolabelled TG was extracted, following an initial total lipid isolation with the liquid-liquid (chloroform-methanol, 2:1 v/v) extraction method, using a mobile phase such as hexane/diethtyl ether/formic acid (v/v/v, 70/30/1) on thin layer chromatography (TLC) plate with 10 nmol glyceryltripalmitate (tripalmitin) as a standard. TG bands on TLC plates were transferred into vials of 5ml scintillation fluid for scintillation counter assessment of radioactivity (for 5 minutes) of the synthesized TG fraction, normalized per milligram (mg) protein concentration by Bradford method. The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-No met control; and $ represents significance compared with 25pM-No-met control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

6.3.3 **Effect of metformin on the lipid synthesis pathways in hepatocyte cell line**

6.3.3.1 ***De novo*** **fatty acid (FA) synthesis**

De novo lipogenesis in the liver involves the synthesis of lipids by converting excess products of glycolysis, pyruvate, into FA, which also undergoes esterification into TG for storage. We have initially shown that *de novo* lipogenesis was increased in low B12 hepatocyte cell line. However, following our observation of inefficient lowering of total intracellular TG levels and FA uptake by metformin in low B12 hepatocyte cell line, we further assessed the probable effect of metformin on gene expression of *de novo* lipid synthesis in different B12 conditions.

In the FA synthesis pathway, we observed significantly higher expression of the genes regulating FA synthesis in low B12 hepatocyte cell line (without metformin) compared with control (Fig 34). In control cells with metformin treatment, there was greater reduction of expression of genes - SREBF1 (49.6%), ACLY (21.0%), ACC (23.8%), FASN (36.0%) and ELOVL6 (35.8%) (Fig 44). Similarly, in low B12 hepatocyte cell line with metformin treatment, the nuclear transcription factor and master regulator of lipogenesis, SREBF1, was significantly decreased (34%) in low B12. We also observed a significantly decreased expression of FA synthesis genes such as ACLY (17.6%), FASN (27.4%) and ELOVL6 (29.9%). However, the expression of the rate limiting enzyme, ACC, was not significantly reduced in metformin treated hepatocyte cell line in low B12 (8.9%) compared with control. Although metformin showed significant reduction in FA genes (except ACC) in low B12, these data may suggest that, the lowering effect of metformin in the FA synthesis genes in control may be greater than in low B12 hepatocyte cell line.

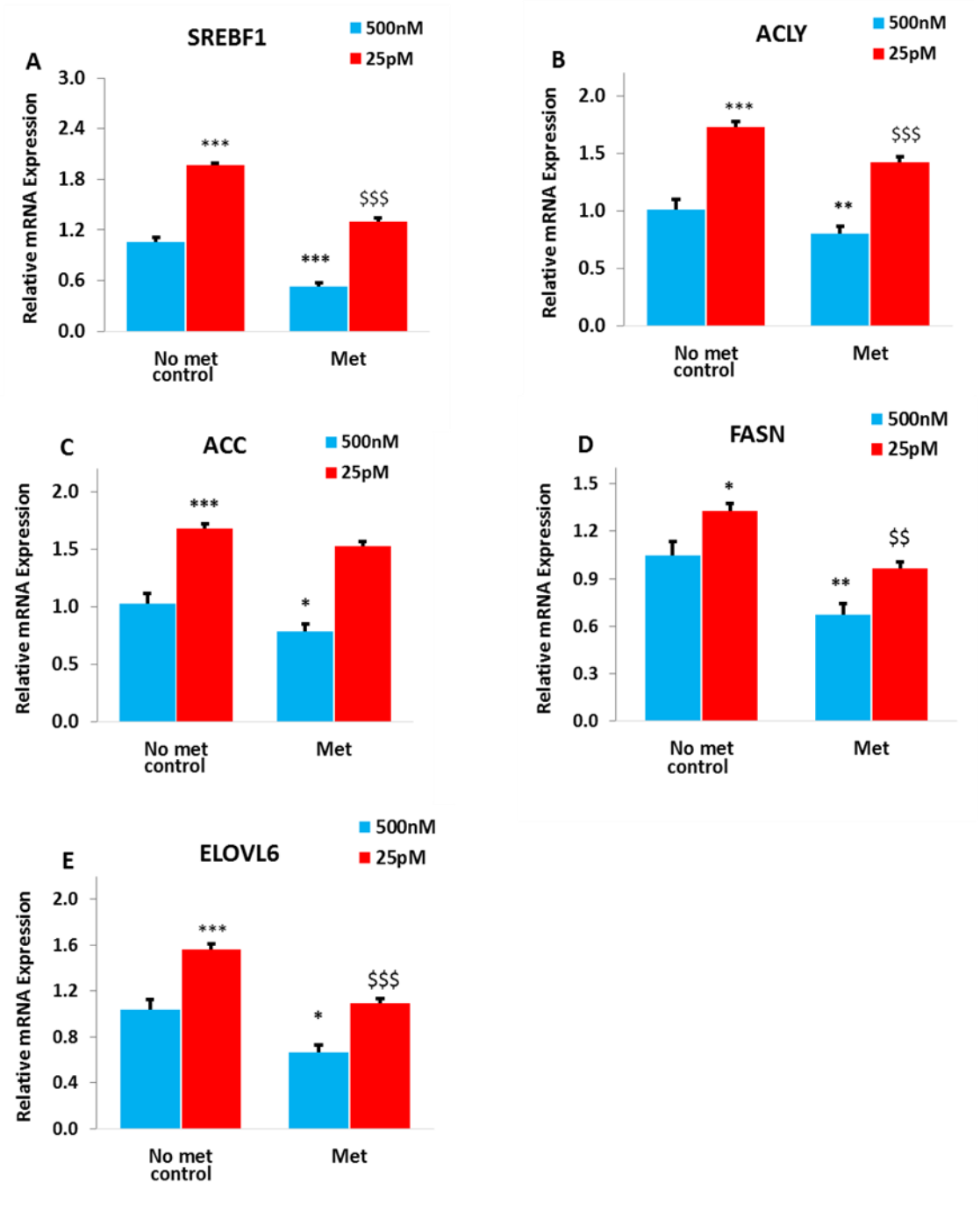


Figure 44: **Effect of metformin on gene expression of enzymes regulating *de novo* FA synthesis:** The mRNA expression of nuclear transcription factor SREBF1 (A) and genes regulating *de novo* FA synthesis: ACLY (B), ACC (C) and FASN (D) and EVOVL6 (E) in different B12 conditions [25pM (low B12) and 500nM (control)] in both metformin (Met) and non-metformin treated (No met) / basal hepatocyte cell line, normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-No-met control; and $ represents significance compared with 25pM-No-met control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

6.3.3.2 **TG biosynthesis**

We subsequently assessed the TG biosynthesis pathway to ascertain whether metformin was effective in the downregulation of TG synthesis due to the observation of impaired lowering of total intracellular TG levels in metformin treated hepatocyte cell line in low B12. TG biosynthesis involves sequential esterification of FA, following synthesis and desaturation, into glycerol-3-phosphate substrate by utilizing enzymes such as SCD1, GPAT (GPAM), AGPAT, DGAT1 and DGAT2.

We observed that the expression of TG biosynthesis genes including SCD1, GPAM, AGPAT, DGAT1 and DGAT2 was significantly higher in low B12 hepatocyte cell line (without metformin) (Fig 45). However, following metformin treatment in control cells, the expression of the TG biosynthesis genes: SCD1 (36.3%), GPAM (26.1%), AGPAT (40.5%), DGAT1 (48.2%) and DGAT2 (29.0%), and in low B12 cells, SCD1 (22.3%), GPAM (21.1%), AGPAT (39.5%), DGAT1 (37.1%) and DGAT2 (23.8%) was lower significantly. Here the finding again suggests that, metformin may decrease gene expression of TG synthesis significantly in both control and low B12 cells, but more efficiently in control cells than the low B12 hepatocyte cell line.

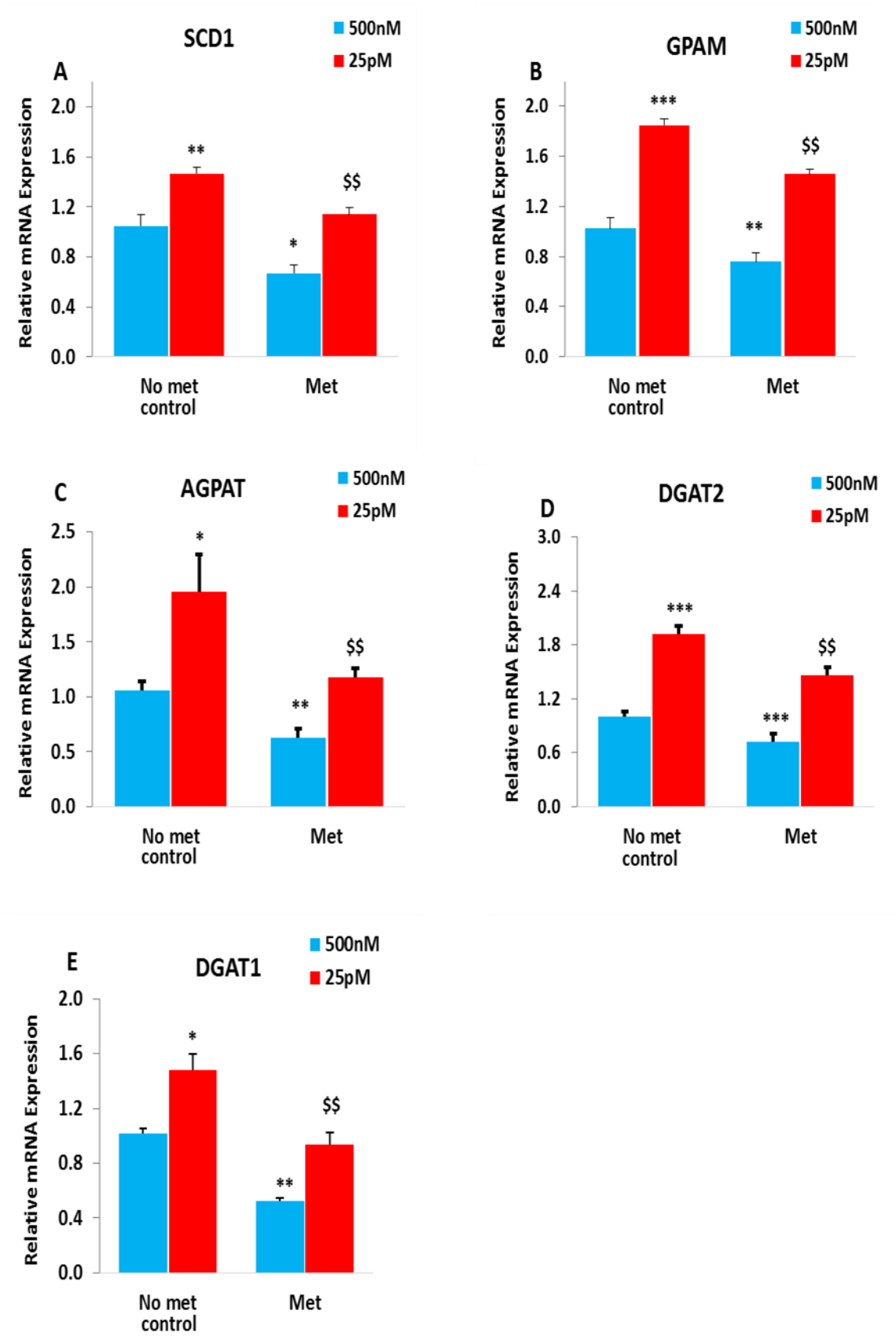


Figure 45: **Effect of metformin on gene expression of enzymes regulating TG synthesis:** The mRNA expression of enzymes regulating TG biosynthesis: SCD1 (A), GPAM (B), AGPAT (C) DGAT2 (D) and DGAT1

(E) in hepatocyte cell line of different B12 conditions [25pM (lowB12) and 500nM (control)] in metformin (Met) and non-metformin treated (No met) /basal hepatocyte cell line, normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-No-met control; and $ represents significance compared with 25pM-No-met control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

6.3.2.3 **Cholesterol biosynthesis**

We next assessed the lowering effect of metformin on cholesterologenesis in hepatocyte cell line in different B12 conditions. Cholesterol biosynthesis is chiefly regulated by the nuclear transcription factor LDLR and the rate limiting enzymes HMGCR and HMGCS [430].

The expression of the cholesterol biosynthesis genes HMGCR, HMGCS1 and LDLR was significantly higher in low B12 hepatocyte cell line (without metformin) (Fig 46). However, in control cells with metformin treatment, we observed significantly decreased expression of HMGCR (32.9 %), HMGCS1 (32.6 %) and LDLR (34.2 %), with similar observation in low B12 hepatocyte cell line which had decreased expression of HMGCR (24.4%), HMGCS1 (27.1%) and LDLR (27.6%). The finding again suggests that, metformin may decrease gene expression of cholesterol synthesis significantly in both control and low B12 cells, but more efficiently in control cells than low B12 hepatocyte cell line.

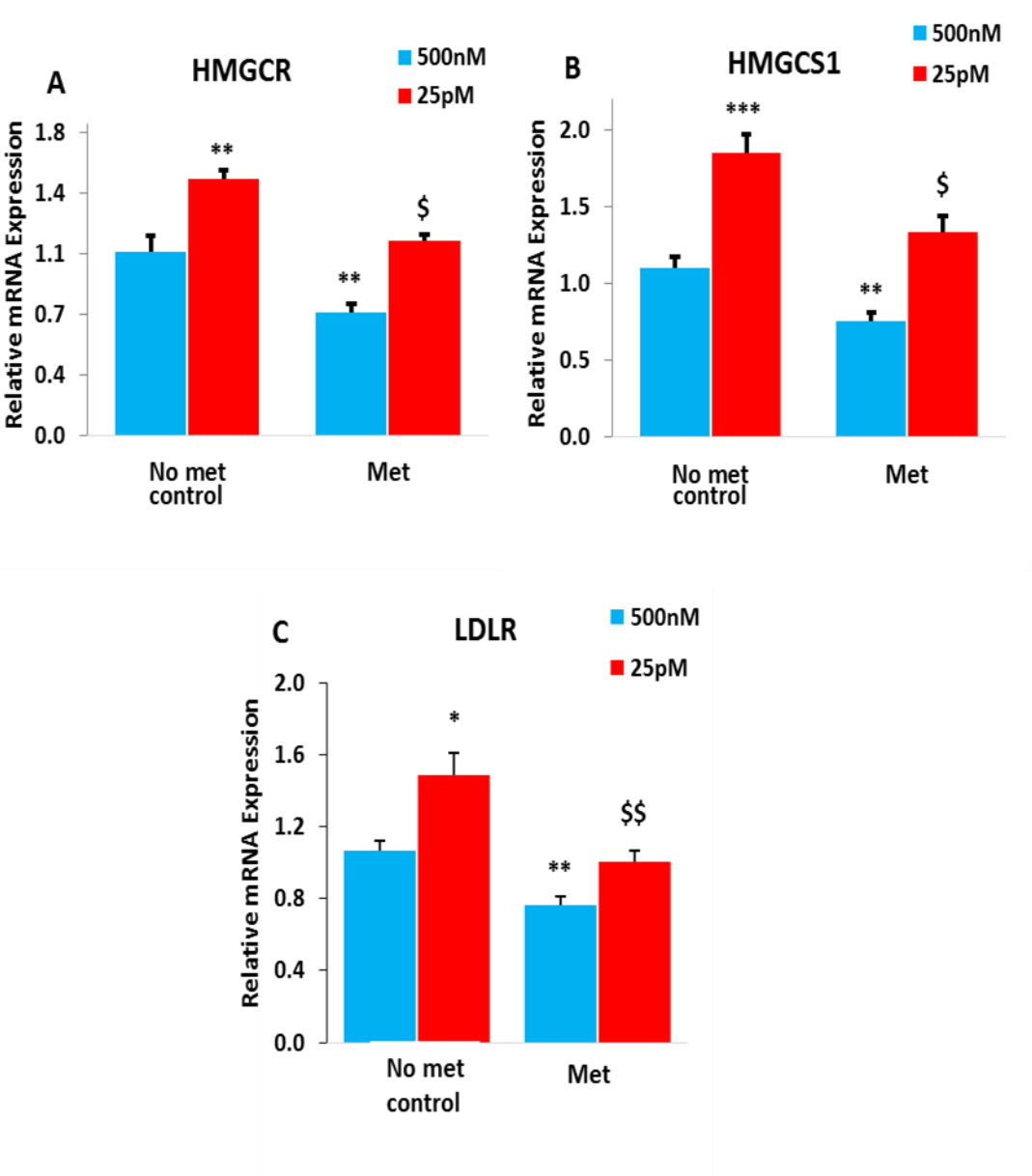


Figure 46: Effect of metformin on gene expression of enzymes regulating cholesterol biosynthesis: The mRNA expression of enzymes regulating cholesterol biosynthesis such as HMGCR (A), HMGCS1 (B) and LDLR (C) in different B12 [25pM (low B12) and 500nM (control)] conditions)] in metformin (Met) and non-metformin treated (No met) / basal hepatocyte cell line, normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-No-met control; and $ represents significance compared with 25pM-No-met control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

6.3.4 **Effect of metformin on fatty acid oxidation (FAO)**

Fatty acid oxidation (FAO), involving the catabolism or breakdown of FA, is crucial in preventing the accumulation of FA in hepatocytes leading to dyslipidaemia and development of NASH and NAFLD. FAO of long chain-FA requires initial activation into fatty acyl-CoA, followed by facilitated transport *via* the mitochondrial membranes utilizing the carnitine system which includes CPT1a, CACT and CPT2. The long chain FAs are then subjected to consecutive processes in the mitochondria involving dehydrogenation, hydration, oxidation and thiolytic cleavage to generate acetyl-CoA (two-carbon units) utilizing enzymes such as ACADL, ACADM, ACADS and HADHA.

Metformin was effective in reducing the expression of various FA synthesis genes in low B12. However, the rate limiting enzyme, ACC, was not significantly decreased in metformin treated hepatocyte cell line in low B12. ACC is also critically involved in the regulation of FAO *via* inhibition of CPT1, resulting from ACC-catalysed generation of methyl malonyl-CoA (MMA) from acetyl-CoA [431]. We therefore assessed whether metformin was effective in upregulating FAO in hepatocyte cell line in low B12 condition. We observed that the expression of CPT1a, CACT, HADHA, ACADS, ACADM and ACADL was significantly decreased in hepatocyte cell line (without metformin) in low B12 compared with control. However, in control cells with metformin treatment, there was a significant increase in the expression of CPT1a (35.5%), CACT (37.8%), HADHA (52.0%), ACADS (79.3%), ACADM (74.3%) and ACADL (27.9%) (Fig 38). Contrarily, there was no significant– increase in CPT1α (2.5%), CACT (14.7%), HADHA (14.1%), ACADS (29.6%), ACADM (23.6%) and ACADL (2.0%) in metformin treated hepatocyte cell line in low B12 (Fig 47). This evidence suggested that in low B12 condition, the expressions of FAO genes were lower and their levels following metformin treatment, were not significantly increased accounting for decreased FAO in low B12 Hep G2 cells treated with metformin.

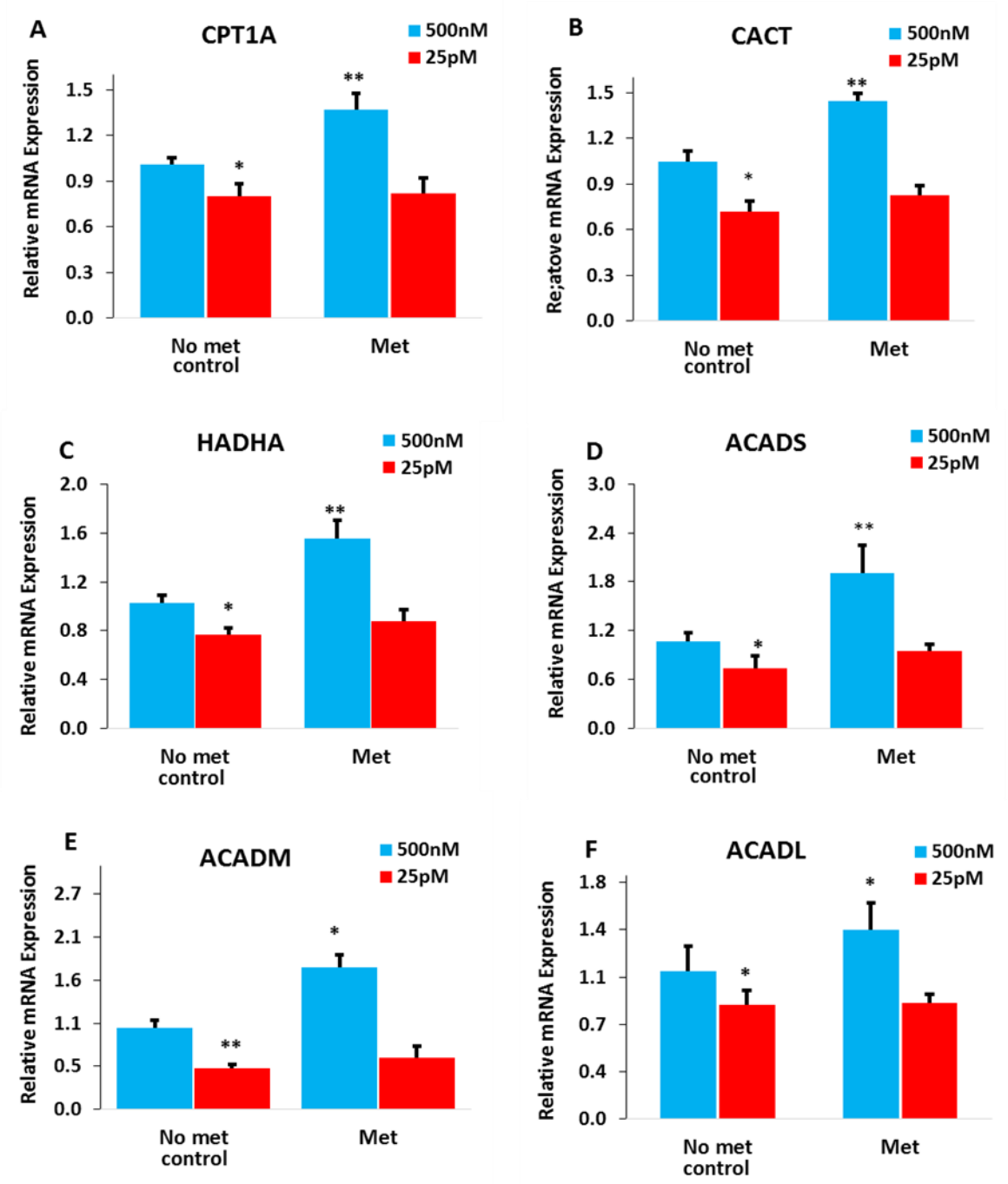


Figure 47: Effect of metformin on gene expression of enzymes regulating fatty acid oxidation (FAO): The mRNA expression of enzymes involved in the regulation of FAO: CPT1a (A), CACT (B), HADHA (C), ACADS (D), ACADM (E) and ACADL (F) normalized to 18S rRNA endogenous control (Applied Biosystems, UK) in metformin (Met) and non-metformin (No met) treated / basal hepatocyte cell line under different conditions of B12: 500nM (control) and 25pM (low B12). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-No met control; and $ represents significance compared with 25pM-No-met control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001

6.3.5 **Effect of metformin on the mitochondria functional integrity**

FAO is functionally interconnected with the mitochondrial respiration involving the electron transport chain (ETC) [432]. Since FAO was impaired in metformin treated hepatocyte cell line in low B12, we further assessed this effect on the mitochondrial functional efficiency with regards to the utilization of substrate supply for mitochondrial respiration in low B12 condition, following metformin treatment.

First, we assessed whether B12 independently affects mitochondria functional integrity in the utilization of substrate supply for energy metabolism, using the seahorse XF24 flux mito-stress assay. We observed that the spare respiratory capacity (SRC) of the mitochondria in hepatocyte cell line in low B12 was significantly lower compared with control (Fig 48). This suggested that low B12 independently decreased the mitochondrial functional potency in hepatocyte cell line by reducing the spare respiratory capacity.

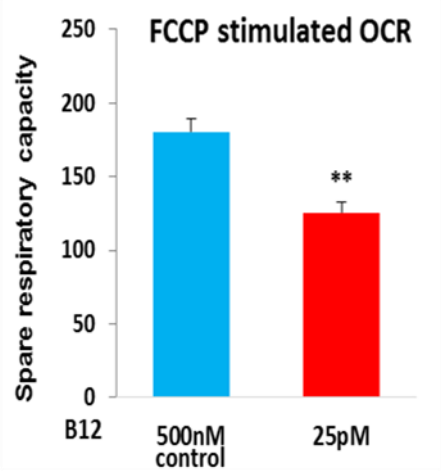


Figure 48: Effect of B12 on the spare respiratory capacity in hepatocytes: The spare respiratory capacity of mitochondria in hepatocyte cell line in different conditions of B12: 500nM (control) and 25pM (low B12). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM control; * p< 0.05, ** p< 0.01, *** p< 0.001

Afterwards, we assessed whether metformin might improve mitochondrial functional efficiency in low B12 hepatocyte cell line. We observed a significant increase (43.6%) in the SRC of metformin treated Hep G2 cells in control, whereas upregulation of the SRC was not significant in low B12 accounting for only 8.6% increase (Fig 49). Therefore, suggesting that restoration of the mitochondrial functional efficiency by metformin treatment was decreased in Hep G2 cells in low B12 compared with control.

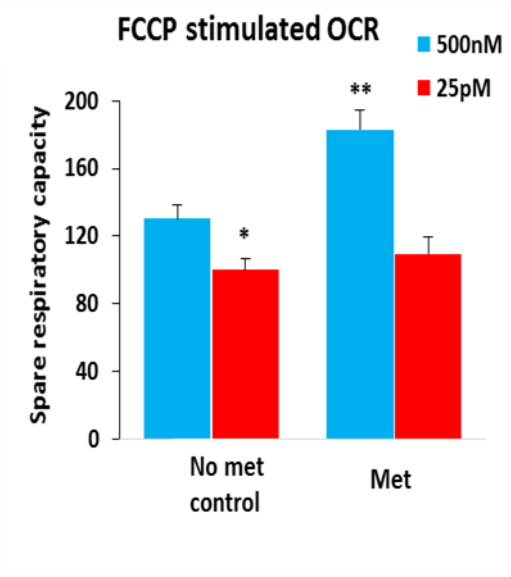


Figure 49: Effect of metformin on the spare respiratory capacity in hepatocytes: The spare respiratory capacity of mitochondria in metformin treated hepatocyte cell line in different conditions of B12: 500nM (control) and 25pM (low B12). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-No-met control; * p< 0.05, ** p< 0.01, *** p< 0.001;

Subsequently, we assessed whether metformin might efficiently improve mitochondrial respiration by utilizing long chain FA, palmitate, in low B12. The upregulation of SRC in metformin treated hepatocyte cell line in palmitate-rich substrate was significantly decreased in low B12 (17.0%) compared with control B12 (60.0%) (Fig 50). Therefore, suggesting that the mitochondrial functional efficiency in metformin treated hepatocyte cell line was compromised in low B12 resulting in the inefficient utilization of palmitate for respiration. This may hence account for the accumulation of long chain FA (palmitate) in metformin treated hepatocyte cell line in low B12 leading to dyslipidaemia.

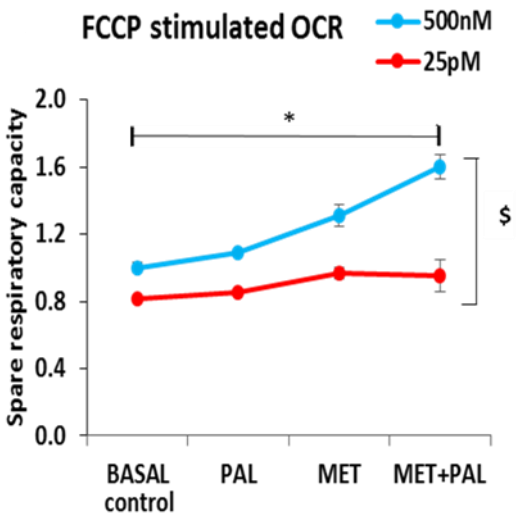


Figure 50: Effect of metformin on the spare respiratory capacity in hepatocyte cell line exposed to palmitate: **T**he spare respiratory capacity of mitochondria in metformin treated hepatocyte cell line supplemented with palmitate in different conditions of B12: 500nM (control) and 25pM (low B12). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 500nM-Met+Pal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

6.3.6 **Effect of B12 on the lipid lowering effect of metformin *via* activation of AMPK**

Reduction in hepatic lipid levels is shown to be achieved *via* the activation of AMPK and subsequent upregulation of FAO [433]. Metformin is known to achieve a lowering effect on lipids *via* the activation of AMPK.[434]. AMPK activation involves phosphorylation of the alpha subunit (pAMPKα) which further results in the phosphorylation (inactivation) of its downstream target, acetyl-CoA carboxylase (pACC).

Therefore, in order investigate the underlying mechanism in lipid regulation through AMPK, we assessed the independent effect of B12 on pAMPKα and pACC levels in hepatocyte cell line to ascertain whether B12 affects the activation of AMPK with subsequent inactivation of ACC. We observed that pAMPKα level in the hepatocyte cell line was significantly decreased in low B12 compared with control (Fig 51A). Similarly, the level of pACC was significantly decreased in low B12 hepatocyte cell line compared with control (Fig 51B), concurrent to the gene expression. Our observation suggests that, the phosphorylation (activation) of AMPK and

subsequent phosphorylation (inactivation) of ACC were decreased in hepatocyte cell line in low B12 compared with control.

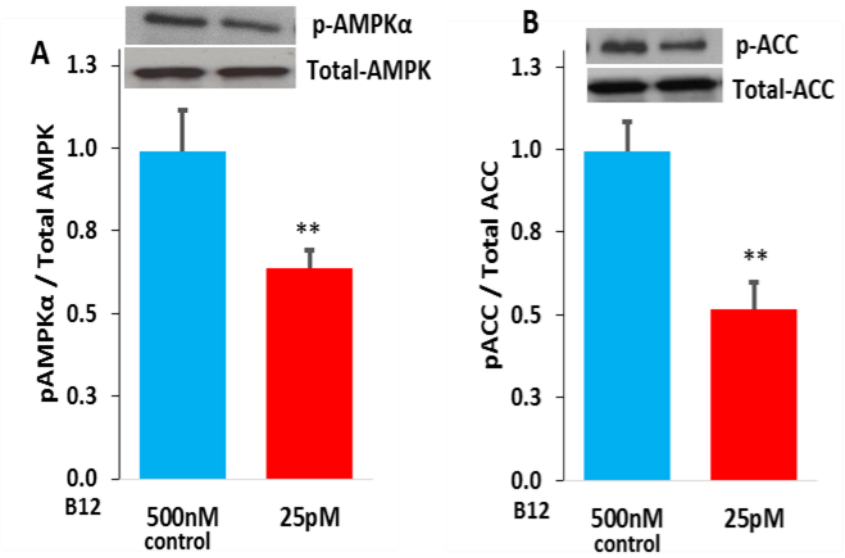


Figure 51: B12 effect on pAMPKα and pACC levels: The level of pAMPKα (A) and pACC (B) proteins in hepatocyte cell line in different conditions of B12: 500nM (control) and 25pM (low B12). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM control; * p< 0.05, ** p< 0.01, *** p< 0.001.

Since the levels of pAMPKα and pACC were significantly reduced in low B12 hepatocyte cell line, we further assessed whether the activation of AMPK and subsequent inactivation of ACC would be restored in low B12 hepatocyte cell line following metformin treatment. We observed that the levels of pAMPKα (Fig 52A) and pACC (Fig 52B) increased significantly (93.1% and 115.6% respectively) in control hepatocyte cell line treated with metformin but not significantly (22.4% and 37.7% respectively) in metformin treated hepatocyte cell line in low B12. This evidence suggests that, metformin did not efficiently restore the levels of pAMPKα and pACC in low B12 therefore confirming that impaired FAO may be mediated through AMPK in low B12 hepatocyte cell line in metformin treated condition.

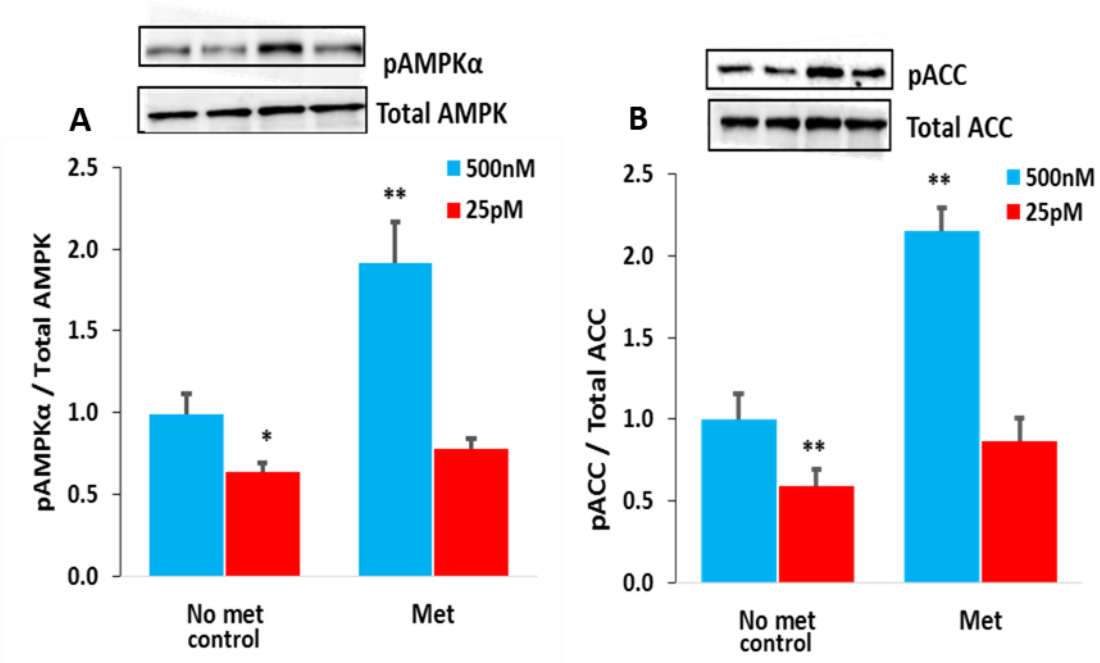


Figure 52: Effect of metformin on pAMPKα and pACC levels: The level of pAMPKα (A) and pACC (B) proteins in metformin treated hepatocyte cell line under different conditions of B12: 500nM (control) and 25pM (low B12). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-No-met control; * p< 0.05, ** p< 0.01, *** p< 0.001.

6.4 Discussion

Metformin treatment in low B12 hepatocyte cell line improved the expression of genes involved in hepatic FAs, TG and cholesterol biosynthesis, but total intracellular TG levels and biosynthesis of TGs remained significantly higher. Likewise, gene expression of FAO and the mitochondrial functional integrity in metformin treated hepatocytes was compromised in low B12 state. Importantly, the ability of metformin to reduce lipids through AMPK was impaired in B12 deficient state.

Our group initially showed that B12 deficiency increased lipogenesis in adipocytes resulting from upregulation of genes regulating fatty acid (FAS and ACACA) [189] and cholesterol (SREBF1, SREBF2, HMGCS1 and HMGCR) biosynthesis [196]. We have also shown clinical evidence that B12 deficiency is associated with high TG levels in diabetes patients of both Indian and European ethnic groups [435]. Similar to this, we have shown in hepatocyte cell line in the previous chapter, that lipid metabolism is dysregulated. The nuclear transcription factor SREBF1 and downstream genes regulating fatty acids (ACLY, ACC, FASN

and ELOVL6), triglycerides (SCD, GPAM, AGPAT, DGAT1 and DGAT2) and cholesterol (HMGCS1 and HMGCR) biosynthesis were upregulated in low B12 hepatocyte cell line. We also observed that upregulation of the rate limiting enzyme of the FAO pathway, CPT1α, and downstream genes (CACT, HADHA, ACADM, ACADS and ACADL) were impaired in low B12 hepatocyte cell line. In this study, we found that metformin treatment in low B12 state resulted in significant reduction in hepatic expression of SREBF1 and downstream genes regulating fatty acids (ACLY, ACC, FASN and ELOVL6), triglycerides (SCD, GPAM, AGPAT, DGAT1 and DGAT2) and cholesterol (HMGCS1 and HMGCR) biosynthesis. However, the total intracellular TG and radio-labelled TG synthesized in metformin-treated low B12 hepatocyte cell line did not reduce significantly. Likewise, the mitochondrial functional potency assessed by its spare respiratory capacity was compromised in low B12 hepatocyte cell line exposed to palmitate-rich substrate. Similarly, metformin treatment could not improve the mitochondrial dysfunction in low B12 hepatocyte cell line. Our study therefore suggests that both FAO enzymes and mitochondrial functional efficiency in hepatocytes treated with metformin may be hampered under low B12 condition. Therefore, implying that B12 deficiency may decrease the desired lipid lowering effect of metformin in human hepatocyte cell line through downregulation of FAO.

It is known that metformin action activates AMPK, resulting in the inactivation and phosphorylation of ACC leading to reduction in lipogenesis, thus alleviates hepatic accumulation of lipids [436]. Several studies involving animal models and cell lines have reported the lipid lowering effect of metformin via reduction of SREBP1 [416, 419, 437, 438], ACC [417, 437, 439], FAS [419, 438, 440], ELOVL6 [438], SCD1 [419, 440], HMGCS1 [438] and HMGCR [438] leading to reduction of intrahepatic triacylglycerol (IHTAG) content [415]. Studies also show that metformin reverses hepatic steatosis by blocking acetyl-CoA carboxylase (ACC) *via* AMPK activation which subsequently minimizes build-up of malonyl-CoA (inhibitor of carnitine palmitoyl transferase 1, CPT1), therefore upregulating hepatic oxidation of fatty acids (FAO) [418, 434, 441]. Currently, clinical studies targeting the hepatic lipid lowering effect of metformin have also shown that intrahepatic TG level in humans remains unaffected following metformin treatment [415]. Some studies involving humans [442], animals [443] and cell lines [444] have also shown that metformin failed to increase FAO in subjects. A study in diabetes cohort similarly showed that compromised hepatic and whole body oxidation of fatty acids was not improved after metformin treatment [442]. As metformin administration has been associated with circulating low B12 levels [428, 445], it may be

conceivable that metformin treatment in humans might result in probable reduction of B12 in hepatic stores. This may explain its ineffective lipid lowering impact on intrahepatic TG levels in a subgroup of patients, due to hepatic B12 deficiency. Activation (phosphorylation) of AMPKα was shown to minimize lipid levels in the liver [433]. We observed decreased levels of pAMPKα in low B12 hepatocyte cell line, an indication that B12 independently might play an epigenetic role in the in-activation of AMPK in the liver. B12 is a well-known methyl donor [446], and similar studies have shown that methyl donor supplementation in mice resulted in the activation (phosphorylation) of AMPK leading to reduction of hepatic lipid accumulation [447]. The probable epigenetic mechanism underlying B12 activation of AMPK requires further investigation. However, derivatives of the axial ligand of cobalamin (B12), 5,6-dimethyl benzimidazole [448] have been reported to prevent dephosphorylation as well as engage in allosteric activation of AMPK [449-451]. Similarly, we observed that phosphorylated-acetyl-CoA carboxylase (pACC) was decreased in low B12 hepatocyte cell line. It has been reported that the inhibitory effect of AMPK on lipogenesis may be achieved *via* phosphorylation (inactivation) of acetyl-CoA carboxylase (ACC) [452]. This may confirm our observation that decreased pAMPKα in low B12 accounted for reduced pACC in the hepatocyte cell line. In addition, we observed that protein expression of pAMKα and pACC in low B12 hepatocyte cell line, following metformin treatment, was impaired. Metformin treatment resulted in the decline of circulating B12 level and altered B12 distribution in rat tissues [453] and humans [454]. However, metformin has been shown to elevate phosphorylation or activation of AMPK in tissues including duodenum [414], skeletal muscles [455] and the liver [456]. Activation of AMPK by metformin in the liver is known to be achieved via elevation of AMP: ATP and ADP: ATP ratios [457]. However, an earlier study also showed that ethanol ingestion together with diet deficient in methyl donor (B9) accounted for downregulation of pAMPK resulting in increased lipogenesis [458]. Our findings may also be supported by the observation of another study in which mice fed with methyl donor deficient diet had decreased levels of AMP, ADP and ATP [459]. In support to these evidences, our findings may suggest that metformin action through AMPK is impaired in B12 deficient condition and hepatic accretion of fat may be partly mediated by AMPK.

6.5 **Conclusion**

B12 deficiency may be associated with dysregulated metabolism of lipids resulting from increased lipogenesis and impaired oxidation of fatty acids in the liver. Risk of developing B12 deficiency is associated with metformin treatment in type 2 diabetes patients. In this study, metformin treatment in low B12 hepatocyte cell line improved hepatic fatty acid biosynthesis but showed higher total TG levels and impaired FAO. Metformin, playing a crucial role in the activation of AMPK, failed to restore pAMPK levels in low B12 hepatocyte cell line. The ultimate effect was a decreased downregulation of lipogenesis and reduced upregulation of mitochondrial oxidation of fatty acids by metformin in low B12 hepatocyte cell line. Therefore, indicating that the desired lipid-lowering effect of metformin in the hepatocyte cell line was compromised by low B12 condition.

7.0 Adiponectin restores the lipid lowering effect of metformin in B12 deficient human hepatocyte cell line

7.1 Introduction

NAFLD generally comprises a range of abnormal liver disorders, extending from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH), which demonstrate variable levels of severity and may result in adverse hepatic conditions including hepatic failure, hepatocellular carcinoma and cirrhosis [460, 461]. NASH may also be associated with elevated risk of developing CVD and malignancy [462]. *De novo* lipogenesis (DNL) is reported to be one of the principal sources of FAs in NAFLD accounting for hepatic lipid accumulation [361]. Clinical studies recently showed that significant elevation in the circulating levels of total cholesterol, VLDL, LDL and TG was observed in 45.7%, 67.1%, 25.7% and 34.3% of NAFLD patients respectively, whereas in 62.9% of patients, decreased concentration of HDL was observed [463]. This may therefore suggest that increasing hepatic lipid accumulation, accounting for higher grades of NAFLD, is associated with significantly higher circulating levels of VLDL, LDL and total-cholesterol as well as decreased HDL levels [463].

Several factors that may account for improvement in hepatic lipid levels include lifestyle interventions such as general weight loss [464], prolonged adherence to calorie-restricted nutrition [465] and increased physical activity [466]. Likewise, insulin sensitizers have been proposed to improve the hepatic lipid levels in conditions of NAFLD or NASH. However, some studies showed that metformin treatment does not result in the significant improvement of liver histology in NAFLD and NASH patients [467]. Adiponectin is known to enhance several valuable effects in the liver. Some studies have shown clinical evidence that adiponectin demonstrates an inverse relationship with NAFLD, obesity and IR [468, 469]. In addition to regression of hepatic generation of glucose due its insulin sensitizing property, adiponectin further reduces inflammation, lowers hepatic fibrosis, increases survival of cells and augments the oxidation of FAs in the liver [468].

Adiponectin has been shown to exert its effects *via* adiponectin receptor subtypes adipoR1 and R2 through stimulation of AMPK (Fig 53). It is proposed that adiponectin has a potential of regulating hepatic metabolism of lipids *via* the activation of AMPK signalling pathway [470, 471]. Thus, investigating the functional role of adiponectin *via* AMPK in B12 deficiency in the presence of metformin will elucidate whether adiponectin can influence the activation status of AMPK and its effect on lipogenesis and lipid oxidation in hepatocytes. In the present study, we

performed to determine (1) whether adiponectin-mediated AMPK regulates the hepatocellular lipid accumulation in B12 deficient state and then, whether (2) treatment of adiponectin and metformin ameliorates lipid synthesis, oxidation and mitochondrial function in hepatocytes treated with low B12.

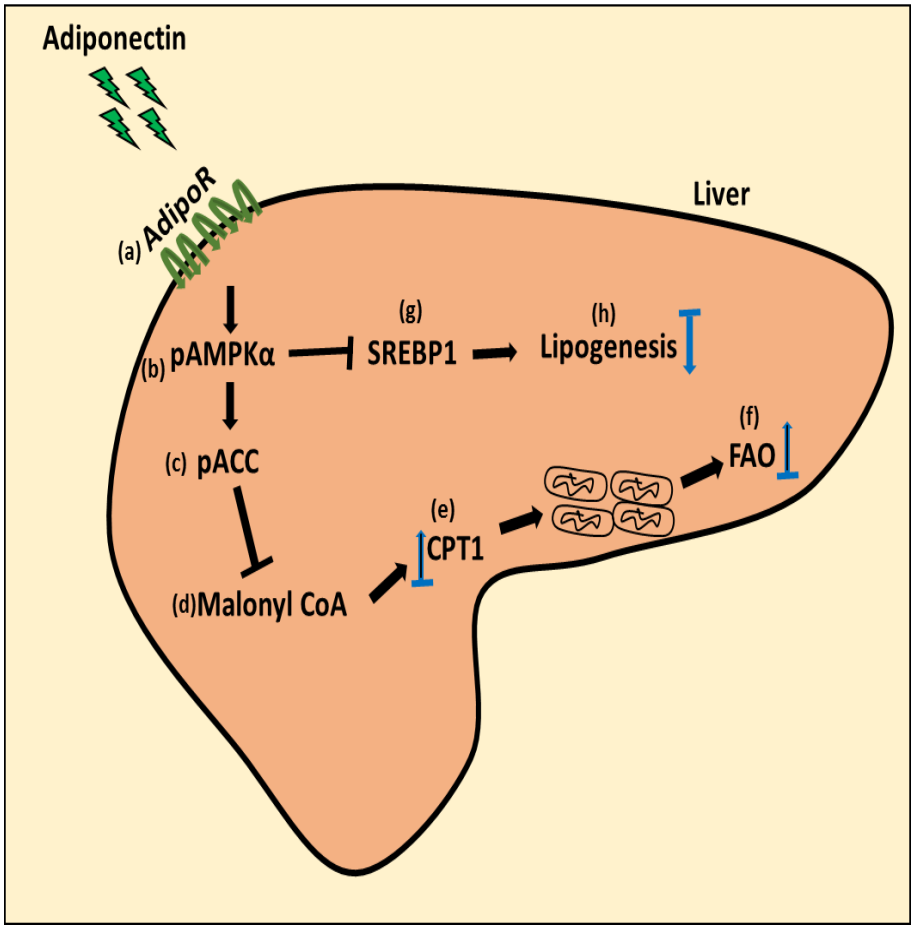


Figure 53: Mechanism of adiponectin impairment of hepatic lipid accumulation: Adiponectin activates adiponectin receptors (AdipoR1 and AdipoR2) (a) in hepatocytes leading to the activation of AMPK (b). Activated AMPK directly phosphorylates acetyl CoA carboxylase (ACC) (c) which results in the inhibition of malonyl CoA synthesis (d). Decreased malonyl CoA levels facilitates the upregulation of carnitine palmitoyl transferase 1 (CPT1) (e) and subsequently upregulates oxidation of fatty acids (FAO) (f) in the mitochondria. Similarly, activation of AMPK results in the inactivation or inhibition of the key nuclear transcription factor, sterol regulatory element binding protein 1 (SREBP1) (g), that subsequently decreases *de novo* lipid synthesis in hepatocytes (h).

7.2 Methods

7.2.1 **Optimisation for the concentration and duration of adiponectin treatment**

To optimize for the concentration and duration of adiponectin treatment, Hep G2 cells were cultured as previously described in 2.2.1. After the last medium change on the 10^{th} day, at 100% confluence, Hep G2 cells were incubated with a serum-free medium for 24-hr. This was followed by treatment with two different concentrations of adiponectin (25ng and 50ng) in a serum-free medium at different durations: 4-hr, 8-hr, 12-hr, 24-hr and 48-hr, and AICAR (positive control) for 24-hr. The cells were then harvested for both RNA and protein extractions and stored at -20^{o}C until utilized. Following the extraction of RNA and cDNA synthesis (described in sections 2.2.2.1-2.2.2.3), the effect of different concentrations and durations of adiponectin treatment on the expression of adipoR1 and R2 receptors (Fig 54), lipogenesis [FA, TG and cholesterol biosynthesis] (Fig 56) and FAO (Fig 57) was assessed using RT-qPCR. Likewise, the effect of different adiponectin concentrations and treatment durations on the levels of pAMPKα and pACC (Fig 55) was also assessed using western blot analysis. Therefore, following the optimization for two different concentrations (25ng and 50ng) of adiponectin at different durations (hours) of treatment: 0hr, 4hr, 8hr, 24hr and 48hr, we used the 25ng concentration and 24hr duration for this study.

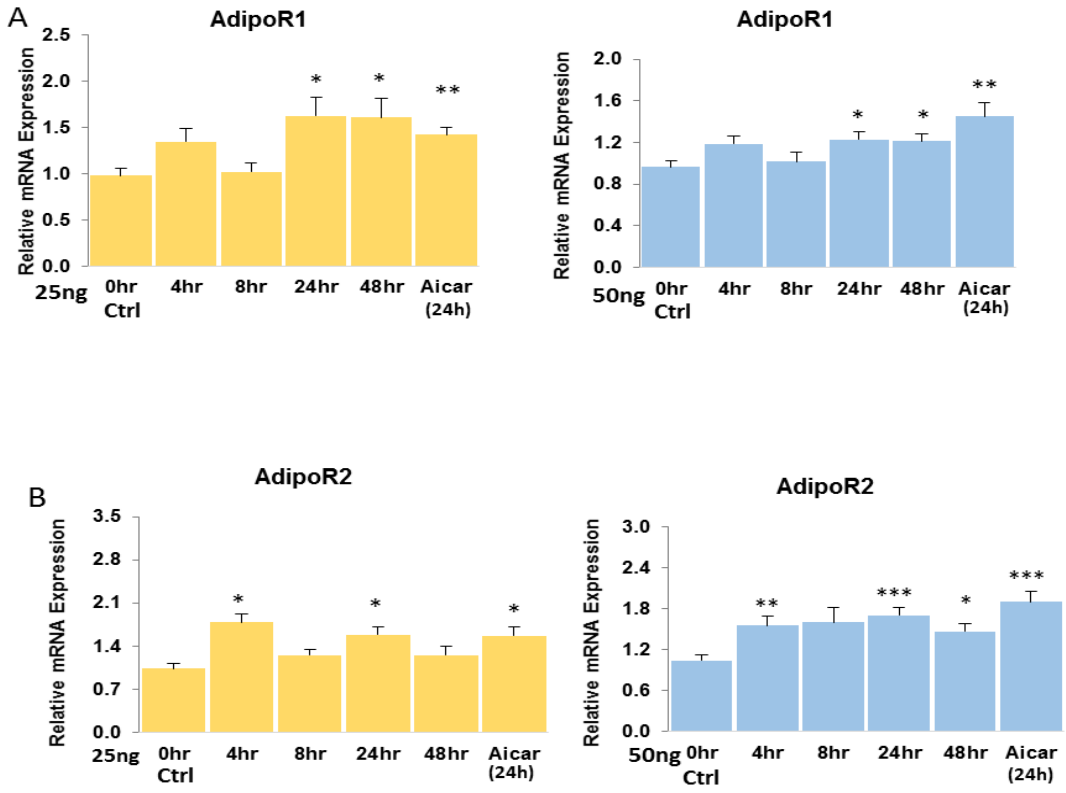


Figure 54: Dose and time-dependent activation of adiponectin receptors by adiponectin: Different concentrations of adiponectin (25ng and 50ng) activates adiponectin receptors AdipoR1 (A) and AdipoR2 (B). The mRNA expressions in hepatocytes were normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), and * indicates significance compared with 0hr (control); * p< 0.05, ** p< 0.01, *** p< 0.001.

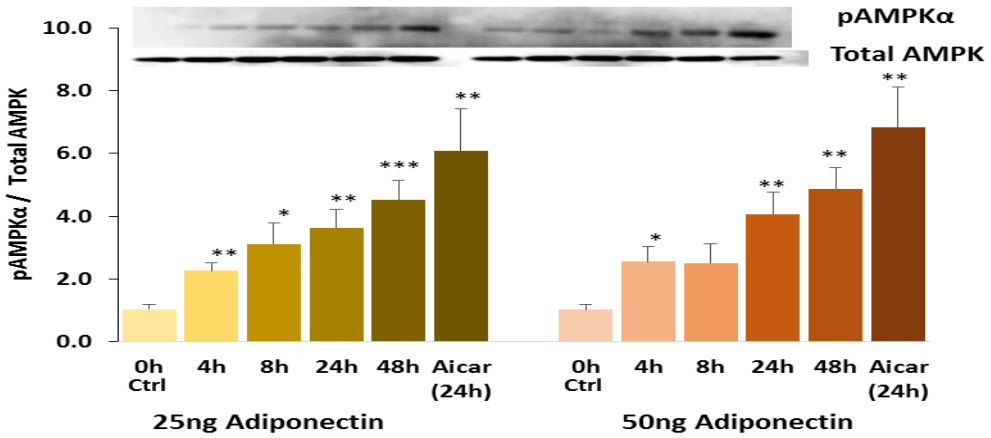


Figure 55: Dose and time-dependent activation of AMPK by adiponectin: Different concentrations of adiponectin (25ng and 50ng) upregulates pAMPKα levels in Hep G2. The data is representative of mean ± SEM (n=6), and * indicates significance compared with 0hr (control); * p< 0.05, ** p< 0.01, *** p< 0.001.

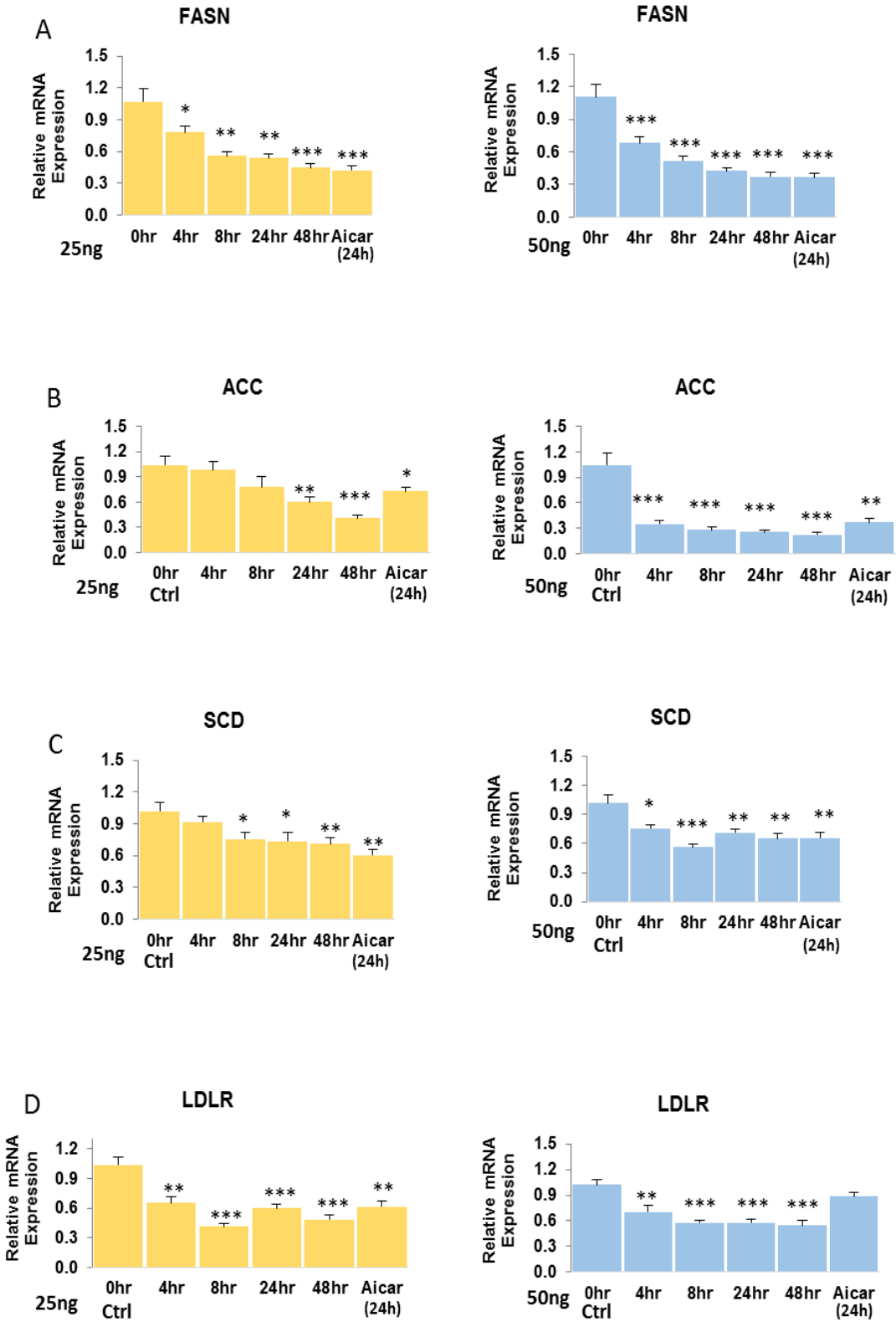


Figure 56: Dose and time-dependent effect of adiponectin on lipogenesis Different concentrations of adiponectin (25ng and 50ng) decreases enzymes involved in fatty acid [FASN (A) and ACC (B)], triglycerides [SCD (C)] and cholesterol [LDLR (D)] biosynthesis. The mRNA expressions in hepatocytes were normalized to

18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), and * indicates significance compared with 0hr (control); * p< 0.05, ** p< 0.01, *** p< 0.001.

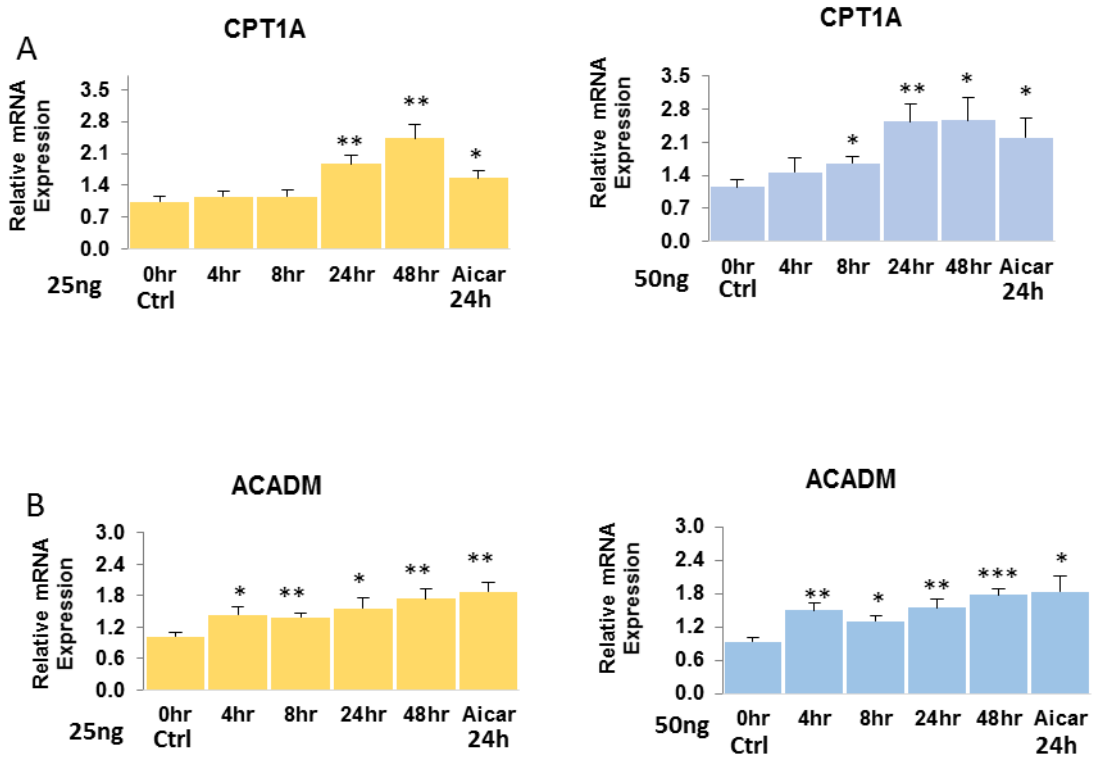


Figure 57: Dose and time-dependent effect of adiponectin on fatty acid oxidation (FAO): Different concentrations of adiponectin (25ng and 50ng) upregulated enzymes CPT1a (A) and ACADM (B) involved in fatty acid oxidation. The mRNA expressions in hepatocytes were normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), and * indicates significance compared with 0hr (control); * p< 0.05, ** p< 0.01, *** p< 0.001.

7.2.2 **Cell culture**

Hep G2 cell culture was done as previously described in section 2.2.1. On the 10th day of cell culture, the B12 (500nM and 25pM) EMEM medium was replaced with a serum-free media (0.1% BSA, 1% L-Glutamine) overnight. The hepatocyte cell line was then maintained in the serum-free media or treated with (a) 25ng adiponectin, (b) combined 25ng adiponectin and 1mM metformin and (c) 1mM AICAR (positive control) for 24-hours, following initial optimization of concentrations and duration (hours) of adiponectin and AICAR. The cells were subsequently harvested and stored at -20^{o}C until utilized for RNA and protein assays. The same treatments were done in the hepatocyte cell line for extracellular seahorse XF24 flux assay using 24-well seahorse plates.

7.2.3 **RNA isolation, cDNA synthesis and gene expression**

Total RNA isolation, cDNA synthesis and gene expression assays were same as previously described in sections 2.2.2.1-2.2.2.4 of chapter 2.

7.2.4 **Western blot analysis**

Protein characterization studies using western blot (pAMPKα and pACC proteins normalized to total AMPK and total ACC respectively), were same as previously described in section 2.2.6.3 of chapter 2.

7.2.5 **Total intracellular TG estimation**

Total intracellular TG in hepatocyte cell line was quantified as previously described in section 2.2.3 of chapter 2. In each condition, the TGs were quantified using the commercial TG quantification kit (ab65336) from Abcam plc, Cambridge, UK, following the manufacturer's protocol.

7.2.6 **Radioactive flux assay for TG biosynthesis**

Upon reaching about 100% confluence in six well plates following the last media change for different B12 conditions such as 500nM and 25pM, Hep G2 cells were treated as previously described (section 7.2.2) for 24 hours and exposed to radiolabelled fatty acid (^{14}C-oleate) for 2 hours. The cells were then harvested and the radiolabelled TGs synthesized within the hepatocyte cell line were extracted and assessed as previously described in section 2.2.4 of chapter 2.

7.2.7 **Mitochondrial dysfunction assessment using seahorse assay**

Following the same protocol in section 2.2.8.1, Hep G2 cells of different B12 conditions were afterwards trypsinized and seeded at 50 000 cells per well of the seahorse XF 24 plate and allowed to settle overnight at 37 ^{o}C and CO_2, followed by replacement of the B12-EMEM medium with a serum-free medium for 24-hours incubation, and subsequent treatment with (a) 25ng adiponectin, (b) combined 25ng adiponectin and 1mM metformin and (c) 1mM AICAR (positive control) for 24-hours in a serum-free medium for 24-hours. The samples, seahorse substrate medium and respiratory inhibitors (oligomycin, FCCP and rotenone /antimycin) were then prepared and used for the seahorse assay as previously described in section 2.2.8.

Following the estimation of OCR in a rich-KHB substrate medium, we further incubated the hepatocyte cell line in a limited-KHB substrate medium containing only 0.5mM L-carnitine and 1.25mM glucose for one hour. After the basal respiration (OCR) was measured in the hepatocyte cell line, the cells were then exposed to 200µM palmitate or 33.3µM BSA (basal control) in the substrate medium to assess how hepatocytes efficiently uptake palmitate for ATP synthesis.

7.3 Results

7.3.1 Effect of B12 on the expression of adiponectin receptors (AdipoR1 and R2)

In this study, we observed that adiponectin was not expressed in Hep G2 cells, however, the expression of adiponectin receptors (adipoR1 and adipoR2) was detected in the hepatocyte cell line. Therefore, we assessed the expression of these receptors in low B12. The gene expression of both adipoR1 and R2 receptors was significantly decreased in low B12 hepatocyte cell line compared with control (Fig 58). This evidence suggested that low B12 impaired and or downregulated the levels of adipoR1 and R2 receptors in the hepatocyte cell line accounting for decreased expression.

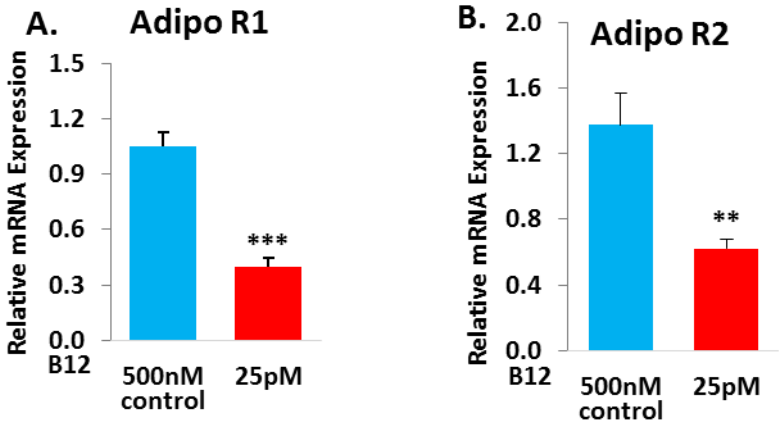


Figure 58: AdipoR1 and R2 receptors were decreased by low B12. Regulation of AdipoR1 (A) and R2 (B) in different conditions of B12: 500nM (control) and 25pM. The mRNA expressions in hepatocyte cell line were normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), and * indicates significance compared with 500nM control; * p< 0.05, ** p< 0.01, *** p< 0.001.

7.3.2 **Effect of adiponectin treatment on Adipo-R1 and R2 expression**

Adiponectin has been shown to induce the activation of signalling pathways leading to regulation of lipid metabolism by binding to adipoR1 and R2 receptors in the liver [472]. We next assessed whether treatment with adiponectin would affect the receptors in the hepatocyte cell line. Following adiponectin treatment in control cells, there was significant increased expression of adipoR1 (43.5%) and R2 (45.0%), as well as similarly increased adipoR1 (67.7%) and R2 (81.0%) receptors in low B12 (Fig 59). This implies that the adiponectin treatment upregulates adipoR1 and R2 receptors significantly in low B12 condition.

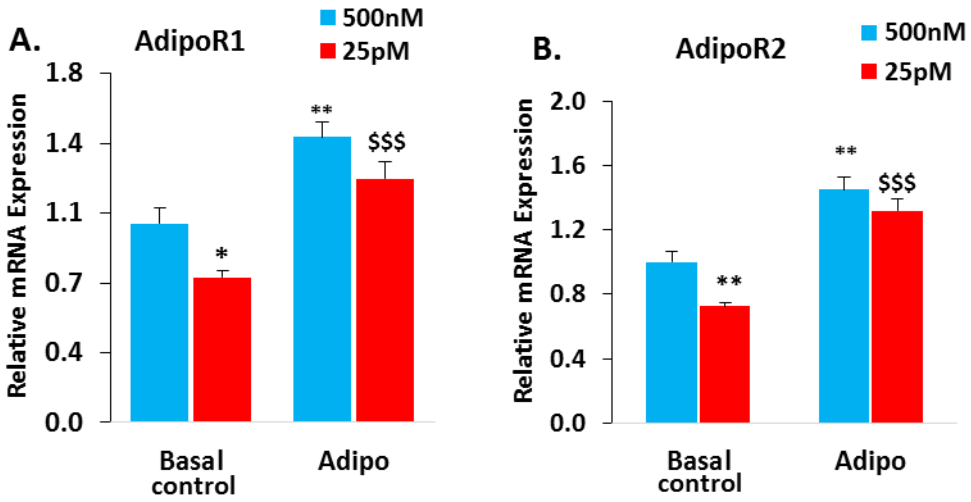


Figure 59: Adiponectin treatment independently increases AdipoR1 and R2 receptors in hepatocytes cell line Decreased expression of AdipoR1 (A) and R2 (B) in low B12 hepatocyte cell line was increased after adiponectin treatment. The mRNA expression, in different B12 conditions: 500nM (control) and low B12 (25pM) in both basal and adiponectin (Adipo) treated hepatocyte cell line, was normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.3 **Effect of adiponectin on pAMPKα and pACC levels in hepatocytes**

We further assessed whether adiponectin treatment and subsequent upregulation of adipoR1/R2 affect the activation of AMPK in B12 conditions. We observed that the levels of pAMPKα and pACC were significantly lower in low B12 compared with control. However, pAMPKα and

pACC levels as observed in control cells (199.6% and 83.3%-increase), were significantly increased in low B12 (426% and 211%) respectively following adiponectin (positive control-AICAR) treatment (Fig 60). Therefore, this suggests that there was significant activation (phosphorylation) of AMPKα and subsequent inactivation (phosphorylation) of ACC in low B12 hepatocyte cell line following adiponectin treatment.

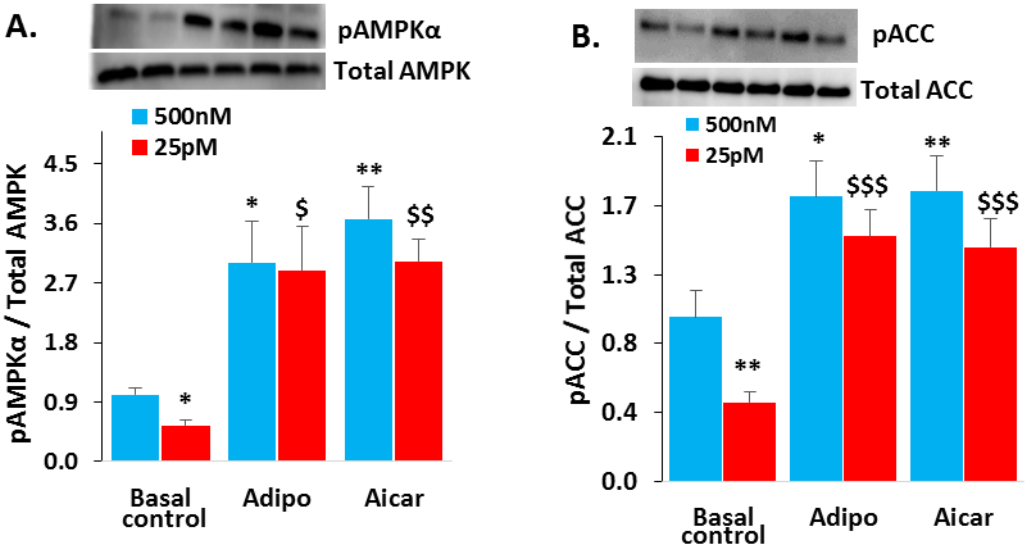


Figure 60: Adiponectin increases pAMPKα and pACC levels in hepatocyte cell line: Level of pAMPKα (A) and pACC (B) proteins in hepatocyte cell line, in different conditions of B12 [500nM (control) and 25pM (low B12)], treated with 25ng adiponectin (Adipo) or 1mM AICAR (positive control). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.4 **Effect of adiponectin on hepatic lipid metabolism**

7.3.4.1 ***De novo*** **FA synthesis**

Observing the significant elevation in the levels of pAMPKα and pACC in low B12 hepatocyte cell line following adiponectin treatment, we therefore assessed this effect on several genes regulating lipogenesis and FAO in hepatocyte cell line. In basal condition (without adiponectin), we observed that the gene expression of the key nuclear transcription factor SREBF1 and downstream genes regulating FA synthesis (ACC, FASN and ELOVL6) was upregulated in low B12 hepatocyte cell line. However, treatment with adiponectin (positive control -AICAR) in control cells resulted in significantly decreased expression of SREBF1

(22.1%), ACC (41.1%), FASN (36.8%) and ELOVL6 (22.2%) (Fig 61). Likewise, in low B12 hepatocyte cell line, significantly decreased expression of SREBF1 (41.5%), ACC (49.3%), FASN (46.7%) and ELOVL6 (44.6%) was observed. This therefore suggests that adiponectin was effective in reducing FA synthesis in low B12 hepatocyte cell line.

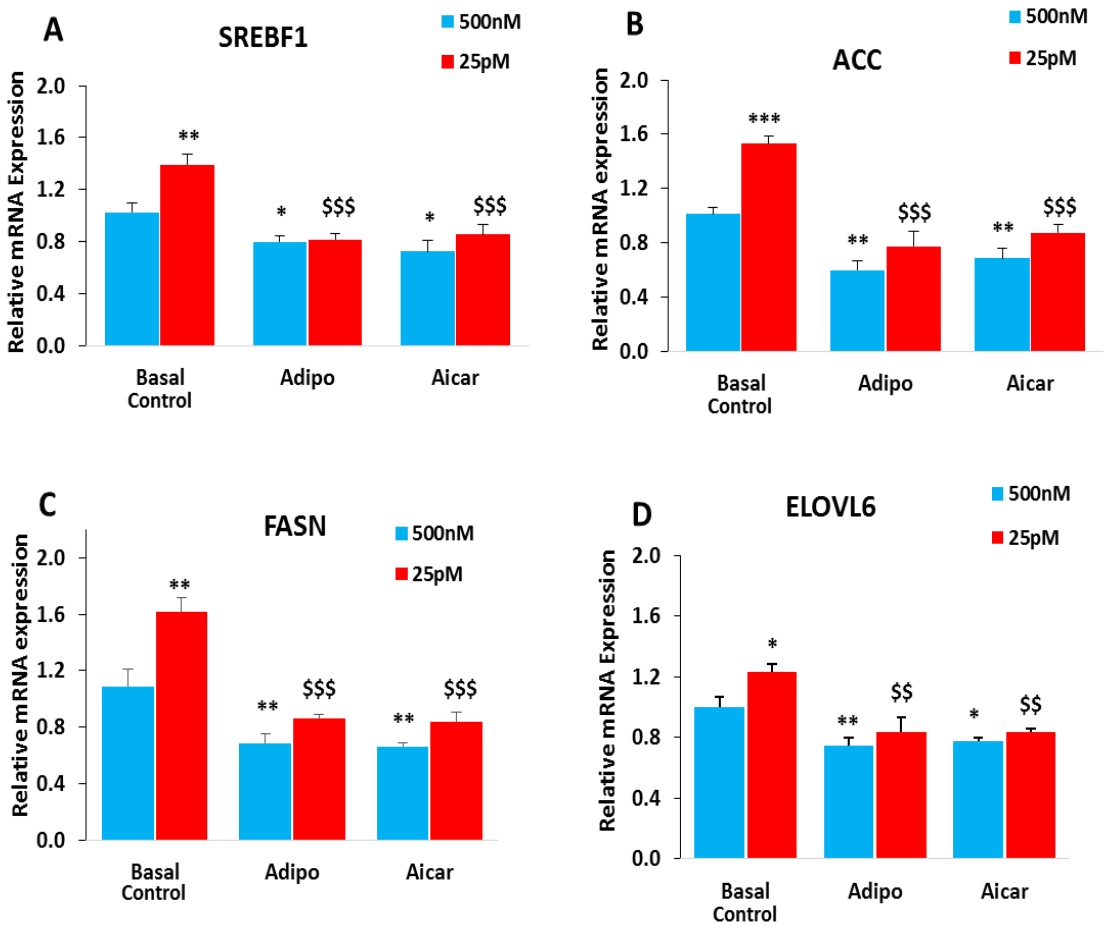


Figure 61: Adiponectin decreases FA synthesis in low B12 hepatocyte cell line. The expression of SREBF1 (A) and fatty acid synthesis genes such was ACC (B), FASN (C) and ELOVL6 (D) in hepatocytes was decreased after adiponectin (positive control-AICAR) treatment. The mRNA expressions in hepatocytes were normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.4.2 **TG and cholesterol biosynthesis**

We observed that the gene expression of enzymes regulating TG synthesis (SCD1, DGAT1 and DGAT2) and the master regulator of cholesterol (LDLR) biosynthesis in basal condition was

increased in low B12 compared with control. However, treatment with adiponectin (positive control AICAR) in resulted in significant reduction of expression of genes - SCD1 (27.2%), DGAT1 (26.3%), DGAT2 (23.1%) and LDLR (24.1%) in control cells. Similarly, in low B12 hepatocyte cell line, significantly decreased expression of genes - SCD1 (39.8%), DGAT1 (39.1%), DGAT2 (41.4%) and LDLR (36.4%) was observed following adiponectin treatment (Fig 62).

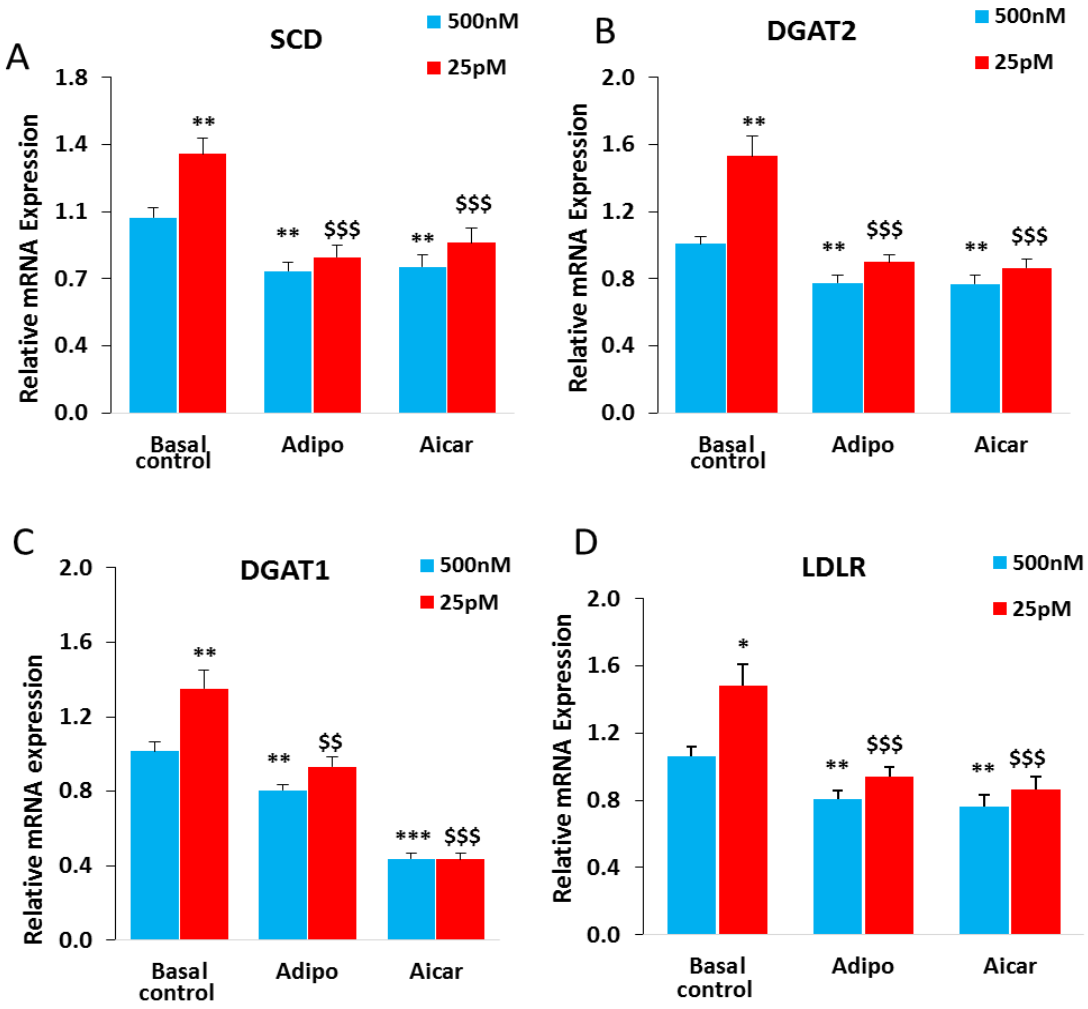


Figure 62: Adiponectin decreases TG and cholesterol synthesis in low B12 hepatocyte cell line. Expression of genes involved in TG [SCD (A), DGAT2 (B) and DGAT1 (C)] and cholesterol [LDLR (D)] biosynthesis was decreased after adiponectin (or AICAR) treatment. The mRNA expressions in hepatocytes were normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.4.3 **TG synthesis using radiolabelled FA (^{14}C-oleate)**

To confirm adiponectin effect on TG synthesis, we performed the radioactive flux assay to assess the uptake and esterification of FA (radiolabelled) for TG synthesis in low B12 hepatocyte cell line. Similarly, in basal condition, the exposure of hepatocyte cell line to radio-labelled FA (^{14}C-Oleate) resulted in significantly high levels of TG in low B12 compared with control. However, adiponectin (positive control - AICAR) treatment resulted in significantly lower level of TG synthesized in low B12 (41.9%) compared with control (23.6%-decrease). This therefore shows that adiponectin treatment alleviates the effect of low B12 on the uptake and esterification of FA for TG synthesis in low B12 hepatocyte cell line (Fig 63).

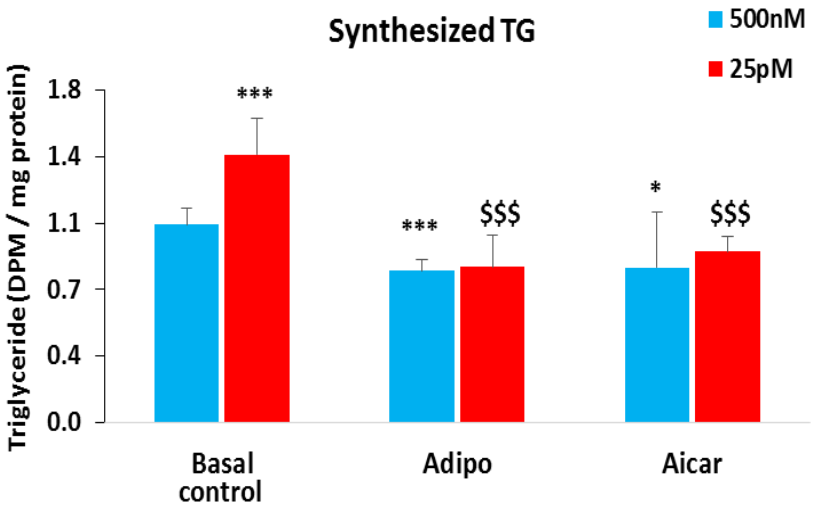


Figure 63: Adiponectin decreased synthesis of TGs: Radioactive flux assay was performed after seeding 75.000 Hep G2 cells under different conditions of B12 [500nM (control), and 25pM] until reaching 100% confluence at 10th day of the cell culture. The cells were then treated with 25ng adiponectin (or 1mM AICAR) in a serum-free media for 24 hours. After this hepatocytes were incubated with ^{14}C-Oleate (0.75mM concentration at 2000 dpm/nmol) and L-carnitine (1mM) to facilitate incorporation of ^{14}C-Oleate into the hepatocyte cell line for two (2) hours in EMEM, against five (5) minutes incubation for background normalizing control at 37^{o}C and 5% CO_2 saturation. The cells were harvested in 2ml methanol and the synthesized radiolabelled. TG was extracted, following an initial total lipid isolation with the liquid-liquid (chloroform-methanol, 2:1 v/v) extraction method, using a mobile phase such as hexane/diethtyl ether/formic acid (v/v/v, 70/30/1) on thin layer chromatography (TLC) plate with 10 nmol glyceryltripalmitate (tripalmitin) as a standard. TG bands on TLC plates were transferred into vials of 5ml scintillation fluid for scintillation counter assessment of radioactivity (for 5 minutes) of the synthesized TG fraction, normalized per milligram (mg) protein concentration by Bradford method. The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.4.4 **Total intracellular TG level**

Since adiponectin was effective in the reduction of gene expression of lipid (FA, TG and cholesterol) synthesis, we further assessed its effect on the total intracellular TG levels in low B12 hepatocyte cell line. Similarly, we observed that total intracellular TG level in basal condition was initially higher in low B12 compared with control. Following treatment with adiponectin (positive control – AICAR), the total TG level in low B12 was significantly reduced by 33.2% compared with hepatocyte cell line in control B12 (20.5%). This suggests that adiponectin reduces intracellular TG levels significantly in hepatocyte cell line in low B12 (Fig 64).

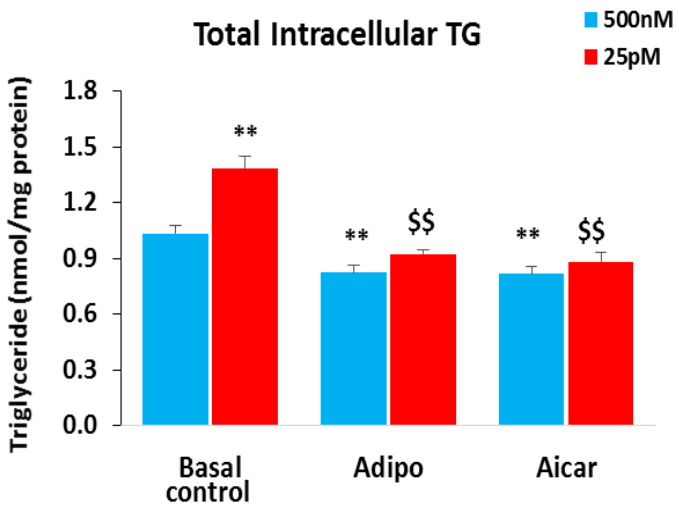


Figure 64: Adiponectin decreased total intracellular TGs. Total intracellular level of TG was quantified in Hep G2 cells under different B12 conditions: 25pM and 500nM (control), using the TG kit (ab65336, Abcam plc, UK) and normalized per milligram protein of hepatocyte cell line in each B12 condition: 500nM (control) and 25pM (low B12). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.4.5 **Fatty acid oxidation (FAO)**

Since adiponectin decreased lipogenesis significantly in low B12 hepatocyte cell line, we further assessed how the FAO pathway may be affected by adiponectin treatment. In basal condition, the gene expression of CPT1α and ACADM regulating the FAO pathway was decreased in low B12 hepatocyte cell line compared with control B12. However, adiponectin (positive control- AICAR) treatment resulted in significant upregulation of the FAO genes in low B12 accounting for 60.8% increase in CPT1α and 56.6% in ACADM compared with control (CPT1 - 37.2% and ACADM - 47.6% - increase) (Fig 65).

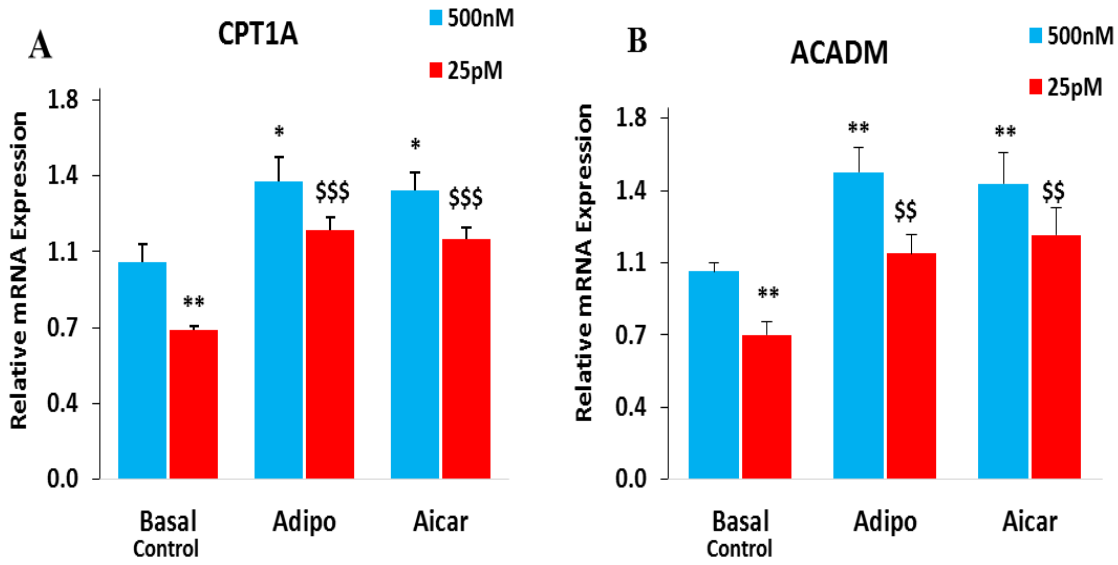


Figure 65: Adiponectin increased FAO genes in low B12 hepatocyte cell line. Expression of genes involved in oxidation of fatty acids such as CPT1a (A) and ACADM (B) were increased after adiponectin (or AICAR) treatment. The mRNA expressions in the hepatocyte cell line were normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.4.6 **Mitochondrial functional assessment**

a. **Effect of adiponectin on the oxygen consumption rate (OCR) and spare respiratory capacity (SRC) in hepatocyte cell line**

Next, we assessed the independent effect of adiponectin on the mitochondrial functional efficiency in the utilization of substrate supply for respiration. We observed that in basal condition, the functional efficiency (OCR and SRC) was decreased in low B12 compared with control. However, treatment with adiponectin (positive control - AICAR) resulted in significant increase in mitochondrial functional efficiency (OCR and SRC) in low B12 (89%-increase) compared with basal control (42%). This evidence indicates that there was significant improvement in the functional efficiency induced by adiponectin in hepatocyte cell line in low B12 (Fig 66).

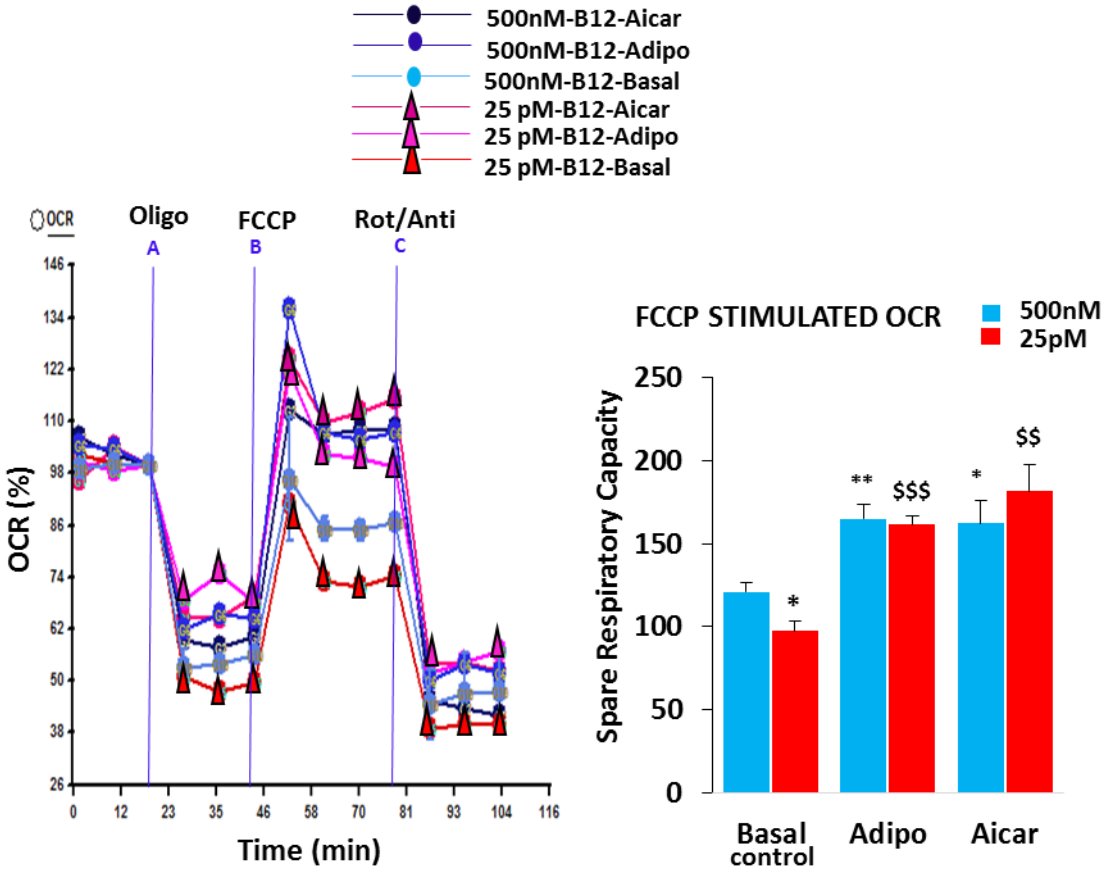


Figure 66: Adiponectin improves mitochondrial functional capacity in low B12 hepatocytes. The oxygen consumption rates and spare respiratory capacities were measured from hepatocytes in a substrate-rich media injected with respiratory inhibitors such as oligomycin, FCCP and rotenone /antimycin, The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents

significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

b. **Effect of adiponectin on the uptake of palmitate for mitochondrial respiration**

Furthermore, we assessed the effect of adiponectin on the uptake of long chain FA palmitate from the substrate medium for mitochondrial respiration in Hep G2 cells. The functional efficiency (OCR and SRC), following adiponectin (positive control - AICAR) treatment, was significantly increased in the hepatocyte cell line exposed to only palmitate in low B12 (122%) compared to control (68%) (Fig 54). Likewise, exposure to palmitate in the presence of respiratory inhibitors (oligomycin, FCCP and rotenone/antimycin) accounted for 108% increase in low B12 compared with control (120%) (Fig 67). This evidence signifies that adiponectin enhanced the uptake and utilization of palmitate in low B12 hepatocyte cell line for mitochondrial respiration, therefore, preventing the accumulation of lipids in the hepatocyte cell line.

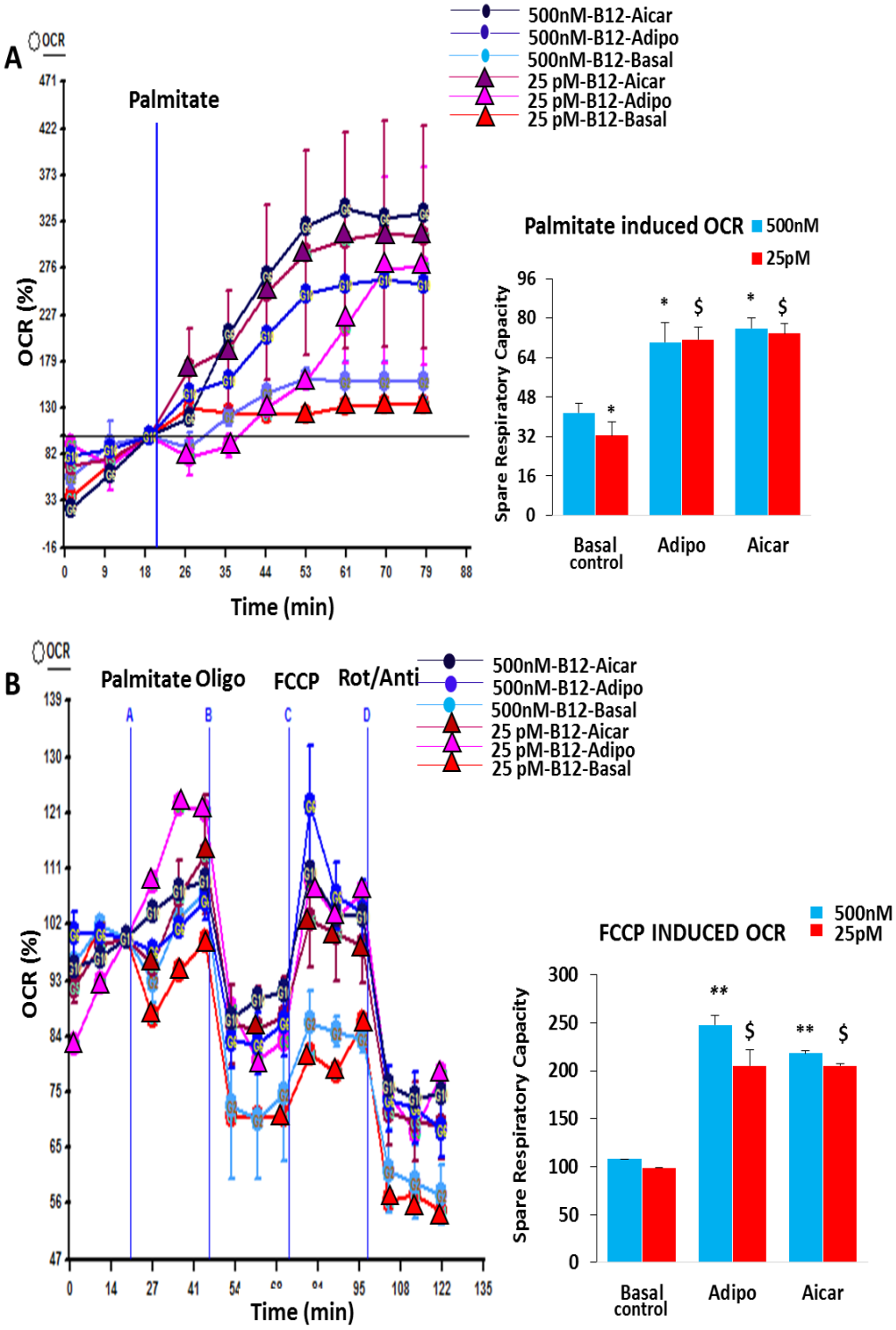


Figure 67: Adiponectin improves mitochondrial utilization of palmitate in low B12 hepatocyte cell line. The maximal respiratory capacity, assessed by the OCR, and the SRC were measured from Hep G2 cells (A) in substrate-limited media injected with palmitate and (B) in substrate-limited media injected with palmitate followed by respiratory inhibitors such as oligomycin, FCCP and rotenone / antimycin. The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents

significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.5 **Combined adiponectin-metformin treatment effect on lipid metabolism**

7.3.5.1 **Effect of adiponectin and metformin co-treatment on adiponectin receptors**

Since adiponectin treatment independently reduced lipid accumulation in low B12 hepatocyte cell line, we further assessed the effect of combined adiponectin and metformin treatment on lipid metabolism of the hepatocyte cell line in low B12. We observed in our previous chapters that metformin alone had no effect on expression of AdipoR1 and R2 receptors in Hep G2. However, the gene expression of adipoR1 and R2 receptors was significantly upregulated in low B12 (56.6% and 56.9%) compared with control (28.3% and 26.8%- respectively) following the combined adiponectin-metformin treatment in hepatocyte cell line (Fig 68).

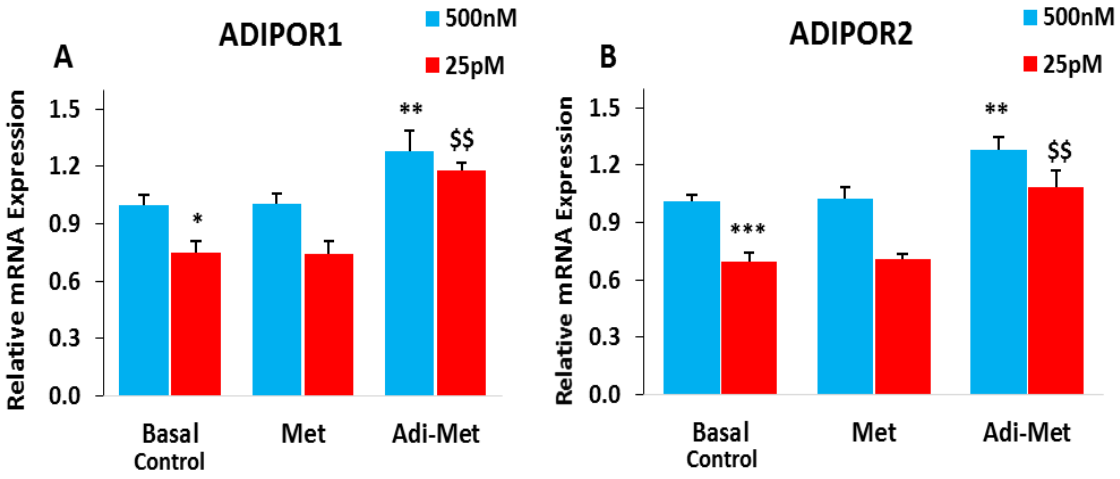


Figure 68: Adiponectin and metformin co-treatment increased AdipoR1 and R2 receptors in low B12 hepatocyte cell line. Expression of AdipoR1 (A) and R2 (B) receptors were unaffected by metformin but increased after adiponectin-metformin co-treatment. The mRNA expressions in hepatocytes were normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.5.2 **Effect of adiponectin and metformin co-treatment on the activation of AMPKα in low B12 hepatocyte cell line:**

Further to the upregulation in adipoR1 and R2 by the combined adiponectin-metformin treatment in low B12 hepatocyte cell line, we subsequently assessed how this affects the phosphorylation of AMPK and ACC. We observed that the combined adiponectin-metformin treatment resulted in significant upregulation in pAMPKα and pACC levels (257.0% and 229.0%) in low B12 compared with control (75.0% and 69.4%) (Fig 69). This evidence suggests that the combined adiponectin-metformin treatment resulted in a significant activation of AMPKα and subsequent inactivation of ACC in low B12 hepatocyte cell line.

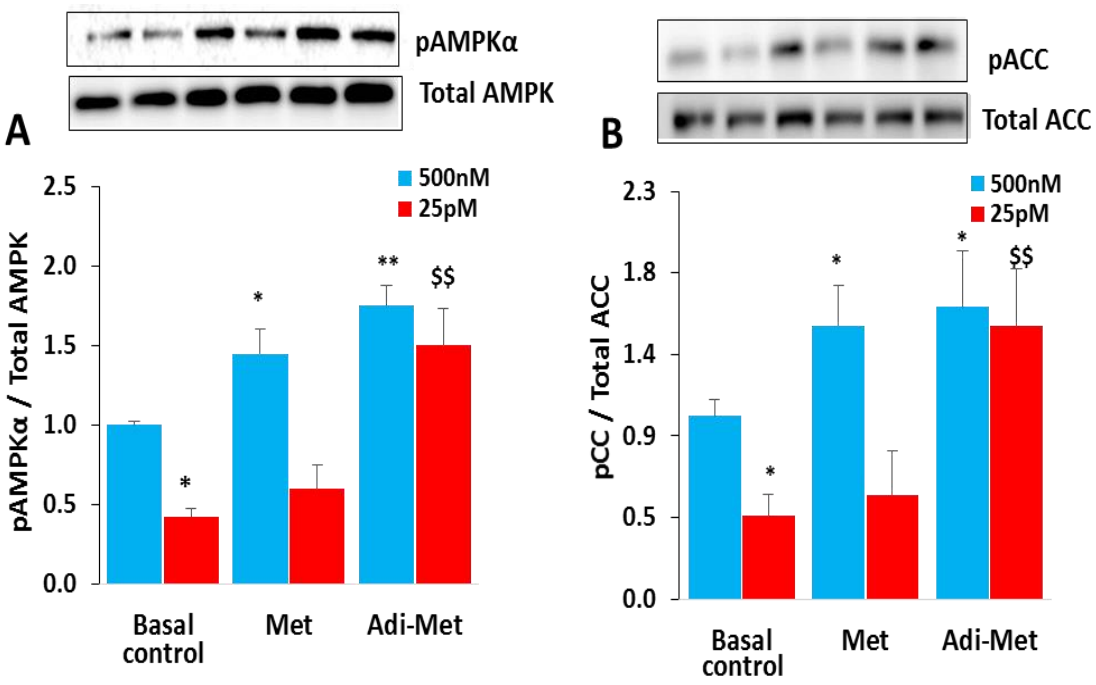


Figure 69: Adiponectin and metformin co-treatment increased pAMPKα and pACC levels in hepatocyte cell line: Level of pAMPKα (A) and pACC (B) proteins in hepatocytes significantly increased in combined adiponectin-metformin treated hepatocytes in different conditions of B12: 500nM (control) and 25pM. The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.6 **Effect of adiponectin and metformin co-treatment on lipogenesis**

7.3.6.1 ***De novo* FA synthesis**

Following the increased levels of activated AMPK (pAMPKα) in hepatocyte cell line after the combined treatment with adiponectin and metformin, we further assessed the subsequent effect on lipogenesis. The expression genes regulating *de novo* FA synthesis was significantly downregulated in low B12, accounting for the respective decrease of - SREBF1 (47.7%), ACC (40.0%), FASN (46.7%) and ELOVL6 (42.5%), compared with control [SREBF1 (41.8%), ACC (32.4%), FASN (45.6%) and ELOVL6 (40.2%)-decrease] in the combined adiponectin-metformin treated hepatocyte cell line (Fig 70).

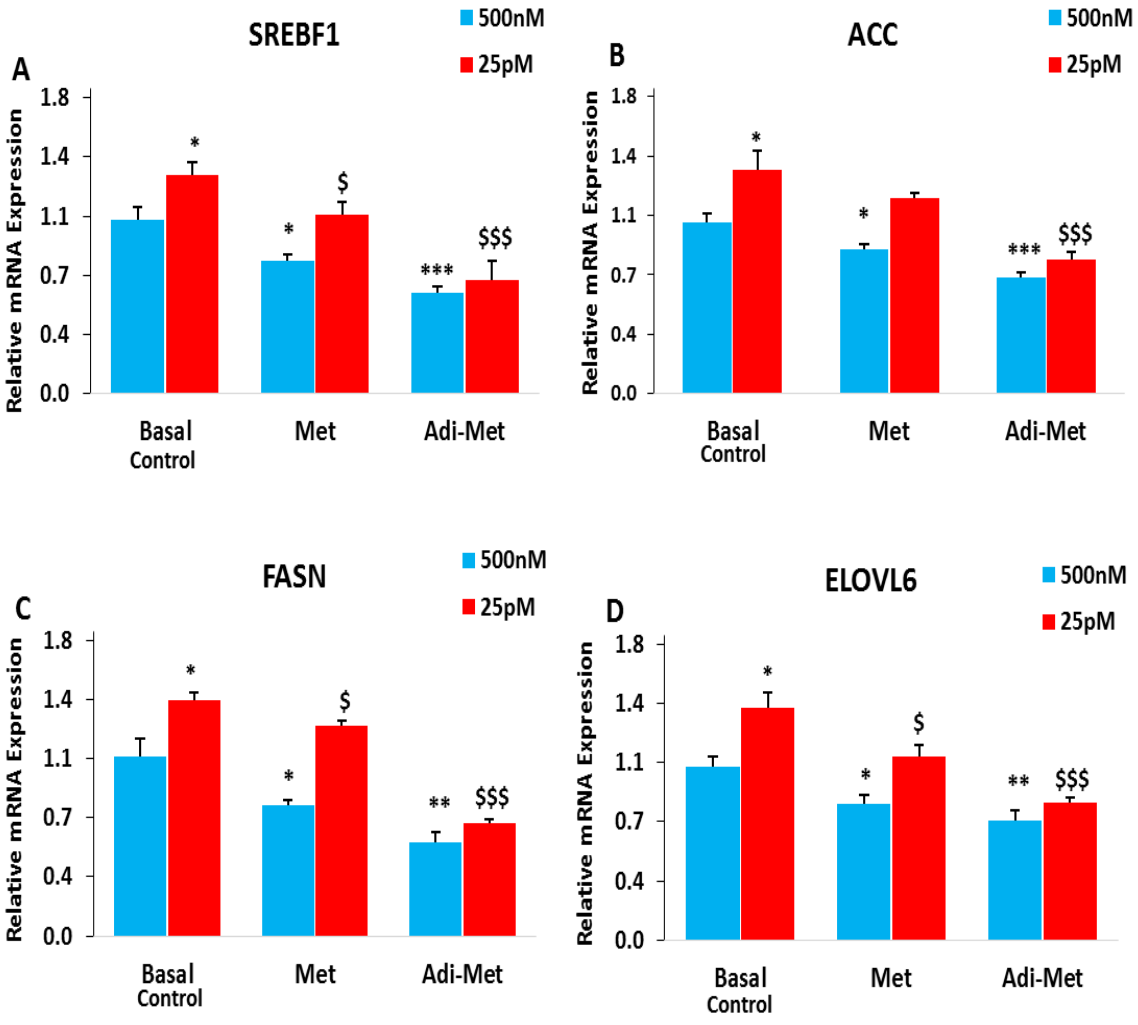


Figure 70: Adiponectin and metformin co-treatment decreased FA synthesis. The nuclear transcription factor SREBF1 (A) and downstream genes ACC (B), FASN (C) and ELOVL6 (D) involved in *de novo* FA synthesis were decreased after adiponectin-metformin co-treatment. The mRNA expressions in hepatocyte cell line were normalized to 18S rRNA endogenous control (Applied Biosystems, UK. The data is representative of mean ± SEM

(n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.6.2 **TG and cholesterol biosynthesis**

Similarly, assessment of the TG and cholesterol synthesis showed that combined adiponectin-metformin treatment significantly downregulated the expression of genes regulating the synthesis of TG [SCD1 (49.7%), DGAT1 (45.6%) and DGAT2 (40.6%)-decrease] and the master regulator of cholesterol LDLR (44.2%) biosynthesis in low B12, than control [SCD1 (36.6%), DGAT1 (38.2%), DGAT2 (30.5%) and LDLR (31.1%)-decrease] in the combined adiponectin-metformin treated hepatocytes (Fig 71).

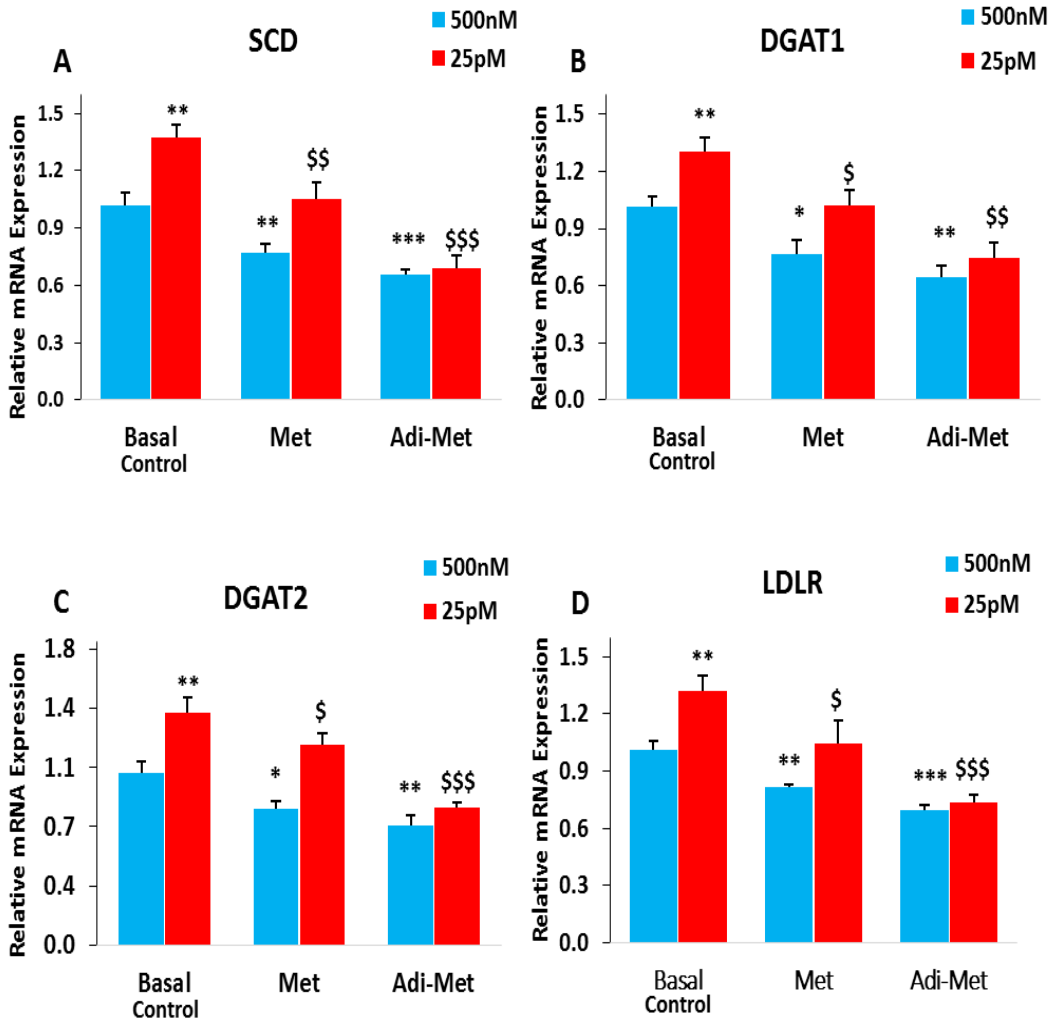


Figure 71: Adiponectin and metformin co-treatment decreased genes regulating TG and cholesterol synthesis: Expression of genes involved in triglyceride [SCD (A), DGAT2 (B) and DGAT1 (C)] and cholesterol [LDLR (D)] biosynthesis was decreased by adiponectin-metformin treatment. The mRNA expressions in

hepatocyte cell line were normalized to 18S rRNA endogenous control (Applied Biosystems, UK). The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.6.3 **FA (radiolabelled) uptake for TG Synthesis**

To further validate the evidence of decreased expression of TG biosynthesis genes in low B12 hepatocyte cell line resulting from the combined adiponectin-metformin treatment, we further assessed the level of radiolabelled TGs synthesized following exposure of the hepatocyte cell line to radiolabelled long chain fatty acid (^{14}C-oleate) and L-carnitine. We observed that the level of radiolabelled TG synthesized in the hepatocyte cell line was significantly decreased (49.1%) in low B12 compared with control (38.5%) in the combined adiponectin-metformin treated hepatocyte cell line (Fig 72). This therefore suggests that combined adiponectin-metformin treatment decreased the utilization of free long chain FA (oleate) for TG synthesis in low B12 hepatocyte cell line.

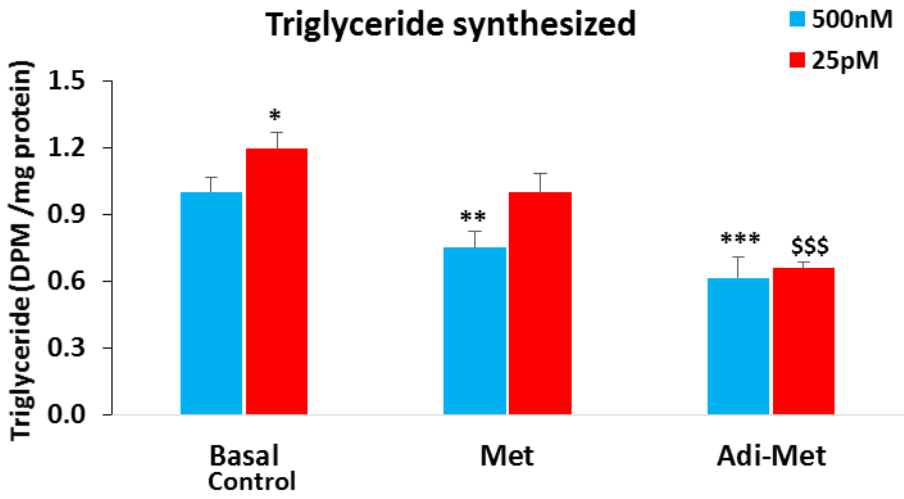


Figure 72: Adiponectin-metformin co-treatment decreased synthesis of TGs: Radio-active flux assay was performed after seeding 75.000 Hep G2 cells under different conditions of B12 [500nM (control), and 25pM] until reaching 100% confluence at 10th day of the cell culture. The cells were then treated with combined 25ng adiponectin and 1mM metformin in a serum-free media for 24-hours. After this, the cells were incubated with ^{14}C-Oleate (0.75mM concentration at 2000 dpm/nmol) and L-carnitine (1mM) to facilitate incorporation of ^{14}C-Oleate into hepatocytes for two (2) hours in EMEM, against five (5) minutes incubation for background normalizing control at 37ºC and 5% CO_2 saturation. Hep G2 cells were harvested in 2ml methanol and the synthesized radiolabelled. TG was extracted, following an initial total lipid isolation with the liquid-liquid (chloroform-

methanol, 2:1 v/v) extraction method, using a mobile phase such as hexane/diethtyl ether/formic acid (v/v/v, 70/30/1) on thin layer chromatography (TLC) plate with 10 nmol glyceryltripalmitate (tripalmitin) as a standard. TG bands on TLC plates were transferred into vials of 5ml scintillation fluid for scintillation counter assessment of radioactivity (for 5 minutes) of the synthesized TG fraction, normalized per milligram (mg) protein concentration by Bradford method. The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.6.4 **Total intracellular triglyceride level**

To verify the impact of decreased lipogenesis resulting from the combined adiponectin-metformin treatment in low B12 hepatocyte cell line, the total intracellular TG levels in Hep G2 cells were subsequently quantified and assessed. We observed that the total intracellular TG level was significantly decreased (36.1%) in low B12 compared to control (24.9 %) in the combined adiponectin-metformin treated hepatocyte cell line (Fig 73). This evidence therefore suggests that the combined therapy reduced the total TG levels significantly in low B12 hepatocyte cell line.

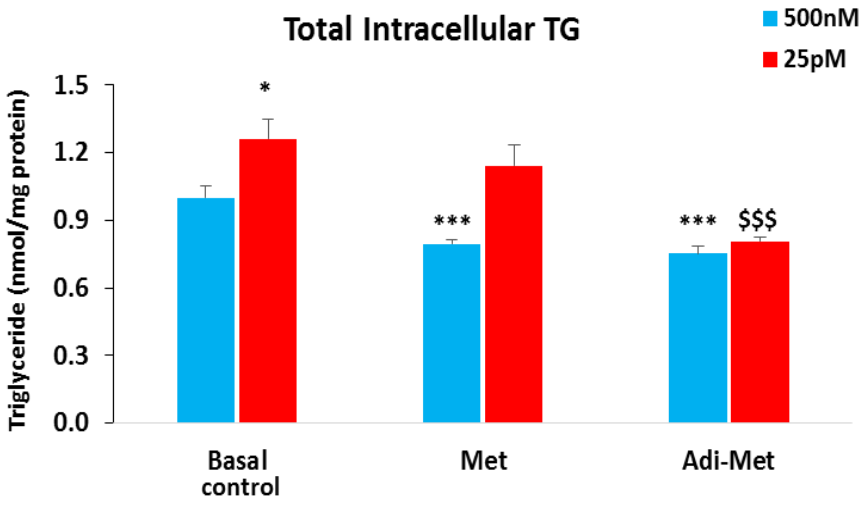


Figure 73: Adiponectin-metformin co-treatment decreased total intracellular TGs. Total intracellular levels of triglycerides following combined adiponectin and metformin treatment were quantified in hepatocyte cell line under different B12 conditions such as 25pM and 500nM (control) using the TG kit (ab65336, Abcam plc, UK) and normalized per milligram protein of hepatocytes under each B12 condition. The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.7 **Effect of combined adiponectin-metformin treatment on FAO in low B12 hepatocyte cell line**

Following the observation of significantly decreased lipogenesis by adiponectin-metformin co-treatment in low B12 hepatocyte cell line, we further assessed the effect on the FAO. We observed that, the rate limiting enzyme CPT1a as well as ACADM were significantly upregulated, accounting for 68.8% (CPT1a) and 112.8% (ACADM) -increase in low B12 compared with control [45.1% (CPT1a) and 53.7% (ACADM)] in the combined adiponectin-metformin treated hepatocyte cell line (Fig 74). This suggests that FAO in the hepatocyte cell line was significantly improved by adiponectin-metformin combined treatment in low B12 hepatocytes.

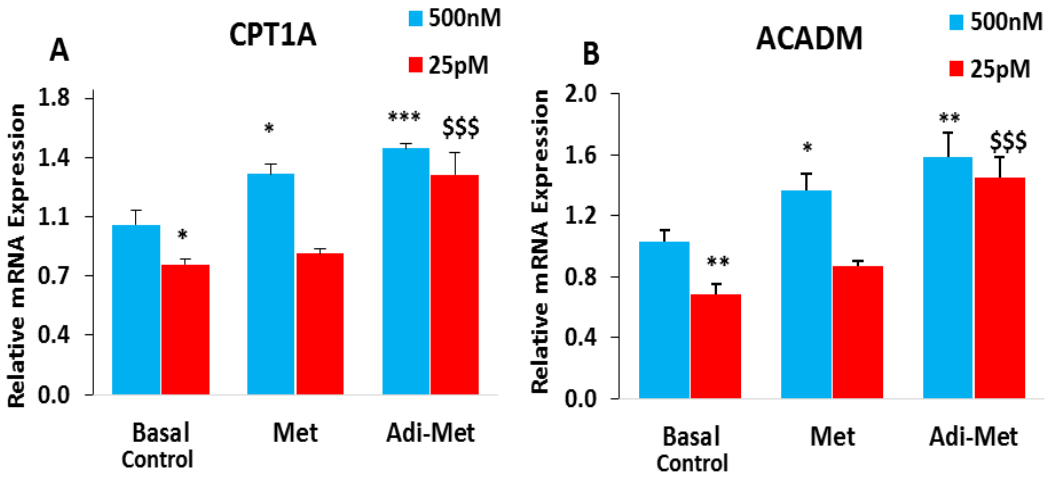
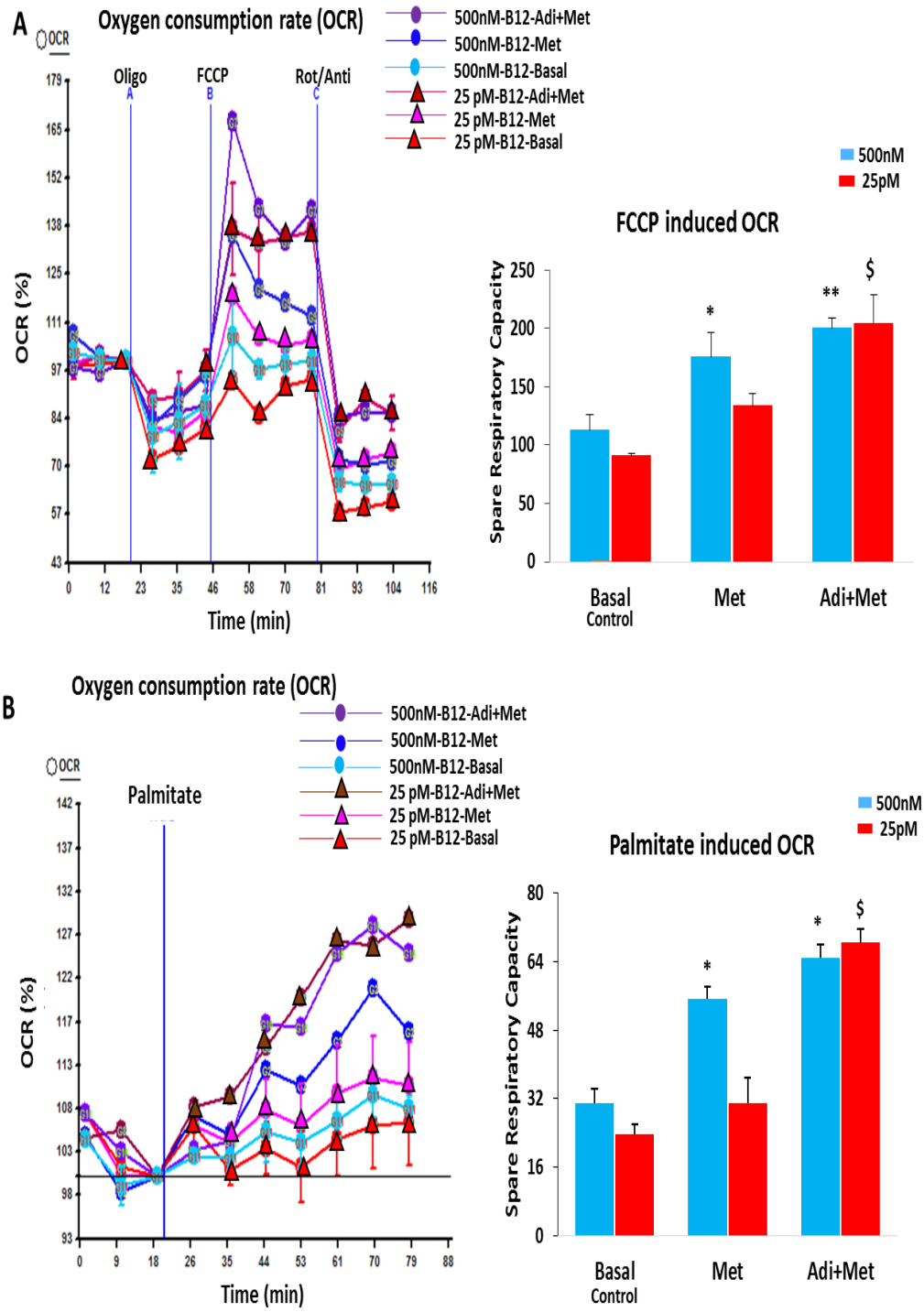


Figure 74: Adiponectin-metformin co-treatment increases CPT1a and ACADM regulating FAO: The mRNA expression of enzymes involved in the regulation of FAO such as CPT1a (A), and ACADM (B) normalized to 18S rRNA endogenous control (Applied Biosystems, UK) in hepatocytes with a combined adiponectin-metformin treatment. The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.3.8 **Effect of combined adiponectin-metformin treatment on mitochondrial functional potency**

To obtain further evidence in support of the FAO, the mitochondrial functional integrity was assessed using the extracellular seahorse XF24 flux assay. Following the combined adiponectin-metformin treatment, we observed that the mitochondrial functional efficiency (OCR and SRC) of hepatocyte cell line in the presence of respiratory inhibitors (Oligomycin,

FCCP and rotenone/ antimycin) was significantly increased in low B12 (125%) compared with control (77%) (Fig 75A). The basal uptake of long chain FA (palmitate) by the hepatocyte cell line after adiponectin-metformin co-treatment resulted in significantly increased functional efficiency (OCR and SRC) in low B12 (186%) compared with control (109%) (Fig 75B). Lastly, the functional efficiency of adiponectin-metformin treated hepatocyte cell line exposed to palmitate in the presence of the respiratory inhibitors, was significantly higher in low B12 (75%) compared to control (66%) (Fig 75C). This evidence shows that the efficiency of mitochondrial respiration utilizing palmitate in the substrate was significantly improved following combined adiponectin-metformin treatment, therefore, suggesting that accumulation of long chain FA (palmitate) in low B12 can be prevented by adding adiponectin treatment to metformin treated hepatocyte cell line..


A
Oxygen consumption rate (OCR)
OCR
Oligo
FCCP
Rot/Anti
A
B
C
500nM-B12-Adi+Met
500nM-B12-Met
500nM-B12-Basal
25 pM-B12-Adi+Met
25 pM-B12-Met
25 pM-B12-Basal
OCR (%)
Time (min)
500nM
25pM
FCCP induced OCR
Spare Respiratory Capacity
Basal
Control
Met
Adi+Met
B
Oxygen consumption rate (OCR)
Palmitate
Palmitate induced OCR

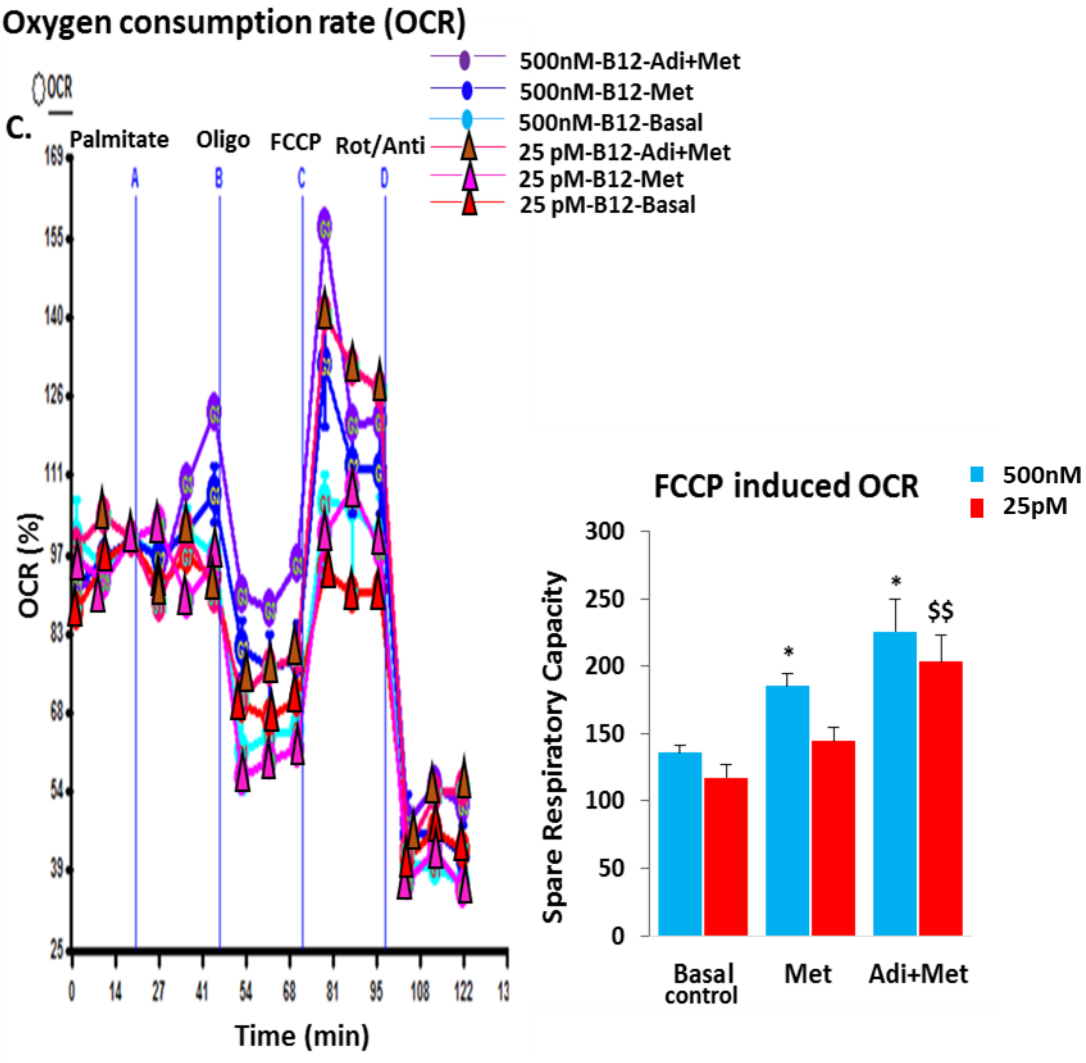


Figure 75: Adiponectin-metformin co-treatment improves mitochondrial functional capacity in low B12 hepatocyte cell line. The oxygen consumption rates and spare respiratory capacities were measured from hepatocyte cell line; (A) in a substrate-rich media injected with respiratory inhibitors such as oligomycin, FCCP and rotenone /antimycin, (B) in substrate-limited media injected with palmitate and (C) in substrate-limited media injected with palmitate followed by respiratory inhibitors such as oligomycin, FCCP and rotenone / antimycin. The data is representative of mean ± SEM (n=6), whereby * represents significance compared with 500nM-basal control; and $ represents significance compared with 25pM-basal control; * p< 0.05, ** p< 0.01, *** p< 0.001; $< 0.05, $$< 0.01, $$$< 0.001.

7.4 **Discussion**

In the current study we observed that; (1) adiponectin was not expressed in Hep G2 cell line, however, the expression of adiponectin receptors (AdipoR1 and AdipoR2) in the hepatocyte cell line was detected but decreased in low B12 compared with control. (2) Adiponectin treatment however increased adipo-R1 and R2 receptors in low B12 hepatocyte cell line and increased pAMPKα and pACC levels. (3) This resulted in downregulation of genes involved in FA, TG and cholesterol synthesis. (4) Similarly, adiponectin increased expression of key genes involved in FAO in low B12 hepatocyte cell line and improved mitochondrial functional efficiency. Observing the independent lipid lowering effect of adiponectin in low B12 hepatocyte cell line, we therefore combined adiponectin with metformin treatment in low B12 condition and (5) observed significant elevation in AdipoR1 and R2 receptors leading to increased pAMPKα and pACC levels. (6) This resulted in decreased FA, TG and cholesterol synthesis with increased FAO. (7) Finally, the mitochondrial functional integrity was also improved in low B12 hepatocyte cell line and therefore accounting for decreased accumulation of lipids in the cells.

Recent studies in bovine hepatocytes [470] and pancreatic beta cells [473] have demonstrated that adiponectin activates AMPK signalling pathway, mediates suppression of lipid synthesis and promotes lipid oxidation. Studies also showed that adiponectin decreased TG and FA levels in muscle and liver [474]. In this study, though adiponectin was not expressed in Hep G2 cells, the expression of adipoR1 and R2 receptors was detected but found reduced in low B12 condition. Following treatment with adiponectin, we observed an increase in the expression of adipoR1 and R2 receptors leading to higher activation of AMPK and pACC levels in low B12. A similar study confirmed that mRNA expression of adiponectin was undetectable in liver tissues of rat, humans and primary hepatocytes [475]. Some studies have also confirmed the detection of adipoR1 and R2 in the liver [476-479] and others report a reduction of both receptors in obesity [480] which is also clinically associated with low B12. In addition to increased phosphorylation of AMPK, we also observed that adiponectin treatment resulted in elevation of pACC levels in low B12 hepatocyte cell line. AMPK is reported to induce inhibition of lipid biosynthesis through phosphorylation of acetyl-CoA carboxylation (ACC) [479, 481]. Assessing the effect of the upregulation in AdipoR1 and R2, pAMPKα and pACC levels by adiponectin in low B12 state, we observed that the master regulator of lipogenesis SREBF1 and downstream genes involved in *de novo* FA (ACC, FASN and ELOVL6), TG (SCD, DGAT1 and DGAT2) and cholesterol (LDLR) synthesis were significantly decreased. Suppression of SREBP1c by adiponectin *via* AdipoR1 and AMPK dependent pathway has been

shown in a mice study [482, 483]. Similar studies have shown that AMPK activation accounts for inhibition of FA synthesis in the liver due to downregulation of genes such as ACC [484, 485], FASN [485, 486] and ELOVL6 [485]. Similarly, activation of AMPK has been reported to downregulate enzymes involved in TG (SCD [485] and DGAT [487]) and cholesterol (LDLR [488, 489]) synthesis. In this study, we also observed that adiponectin promoted FAO *via* increasing the expression of CPT1a and ACADM in low B12 hepatocyte cell line. Adiponectin also improved the mitochondrial functional integrity in the utilization of substrates such as palmitate for energy metabolism in this study. This is consistent with findings in skeletal muscles demonstrating that adiponectin upregulated FAO by increasing the expression of CPT1 [490] as well as elevated mitochondrial number and function, leading to increased oxidation of palmitate [491]. A mice model has also shown that activation of AMPK with subsequent facilitation of FAO was achieved *via* binding of adiponectin to AdipoR1 and R2 [492], therefore, confirming our observation that adiponectin in circulation might bind to these adipoR1/R2 in low B12 state and might activate AMPK and FAO.

Observing the independent lipid lowering effect of adiponectin in low B12 hepatocyte cell line, we further assessed the combined effect of adiponectin and metformin on hepatic lipid metabolism in low B12 state. Combined adiponectin and metformin treatment resulted in a significant increase in adipoR1 and R2, pAMPKα, pACC, lipid synthesis and reduced FAO. An earlier study has shown that combination of adiponectin and metformin demonstrate a higher effect on blood glucose in animals [493]. Similarly, combination of metformin with another potent activator of AMPK, lactoferrin, resulted in significant alleviation in the accumulation of lipids in the liver by increasing pAMPK/AMPK ratio [494]. The authors also observed a significant reduction in the protein levels of enzymes involved in the synthesis of FA and cholesterol, therefore suggesting for obesity prevention and improvement in metabolism of lipids [494]. Adiponectin within circulation interacts with AdipoRs expressed in the liver leading to the regulation of hepatic metabolism of lipids through AMPK and SREBP-1 signalling [495]. Activation of AMPK by adiponectin involves direct phosphorylation of threonine 172 (Thr172) located on the alpha subunit [495]. It has been reported that AMPK can also be indirectly activated by preservation of the alpha subunit from Thr172 phosphatase through allosteric modulation induced *via* high AMP/ATP ratio by metformin [414, 496]. In this study, it implies that metformin alone had no up-regulatory effect on adipoR1/R2 receptors. Therefore, activation of AMPK by metformin was achieved through a different axis resulting from high AMP/ATP levels. Moreover, AMPK activation *via* the latter mechanism has been

shown to be less than 5-fold [496, 497], whereas combination of both phosphorylation and allosteric mechanisms accounts for >1000-fold activation of AMPK [496, 498]. Therefore, the inclusion of adiponectin, rather than metformin only, accounted for the significant upregulation of adipoR1/R2 receptors, with subsequent activation of AMPK. It, therefore, suggests that the significant lipid lowering effect observed in low B12 condition was principally due to the presence of adiponectin in the combined treatment, rather than an equal additive effect of both adiponectin and metformin. This may therefore explain our observation that, co-treatment of adiponectin-metformin in low B12 state activates AMPK and pACC, which subsequently accounted for reduced hepatic lipid synthesis, upregulated oxidation of FA and improved mitochondrial functional integrity.

7.5 **Conclusion**

B12 deficiency is associated with dysregulated metabolism of lipids resulting from increased lipogenesis and impaired oxidation of fatty acids in the liver. Risk of developing B12 deficiency is associated with metformin treatment in type 2 diabetes patients. In this study, metformin treatment in low B12 hepatocyte cell line improved hepatic fatty acid biosynthesis but showed higher total TG levels and impaired FAO, Metformin playing a crucial role in the activation of AMPK, failed to restore pAMPK levels in low B12 hepatocyte cell line. The ultimate effect was an impairment of both the downregulation of lipogenesis and upregulation of mitochondrial oxidation of fatty acids in by metformin in low B12 condition. Therefore, indicating the desired lipid-lowering effect of metformin in hepatocyte cell liine was compromised by low B12 condition. However, we showed that the lipid lowering effect of metformin in low B12 was mediated through adiponectin dependant AMPK signalling pathway. Treatment with adiponectin in Hep G2 cells resulted in significant upregulation of both receptors which accounted for increased pAMPKα and pACC protein levels in low B12 condition. The subsequent effect was a reduction in the expression of the master regulator of lipogenesis, SREBF1, and downstream genes regulating fatty acids (ACC, FASN and ELOVL6), triglyceride (SCD, DGAT1 and DGAT2) and cholesterol (LDLR) synthesis. Adiponectin also increased FAO enzymes CPT1a and ACADM as well as improved mitochondrial functional integrity by utilizing long chain FA (palmitate) for energy metabolism. Similarly, the combined adiponectin-metformin treatment restored the desired lipid lowering effect of metformin in hepatocyte cell line in low B12 condition. These findings may therefore suggest a key role for adiponectin-AMPK axis in hepatic accumulation of lipids in low B12 state. It also highlights

that in non-responsive patients to metformin treatment, the desired lipid lowering effect as suggested by this study may be achieved by adiponectin therapy. This study also suggests developing potential therapeutic agents targeting adiponectin-AMPK in low B12 state. However, validation of this evidence in vivo using animal and human models is recommended for future studies.

Chapter four – General discussion

8.1 Discussion

The global epidemic of obesity is constantly increasing and currently estimated, by the World Health Organisation, that over 650 million (13% of adult population) adults have obesity [499]. Likewise, about 1.9 billion (39% of adult population) adults are overweight [499]. Equivalently, the incidence of complications associated with obesity, including non-alcoholic fatty liver disease (NAFLD), is also increasing. Moreover, NAFLD has become one of the principal causes of hepatic diseases globally. Growing evidence have also shown the association of vitamin B12 (B12) deficiency with obesity [172], GDM [500] and T2DM [501]. We earlier showed that in experimental studies, B12 deficiency was associated with altered lipid handling by adipocytes. This essentially resulted from increased TG accumulation as well as hypomethylation of the master regulators of lipid biosynthesis. We also showed in clinical studies that B12 deficiency was associated with high TG levels in both Whites and South Asians. As adipocytes contribute only small amount to the circulating levels of TGs, we designed the current study to assess whether similar observations occur in hepatocytes. If true, this will explain the clinical observations and may add further evidence that these associations may be clinically linked. In the current study, we assessed the role of B12 on hepatic metabolism of lipids with the elucidation of probable molecular mechanisms underlying B12 deficiency.

First, we assessed the hepatic regulation of B12 uptake and storage in varying circulating B12 levels. Hep G2 cells treated with low B12 resulted in 2-3-fold increase in intracellular levels of B12 in Hep G2 cells. We also observed increased gene and protein expression of B12 receptor (CD320) and transporter (TCN2) in hepatocyte cell line in low concentrations of B12. However, progressively increasing concentrations of extracellular B12 resulted in decreased uptake of B12 and lower expressions of CD320 and TCN2. Similar results were observed in the liver tissue with low B12 levels compared with high circulatory B12 levels. This indicated that compensatory upregulation of transporter activities is happening in low B12 condition to maintain intracellular B12 levels. This may compromise other intracellular activities in such conditions.

Next, we investigated the effect of B12 deficiency on hepatic lipid metabolism, targeting *de novo* lipid (FA, TG and cholesterol) synthesis and the fatty acid oxidation (FAO). Low B12 resulted in increased lipid droplets and higher intracellular TG levels in hepatocyte cell line, confirmed by increased synthesis of radio-labelled TGs. The gene expression of the master regulator of lipogenesis (SREBF1) and cholesterol (LDLR) synthesis was significantly higher

in low B12 with subsequent increase in the expression of key genes regulating FA, TG and cholesterol synthesis. Low B12 hepatocyte cell line had higher levels of total fatty acids (TFAs), saturated fatty acids (SFAs), monounsaturated fatty acids (MUFA) and trans-fatty acids compared with control. PUFAn-6, LC-PUFAn-6 and PUFA n-6/n-3 ratio were significantly higher in low B12 hepatocyte cell line compared with control. Fatty acids were significantly higher predominated by even chains (C16, C18 and C18:1) and to a lesser extent by odd chains (C17, C21, C23) in low B12 hepatocyte cell line compared with control.

In the FAO pathway, low B12 resulted in significant reduction in expression of the rate limiting enzyme CPT1α as well as downstream genes crucial in the FAO pathway. The mitochondrial functional integrity was compromised in low B12 accounting for impaired utilization of long chain FA (palmitate) for energy metabolism, suggesting the likelihood of accumulation in the hepatocyte cell line.

Prolonged oral therapy with metformin can cause B12 deficiency in significant proportion of T2DM patients. This has been shown to exacerbate the existing diabetic neuropathy and may indeed cause neuropathy [502]. Whether such B12 deficiency in metformin treated patients is causally related to the coexisting hypertriglyceridemia [503], is not known. Therefore, we assessed the probable effect of low B12 on the lipid lowering effect of metformin in hepatocyte cell line. We observed that B12 independently affected the activation of the master regulator of energy homeostasis AMPK, with decreased pAMPKα and pACC levels observed in low B12 hepatocyte cell line. Treatment with metformin failed to restore pAMPKα and pACC levels significantly in low B12 hepatocyte cell line. We also observed higher levels of the total intracellular TG although *de novo* synthesis of lipids (FA, TG and cholesterol) was significantly decreased in metformin treated hepatocyte cell line in low B12 compared with basal control. In addition, low B12 impaired FAO significantly in metformin treated hepatocyte cell line by limiting CPT1α and other key genes crucial in the FAO pathway. Likewise, the mitochondrial functional integrity in metformin treated hepatocyte cell line was significantly impaired in low B12 compared with control, suggesting that the desired lipid lowering effect of metformin may be compromised by low B12.

Finally, adiponectin is a well-known adipocytokine and potential therapeutic agent in several metabolic disorders including obesity and T2DM. Likewise, adiponectin demonstrates an independent lipid lowering effect in the liver *via* the AMPKα signalling axis. We therefore hypothesized that adiponectin might improve the compromised lipid lowering effect of

metformin in low B12 hepatocyte cell line. We observed that adiponectin was not expressed in the hepatocyte cell line, however, adiponectin receptors adipoR1 and R2 were expressed but significantly reduced in low B12. Adiponectin treatment resulted in the significant upregulation of adipoR1 and R2 receptors with subsequent activation of AMPKα in low B12 hepatocyte cell line. In effect, there was significant decrease in the key genes regulating FA, TG and cholesterol synthesis in low B12 hepatocyte cell line. The FAO and mitochondrial functional efficiency were also significantly upregulated in low B12 hepatocytes treated with adiponectin. In conclusion, we observed a significant lipid lowering effect *via* decreased lipogenesis and upregulated FAO and mitochondrial functional efficiency following a combined adiponectin-metformin treatment in low B12 hepatocyte cell line, suggesting that adiponectin improves the lipid lowering effect of metformin in low B12 hepatocyte cell line.

Our findings may suggest that the liver plays a significant role in the maintenance of intrahepatic B12 levels. B12 is mandatory for normal DNA synthesis, improvement of cognitive function and red blood cells development. In the liver, B12 presenting as methyl-cobalamin and adenosyl-cobalamin serve as coenzymes in two significant pathways [(methionine synthase (MS) and methyl malonyl-CoA mutase (MCM) pathways] regulating energy metabolism. The liver and kidneys are the main organs that store higher levels of B12 due to possession of additional receptors (apart from CD320) such as asialoglycoprotein and megalin respectively, accounting for higher B12 uptake. The regulation of B12 uptake and storage in the liver is however less explored, therefore, our study is the first to characterize the regulation of intrahepatic storage in varying circulating B12 levels. Some studies have proposed that at higher concentrations of B12, some tissues adopt an efflux system that pushes excess of B12 out of cells whereas others modulate the expression of B12 receptors on cellular surfaces [296]. In the current study, when the circulatory level in low B12 medium was 25pM, the intracellular level was 178pM (2.8-fold) but when circulatory level in high B12 medium was 500nM (20,000-fold higher than 25pM), the intracellular level was only 8nM (increased 0.02-fold). Similarly in the animal model, we observed that when the circulatory level in LB was 0.82nM, the intracellular level was 27nM (i.e. 33.8 fold higher) but when the circulatory level in HB was 5.36nM (6.5 fold higher than 0.82nM), the intracellular level was only 46nM (8.6 fold). In addition to studies involving animal (rat) models, similar evidence has also been shown in placental tissues of pregnant subjects presenting with low plasma B12 [504]. It has been reported that excessive supply of B12 may not be internalized by cells, resulting in B12 secretion in the bile [505]. We also observed a significant increase in the hepatic expression of

B12 receptors (CD320) and transporters (TCN2) in low extracellular levels of B12 and vice versa. Our findings confirm the evidence from earlier studies suggesting that B12 receptors (CD320) and transporters (TCN2) are *de novo* synthesized in the liver [296], therefore, hepatic modulations may account for the alterations in B12 uptake. Similarly, due to high foetal demand of B12 in pregnancy, higher serum levels of soluble CD320 were observed in mothers which was suggested to have originated from the placenta [301]. Similar observation was reported for TCN2 in the circulation of pregnant mothers with low B12, accounting for high cord blood and foetal B12 concentrations [278]. The limitation of the present study was its non-validation of the current evidence with further assessment on hepatic uptake of B12 in real-time, probably by monitoring the uptake of radiolabelled-B12. Also, metabolomics, targeting the levels of soluble B12 transporters and receptors released into circulation at different extracellular B12 concentrations, are recommended. Altogether, evidence from this current study shows that hepatocytes modulate the intracellular levels and storage of B12 by regulating the expression of B12 transporters and receptors leading to the maintenance of intracellular B12 balance. A recent study has recommended a physiological dose of 2µg/day B12 for oral supplementation in Indian adolescents [506]. The recommendation was based on evidence from earlier clinical trials, by the same group, which showed that there was adequate absorption in majority of the population as well as improvement in B12 levels after one year of oral supplementation [506]. It is however recommendable to conduct similar trials to establish an optimal physiological dose for different ethnic and age-specific groups of individuals. Oral supplementation with 2µg/day of B12 for eleven months yielded similar clinical results compared with supplementation with a greater dose of 500µg/day B12 for the first six weeks followed by 15µg/day for successive twenty weeks [506, 507]. Apart from being more expensive and not likely to be utilized in a large-scale public health intervention in most developing countries, higher doses of B12, according to evidence from the current study, may not provide any additional benefit to the liver. Our data supports that the optimal physiological levels of B12 may be required rather than overloading with supplements when subjects are B12 deficient, therefore, supporting a public health strategy to tackle the widely predominant status of low B12 in many developing countries. This provides us evidence and stimulates to further study whether dysregulation in hepatic metabolism in pathological conditions could be related to tissue-specific effect of low B12 in liver.

Next, we assessed the effect of B12 deficiency on hepatic metabolism of lipids. Low B12 accounted for higher lipid droplets in hepatocyte cell line with increased level of total

intracellular TG and FA uptake for TG synthesis compared with control. Several studies have also reported the association of B12 with obesity [508] and dyslipidaemia [509] including high TG and cholesterol levels [510]. These evidence have been shown in pre-clinical (cell lines and animal models) and clinical studies. To buttress this observation, we further assessed the expression of the master regulator of lipogenesis (SREBF1) and cholesterol (LDLR) biosynthesis in low B12. We observed that both SREBF1 and LDLR as well as key downstream genes crucial in the FA, TG and cholesterol biosynthesis were significantly increased in low B12 compared with control. It has also been shown recently that epigenetic modification such as hypo-methylation near the promoter regions of SREBF1 and LDLR genes in low B12 adipocytes accounted for their upregulation leading to increased lipid synthesis [510]. *De novo* lipogenesis (DNL) accounts for about 26% of intra hepatic TG levels which increases the risk of NAFLD development [511]. Therefore, low B12 upregulation of DNL could hasten the development of NAFLD, especially in susceptible individuals. Likewise, the contribution of hepatic lipogenesis, compared with adipose tissues, to the total TG stores of the body is much higher [512]. This suggests that low B12-augmented hepatic lipogenesis may exert a greater dysregulation to the total body fat. We also observed that FA profile in hepatocyte cell line presented with higher total fatty acids (TFAs), Saturated FAs (SFAs), monounsaturated fatty acids (MUFAs) and trans-fatty acids in low B12 compared with control. Similarly, PUFAn-6, LC-PUFAn-6 and PUFA n-6/n-3 ratio were all higher in low B12 hepatocytes. In addition, FAs including palmitate (C16), stearate (C18) and oleate (C18:1) predominated in the hepatocyte cell line and were significantly higher in low B12 compared with control. These FA groups have been shown to be associated with non-alcoholic steatohepatitis (NASH) and higher risk of cardiovascular diseases [513-518], therefore, suggesting that low B12 increases hepatic synthesis of 'unsafe' FAs. However, quantifying the level of circulating lipids contributed *via* hepatic secretion in different conditions of B12 may be recommended to appreciate the impact on metabolic risk.

We observed that low B12 impaired FAO by decreasing the expression of the rate limiting enzyme CPT1α and downstream genes crucial in the FAO pathway. Similar studies have shown that methyl donor (B12 and folate) deficiency accounted for impaired FAO in the myocardial cells [398] and adipocytes tissues [393] by decreasing the level of enzymes crucial in FAO the pathway. B12 is known to be involved in the regulation of methyl malonyl-CoA levels. Therefore, low B12 results in methyl malonic academia (MMA) which is known to be a potent inhibitor of CPT1. However, the precise mechanism of B12 regulation of downstream FAO enzymes requires further investigation. We also observed that low B12 impaired the

mitochondrial functional integrity resulting in impaired utilization of long chain FA (palmitate) for energy metabolism. Our findings may confirm a recent study which showed that supplementation with B12 improves mitochondrial health, whereas B12 deficiency is associated with dysfunction [519]. Basically, beta oxidation (FAO) and mitochondrial respiration (oxidative phosphorylation) are not entirely independent processes. FAO results in the generation of electron-rich donors (NADH and $FADH_2$) which are required by the electron transport chain (ETC) of the mitochondria for ATP synthesis. Therefore, low B12 impairment of CPT1α and key enzymes in the FAO pathway may affect the supply of electrons to the ETC which compromises the efficiency of mitochondrial respiration. Altogether, the increased lipogenesis with altered FA profile, as well as impaired FAO and mitochondrial dysfunction in low B12, may trigger lipid accumulation in the liver. This may therefore explain the observation in several clinical studies indicating that B12 deficiency is associated with obesity and dyslipidaemia, predisposing subjects to a higher risk of GDM, T2DM and CVD.

Several clinical studies have shown that prolonged treatment with metformin results in the development of B12 deficiency in patients [503], we therefore assessed whether B12 deficiency affects the lipid lowering effect of metformin in human hepatocyte cell line. We observed that the total intracellular TG level was not significantly reduced in metformin treated hepatocytes in low B12 compared with control. Likewise, although the synthesis of lipids (FA, TG and cholesterol) was decreased, the FAO and mitochondrial functional efficiency were impaired in metformin treated hepatocytes in low B12 accounting for hepatic accumulation of lipids. Clinically, several studies (about 50%) have shown evidence that metformin treatment had no lowering effect on intrahepatic lipid levels [415]. The development of B12 deficiency after metformin administration, is associated with both dosage and duration (years) of treatment [428, 445]. Evidence shows that hepatic deficiency of B12 may also occur after prolonged deprivation since the liver is able to maintain physiological B12 reserves for 3-5 years [520]. This suggests the non-responsiveness of metformin to hepatic lipid levels may dominate in low B12 state. However, further *in vi*vo studies in humans to validate the current evidence is required. To understand the mechanism underlying this observation, we assessed the AMPK signalling axis by which metformin achieves a lipid lowering effect. We observed that pAMPKα and pACC levels were significantly reduced in low B12, which could be due to epigenetic effect and needs exploration in future. However, treatment with metformin could not restore pAMPKα and pACC levels significantly in low B12 compared with control. This data therefore confirms that efficacy of metformin drug is less efficient in low B12 state and could be mediated through AMPK pathway. Recently, a study showed that the liver of mice when

supplemented with methyl donors including B12 resulted in a significant activation of AMPK [521] leading to NAFLD regression.

Metformin activation of AMPK is achieved by higher levels of AMP: ATP or ADP: ATP ratio. However, the process leading to the generation of AMP, ADP and ATP such as TCA cycle, beta oxidation and oxidative phosphorylation [522] may not be entirely independent of methyl donors (such as B12). This may be due to their epigenetic effect as well as cofactor roles in these pathways [523]. Derivatives of the benzimidazole ligand of cobalamin (B12) [524], have been shown to be potent activators of AMPK by binding to its beta subunit [525, 526]. It therefore suggests that unlike metformin, B12 may independently activate AMPK. However, elucidation of the precise mechanism of B12 activation of AMPK was not shown in the current study, therefore, requires further investigations.

Adiponectin action through AMPK axis has the potential to regulate lipid metabolism. Finally, we proposed that adiponectin-AMPK axis might improve the lipid lowering effect of metformin in low B12 hepatocyte cell line. We observed that although adiponectin was not expressed in hepatocyte cell line, the expression of adiponectin receptors adipoR1 and R2 was detected but significantly reduced in low B12 compared with control. Some studies have also shown similar evidence [475] and others have reported reduced expression of adipoR1 and R2 in obese [480] and methyl donor deficient [477] subjects. However, evidence from an animal study showed that reduction in the expression of adipoR1 and R2 was not as a result of methylation in methyl donor deficient subjects [477], suggesting the need for further investigations. Adiponectin is synthesized chiefly by the adipose tissues and therefore, might account for its non-detection in the liver. We also, observed that adiponectin treatment resulted in increased expression of adipoR1 and R2 receptors and subsequent increase in pAMPKα and pACC levels in low B12 hepatocyte cell line. In effect, there was significant reduction in the total intracellular TG levels in low B12 hepatocyte cell line. The expression of the master regulator of lipogenesis SREBF1 and cholesterol (LDLR) as well as genes involved in lipogenesis was also decreased in low B12. Adiponectin also increased the FAO and mitochondrial functional efficiency, preventing the accumulation of long chain FA (palmitate) in low B12 hepatocyte cell line. This evidence confirms the potent role of adiponectin in activating AMPK and subsequent target genes in lipid regulation in low B12 hepatocyte cell line. Studies have also shown evidence of improvement in mitochondrial function [527] and reduction in hepatic lipid synthesis [484] resulting from adiponectin treatment. In addition, we observed that a combined adiponectin-metformin treatment resulted in a significant increase in pAMPKα and pACC levels in low B12 hepatocyte

cell line due to adiponectin. This accounted for a significant reduction in the total intracellular TG levels as well as radiolabelled-FA uptake fore TG synthesis in low B12 hepatocyte cell line. We also observed significant reduction in lipogenesis *via* decreased expression of genes involved in the FA, TG and cholesterol biosynthesis. Likewise, there was significant increase in FAO and mitochondrial functional efficiency in low B12. This evidence shows that adiponectin significantly alleviates the accumulation of lipids in metformin-treated hepatocyte cell line in low B12 condition. Clinical evidence showed that adiponectin synthesis from adipose tissues was inhibited in patients on metformin treatment [528] and low levels of adiponectin was associated with obesity and high serum lipids [529]. However, a combination of metformin and adiponectin was also shown to improve serum glucose levels in animal models [493], whereas combination with other potent activators of AMPK (such as lactoferrin) regressed hepatic accumulation of lipid [494]. Adiponectin activation of AMPK results from direct phosphorylation of 172 (Thr172) in the alpha subunit. However, metformin achieves activation *via* allosteric modification leading to preservation of the phosphorylated alpha subunit resulting from AMP binding to the gamma subunit of AMPK. Evidence shows that utilisation of AMP (by metformin) accounts for 5-fold less activation of AMPK, whereas a combination of both mechanisms (direct phosphorylation and allosteric modulation) results in >1000-fold higher activation of AMPK [496, 498]. This may therefore explain our observation that, inclusion of adiponectin to metformin treatment may offer a greater additive action *via* direct phosphorylation of AMPK by adiponectin, to the allosteric modulation induced by metformin. This effect could significantly increase the activation of pAMPK/pACC leading to reduction of lipid levels in low B12 hepatocyte cell line.

8.2 Conclusion and future direction

The current thesis provides novel evidence of the potential role of B12 deficiency in the hepatic metabolism of lipids which could contribute to the development of metabolic disorders such as simple steatosis of the liver, NAFLD, obesity, T2DM and metabolic syndrome. The association of B12 deficiency and the manifestation of metabolic risk have been shown in clinical and pre-clinical studies. In recent studies involving human adipocyte models, the probable underlying epigenetic mechanisms, such as DNA methylation and microRNAs, leading to dysregulated metabolism of lipids by low B12 has been shown. The current study provides novel evidence of hepatic regulation of B12 uptake and storage *via* modulation of B12 transporters and receptors in various circulatory levels including low and high B12. Low B12 accounted for

increased hepatic lipogenesis with evidence of higher lipid droplets, total intracellular TG level and utilization of radiolabelled-FA for TG synthesis. Hepatocyte cell line profile of FA in low B12 showed significantly higher levels of total FAs and predominant groups such as SFAs, MUFAs, trans-FAs and PUFAn-6/n-3 ratio, as well as individual FAs such as palmitate (C16), stearate (C18) and oleate (C18:1). This observation was buttressed by elevation in the master regulator of lipogenesis SREBF1 and cholesterol LDLR accounting for increased expression of genes regulating FAs, TGs and cholesterol biosynthesis under low B12. Concurrently, oxidation of FAs (FAO) and mitochondrial functional efficiency were decreased in low B12 accounting for lipid accumulation in hepatocytes (Fig 76). Prolonged treatment with metformin induces B12 deficiency in T2DM patients and several clinical studies have reported that metformin does not improve intrahepatic TG levels in patients. The current study also provides novel evidence that low B12 impairs the activation (phosphorylation) of the master regulator of energy homeostasis, AMPKα, by metformin treatment. This accounted for decreased pACC levels which significantly impaired the reduction of total intracellular TG levels, FAO and mitochondrial functional efficiency leading to hepatic accumulation of lipids. However, we observed that adiponectin treatment *via* the upregulation of adipoR1 and R2 receptors intensified the activation (phosphorylation) of AMPKα in low B12 hepatocytes, therefore, improving hepatic lipid metabolism by decreasing lipogenesis and increasing FAO and mitochondrial respiration. Likewise, the combined adiponectin-metformin treatment also significantly alleviated hepatic accumulation of lipids by increasing AMPK activation as well as elevating the FAO and functional efficiency of the mitochondria in low B12 condition. Validation of the current evidence in primary hepatocytes in different pathologies and animal models with respect to B12 deficiency is recommended.

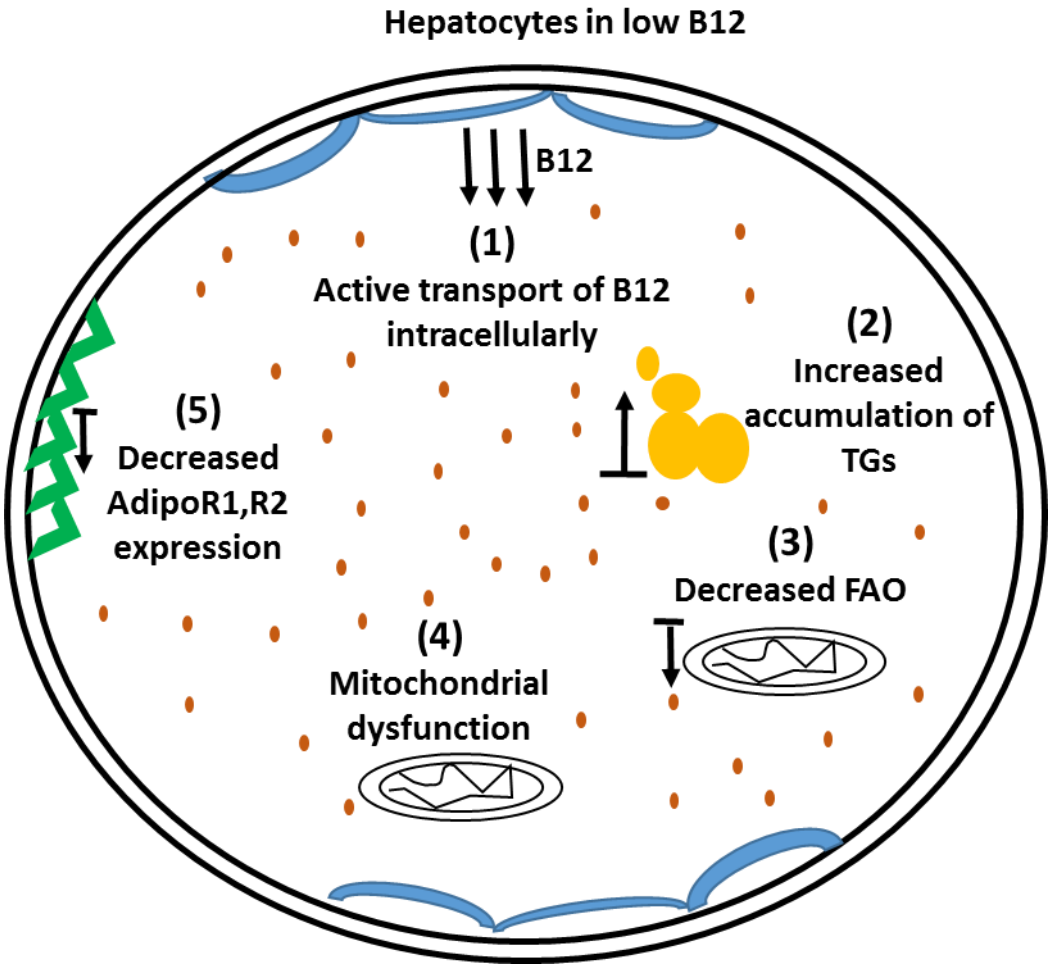


Figure 76: Modulations observed in hepatocytes in low B12 condition: Low B12 condition accounted for (1) active transport of B12 intracellularly in hepatocytes resulting from modulations in B12 transporters and receptors. (2) Hepatic lipogenesis is also increased in low B12 accounting for increased accumulation of intrahepatic TG levels. (3) Beta oxidation (FAO) in low B12 hepatocytes is significantly decreased resulting from decreased levels of key enzymes involved in the FAO pathway. (4) Likewise, the mitochondrial functional efficiency is impaired in low B12 and finally, (5) the expression of adiponectin receptors (adipoR1 and R2) are decreased in low B12 hepatocytes.

In conclusion, since the contribution of hepatic lipogenesis to the total body metabolism of lipids is significantly higher compared with adipose tissue, the current evidence explains the observation of increased lipogenesis accounting for higher adiposity and T2DM risk in low B12 subjects. Hepatic deficiency of B12 resulting from prolonged metformin treatment in humans may account for the clinical observation of unimproved intrahepatic lipid levels. Therefore, combined metformin treatment with adiponectin is suggested to improve the lipid lowering effect of metformin in low B12 hepatocytes. This study therefore provides a novel model for

developing potential therapeutic agents to target B12-adiponectin-AMPK axis in subjects developing IR, obesity and CVD.

Nevertheless, the current study was conducted principally using an immortal cell line of the liver, Hep G2. The use of cell lines, instead of primary cells, have some advantages including the provision of a wholesome cell population accounting for reliable samples that generates reproducible results. Other advantages include cost effectiveness, simplicity to use and higher likelihood to bypass ethical concerns governing the tissue usage. However, the behaviour of cell lines may not exactly mimic primary cells. The former, as result of series of passages over a lengthy period of time, could be genetically manipulated leading to generation of variations in both genotypic and phenotypic properties, therefore, making it difficult to adequately replace primary cells. However, similar evidence on the effect of B12 on the metabolism of lipids in adipocyte cell line (Chub-S7) has been investigated and endorsed in human adipose tissues collected at delivery from pregnant women by our group. Therefore we recommend future studies to validate our current findings in primary human liver cells and subsequently in animal and human liver tissues. Successful replication of the current evidence in the different models would be useful for clinical trials that could provide the strongest evidence in support of the cause-effect relationship. The latter could further serve as basis for clinical and public health policy.

Regardless of the novel evidence shown by the current study, few questions remain unanswered therefore requiring further investigation. It is therefore suggested that future studies should be designed in similar models (Hep G2) and also in primary cells, animal and human liver tissues to examine;

1. The *in vivo* modulation of liver B12 transporters and receptors regulating B12 uptake and storage. Further exploration of tissue-specific regulation of B12 uptake in other metabolic tissues should also be investigated.

2. The probable epigenetic mechanism underlying dysregulation of hepatic lipid metabolism by low B12.

3. The effect of hepatic lipid dysregulation by low B12 on circulating levels of lipid fractions

4. The epigenetic mechanism underlying B12 regulation of AMPKα activation in the liver.

5. The mechanism accounting for reduction in the hepatic expression of adipoR1 and R2 receptors in low B12.

List of manuscripts (abstracts) awaiting publication

1. Role of vitamin B12 on lipid metabolism

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Abstract

Obesity is currently a worldwide epidemic responsible for 5% of mortality globally. Obesity increases the risk of developing metabolic disorders like diabetes, hypertension and cardiovascular diseases (CVDs) which causes great public health concern. A series of studies involving many animal models and epidemiological investigations have demonstrated a relationship between importance of vitamin B12 (B12) and various components of metabolic syndrome. High prevalence of vitamin B12 deficiency has been shown in Europeans (27%) and South Indians (32%) with type 2 diabetes (T2D) patients. A longitudinal prospective study in pregnant women has shown that B12 deficiency could independently predict development of T2D after a period of five years post-delivery. Children born to B12 deficient mothers have augmented fat accumulation that may consequently lead to elevated resistance to insulin and risk of developing T2D and/or CVD in adulthood. However, the role of B12 in metabolism of lipids is not explored. In this review, we provide evidences on the role of B12 deficiency in lipid metabolism and insights on the possible epigenetic markers such as DNA methylation, miRNAs and histone modifications. This will provide probable strategies for therapeutic targets in dyslipidaemia related to a micronutrient deficiency, such as vitamin B12.

2. Uptake and storage of vitamin B12 is modulated by CD320 receptor and TCN2 transporter in hepatocytes in various extracellular B12 concentrations

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Abstract

Background: The liver is the principal storage organ for cobalamin (B12). Hepatocytes make up the bulk of the liver mass expressing specific receptors for B12-bound transporters in circulation that may regulate cellular uptake and storage of B12 in the liver. We assessed the regulation of cellular uptake and storage of B12 in hepatocytes in various concentrations of circulating B12.

Methods: Hep G2 cell line was cultured using custom made B12 deficient Eagles' Minimal Essential Medium (EMEM) and seeded in ten different concentrations of B12 media. Liver tissues from two different mice groups: (1) high and (2) low levels of B12 in plasma were used. Intracellular and circulating B12, gene and protein expression of B12 receptors and transporters were determined.

Results: HepG2 cells treated with low B12 resulted in 2-3fold increase in intracellular levels of B12 in Hep G2 cells. We also observed increased gene and protein expression of B12 receptor (CD320) and transporter (TCN2) in hepatocytes under low concentrations of B12. However, progressively increasing concentrations of extracellular B12 resulted in decreased uptake of B12 and lower expressions of CD320 and TCN2. Similar, results were observed in the liver tissue with low B12 levels compared to high circulatory B12 levels.

Conclusion: This study shows that the active transport of B12 in both HepG2 cell line and liver tissues are higher at low concentration, which might be due to the higher expression of B12 receptors/transporters in the membrane of hepatocytes but not the same when high B12 levels are present in circulation. Our data suggests that the optimal physiological levels of B12 is required rather than overloading with supplements, when subjects are B12 deficient.

3. Vitamin B12 deficiency alters metabolism of lipids triggering accumulation in the liver

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Abstract

Introduction: The impact of higher adiposity on metabolic health is greatly explored in recent times due to elevation in the global incidence of obesity. Evidence of association between low vitamin B12 (B12) in pregnancy and elevated BMI of mothers has been established. Dysregulation of lipogenesis by low B12 via hypomethylation of the master regulator of cholesterol biosynthesis was observed in human adipocytes. However, the contribution of the liver to the lipogenesis of the entire body is higher than adipose tissue. If similar effects happen in hepatocytes, this may explain the observation of dyslipidaemia in humans. Therefore, we investigated whether B12 deficiency affect hepatic metabolism of lipids.

Methods: Hep G2 cell line was cultured using custom-made B12 deficient Eagle's Minimal Essential Medium (EMEM) and seeded in four different concentrations of B12 media such as 500nM (control), 1000pM (1nM), 100pM and 25pM (low) B12. Oil Red O (ORO) staining, RT-qPCR, total intracellular triglyceride (TG), radioactive flux assay, fatty acid profiling using gas chromatography and extracellular seahorse XF24 flux assay were employed to examine the effect of B12 on lipid metabolism.

Results: Hep G2 cells in low B12 had more lipid droplets that were intensely stained with ORO compared to control. Total intracellular TG and incorporation of radio-labelled-FA for TG biosynthesis were also increased. Gene expression of the master regulator and key downstream enzymes regulating FA, TG and cholesterol biosynthesis were upregulated. Levels of total, saturated (SFAs), monounsaturated fatty acids (MUFAs) and trans-fatty acids as well as individual even-chain and odd-chain FAs were significantly increased. Lastly, low B12 impaired FA oxidation and mitochondrial functional integrity in hepatocytes compared to control. In conclusion, our data provide novel evidence that B12 deficiency dysregulates lipid metabolism leading accumulation in hepatocytes.

4. B12 deficiency impairs the lipid lowering effect of metformin via adiponectin-AMPK axis in human hepatocytes

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Abstract

Introduction: The global epidemic of obesity is constantly increasing. Likewise, the incidence of obesity-related complications such as non-alcoholic fatty liver disease (NAFLD) which is also associated with T2DM are increasing proportionately. Metformin is the first-line drug widely used for the treatment of T2DM and is known to decrease liver glucose production, body weight and lipid levels in plasma. However, prolonged treatment with metformin was shown to decrease vitamin B12 (B12) levels in T2DM patients, and evidence shows that low B12 is associated with obesity and dyslipidaemia. Several clinical studies (50%) have shown that metformin had no effect on intrahepatic triglyceride levels, whereas adiponectin was shown to regress NAFLD. Therefore, we proposed that low B12 may impair the lipid lowering effect of metformin in human hepatocytes which may however be improved by adiponectin.

Methods: Hep G2 cell line was cultured using custom-made B12 deficient Eagle's Minimal Essential Medium (EMEM) and seeded in different concentrations of B12 medium: 500nM (control) and 25pM (low) B12. Western blotting, RT-qPCR, total intracellular triglyceride (TG) estimation, radioactive flux assay and extracellular seahorse XF24 flux assay were undertaken in the current study.

Results: Low B12 hepatocytes had higher total intracellular TG which was not significantly reduced by metformin treatment. FA uptake for TG synthesis and genes involved in *de novo* lipid [FA (SREBF1, ACLY, ACC FASN and ELOVL6), TG (SCD1, DGAT1 and DGAT2) and cholesterol (LDLR, HMGCS1 and HMGCR) synthesis were decreased by metformin in low B12, however, FAO and mitochondrial functional efficiency were significantly impaired by low B12 in metformin treated hepatocytes. The mechanism underlying this effect resulted from decreased levels of activated AMPK (pAMPKα) and pACC in low B12 hepatocytes which was not significantly restored in metformin treated hepatocytes. However, adiponectin independently improved intrahepatic lipid levels under low B12 *via* the activation of AMPK leading to improved intrahepatic lipid levels. Likewise, combined treatment of adiponectin and metformin treatment increased AMPK activation under low B12 with subsequent reduction in lipogenesis as well as improvement in FAO and mitochondrial respiration. In conclusion, low B12 impaired the lipid lowering effect of metformin, however, intrahepatic lipid levels were significantly decreased *via* combined adiponectin-metformin treatment.

List of conference presentations

Poster presentations**:**

1. **Title(s):** i). Metformin Treatment fails to restore fatty acid oxidation in low vitamin B12 hepatocytes

ii). Hepatocytes regulate the uptake and storage of vitamin B12 by modulating B12 transporters and receptors

- Society for Endocrinology BES conference, Glasgow, UK, 2018 – Poster presentation

2**. Title:** Effect of B12 deficiency on the lipid lowering effect of metformin in the liver

- Society for Endocrinology BES conference, Harrogate, UK, 2017 - Poster presentation

Nominated for early career Researcher's award

3. **Title(s):** i). Low vitamin B12 induces *de novo* lipogenesis in human hepatocytes

ii). B12 deficiency inhibits the lipid lowering effect of metformin in the liver

iii). Hepatocytes regulate the uptake and storage of vitamin B12 by modulating receptors in various circulatory B12 concentrations

- DOHAD 10th World Congress, Rotterdam, Netherlands, 2017 - Oral (poster) presentation

4. **Title:** Low vitamin B12 induces *de novo* lipogenesis in human hepatocytes

- Annual symposium, University of Warwick, UK, 2017 - Poster presentation

Oral presentations:

1. **Title**: Vitamin B12 deficiency limits metformin-facilitated lowering of lipids in the liver

- European Physiological society conference, London, UK, 2018 – Oral presentation

2. **Title:** Low vitamin B12 triggers lipid accumulation in human hepatocytes

- Annual symposium, University of Warwick, UK, 2018 - Oral presentation

Awarded the Best Oral Presentation

Comment from chairperson:

‘’Dear Joseph
Well done on such a clear and interesting presentation. It was a pleasure to chair your session.
Good Luck with the rest of your PhD.
Best wishes
Kate’’

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3. **Title:** Effect of vitamin B12 deficiency on hepatic metabolism of lipids

- PRiDE annual conference, University of Warwick, UK, 2017 - Oral presentation

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