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Original Paper

Vitamin B12 Induces Hepatic Fatty Infiltration through Altered Fatty Acid Metabolism

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Key Words
Vitamin B12 • Lipogenesis • Fatty acid oxidation • Dyslipidaemia • Hepatocyte • Triglyceride

Abstract
Background/Aims: Rise in global incidence of obesity impacts metabolic health. Evidence from human and animal models show association of vitamin B12 (B12) deficiency with elevated BMI and lipids. Human adipocytes demonstrated dysregulation of lipogenesis by low B12 *via* hypomethylation and altered microRNAs. It is known d*e novo* hepatic lipogenesis plays a key role towards dyslipidaemia, however, whether low B12 affects hepatic metabolism of lipids is not explored. ***Methods:*** HepG2 was cultured in B12-deficient EMEM medium and seeded in different B12 media: 500nM(control), 1000pM(1nM), 100pM and 25pM(low) B12. Lipid droplets were examined by Oil Red O (ORO) staining using microscopy and then quantified by elution assay. Gene expression were assessed with real-time quantitative polymerase chain reaction (qRT-PCR) and intracellular triglycerides were quantified using commercial kit (Abcam, UK) and radiochemical assay. Fatty acid composition was measured by gas chromatography and mitochondrial function by seahorse XF24 flux assay. ***Results:*** HepG2 cells in low B12 had more lipid droplets that were intensely stained with ORO compared with control. The total intracellular triglyceride and incorporation of radio-labelled-fatty acid in triglyceride synthesis were increased. Expression of genes regulating fatty acid, triglyceride and cholesterol biosynthesis were upregulated. Absolute concentrations of total fatty acids, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), trans-fatty acids and individual even-chain and odd-chain fatty acids were significantly increased. Also, low B12 impaired fatty acid oxidation and

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mitochondrial functional integrity in HepG2 compared with control. ***Conclusion:*** Our data provide novel evidence that low B12 increases fatty acid synthesis and levels of individual fatty acids, and decreases fatty acid oxidation and mitochondrial respiration, thus resulting in dysregulation of lipid metabolism in HepG2. This highlights the potential significance of *de novo* lipogenesis and warrants possible epigenetic mechanisms of low B12.

Introduction

The impact of higher adiposity on metabolic health has been extensively studied due to alarming increase in the global incidence of obesity. It was recently estimated that the worldwide prevalence of overweight in adults is 39%, obesity is 13% and non-alcoholic fatty liver disease (NAFLD) is 25% [1, 2]. Obesity, characterized by the excessive storage of fat in adipose and hepatic tissues, has been associated with dyslipidaemia, insulin resistance and NAFLD. Hepatic *de novo* lipogenesis is a major factor contributing to plasma triglyceride levels and hepatic steatosis [3]. Similarly, fatty acid oxidation is significant in suppressing lipolysis of adipose triglyceride as well as reducing hepatic fat accumulation [4]. However, increased plasma free fatty acid levels have been associated with insulin resistance, inflammation and gestational diabetes (GDM) [5].

Environmental factors such as nutrition (micronutrients), may affect metabolism of lipids and deregulate the processes of lipogenesis and lipid oxidation. Deficiencies in methyl donors (vitamin B12 (B12) and folate) are associated with obesity [6], liver steatosis [7] and increased risk of metabolic syndrome [8]. Low B12 in pregnancy was associated with elevated BMI, insulin resistance and GDM [9]. Maternal low B12 was associated with higher levels of triglyceride in both maternal and cord blood [10], and higher insulin resistance in the offspring at six years of age [11]. It has also been shown that low serum B12 level was observed in individuals with NAFLD, especially with grade 2 and 3 steatosis [12]. In support to this human studies, B12 restriction in maternal rat models demonstrated that the offspring had higher adiposity, dyslipidaemia, upregulation of enzymes in lipogenesis and lipid oxidation [13]. We have previously shown in a human adipocyte model in low B12, the master regulator of lipogenesis (SREBF - sterol regulatory element binding protein) and cholesterologenesis (LDLR - low density lipoprotein receptor) were upregulated [14] and might lead to higher adiposity and dyslipidaemia via hypomethylation of DNA [14] and altered microRNAs [15]. Compared to the liver, the contribution of adipose tissue to the circulating levels of lipids is considerably lower [16]. Therefore, if the effects of B12 are similar in hepatocytes, this may explain the association observed between low B12 and dyslipidaemia in humans [17] and the causal association observed in animal models [17].

In animal models, apart from the increased triglyceride and cholesterol levels, low B12 was associated with altered levels of long chain polyunsaturated fatty acids (LC-PUFAs) in the plasma and liver of subjects across three generations [18]. Recent evidences suggest that fatty acids in circulation may be implicated in metabolic dysregulation such as GDM [19], postmenopausal obese and overweight women [20], metabolic syndrome [21], type 2 diabetes (T2D) [22] and cardiovascular disease (CVD) [23]. Despite the diverse metabolic roles of these fatty acids and the potential link between low B12 and beta-oxidation of fatty acids [24, 25], there are no studies that explored the relationship between low B12 on the individual fatty acids at tissue levels, especially in an active metabolic tissue such as hepatocytes.

With this foregoing discussion, in this current study we present data on (1) the effects of low B12 on lipogenesis and lipid oxidation in human hepatocyte cell line (HepG2) and (2) the effects of low B12 on the fatty acid concentrations in HepG2.

Materials and Methods

Experimental methods are detailed in Supplemental Methods (for all supplementary material see www.cellphysiolbiochem.com). They are articulated in brief in the following paragraphs.

Cell culture

HepG2 cell culture was done, with slight modifications [26]. Using B12-deficient Eagles' Minimal Essential Medium (EMEM), cells were cultured in T-75 flask and seeded into six well-plates at 75,000 cells/well in different B12 concentrations of EMEM media: 500nM (Control), 1000pM, 100pM and 25pM. Customised B12 medium was changed every 48-hours until 100% confluence.

Oil Red O staining and elution assay

HepG2 cells, following 1-hour fixation with 10% formalin, were stained for 2-hours with ORO. Oil droplet images were captured under 40x objective of light microscope.

RNA isolation, cDNA synthesis and gene expression

RNA was isolated using the Trizol method [27] and gene expression assays were done using qRT-PCR and normalized with 18s rRNA (Applied Biosystems, UK) [14].

Total intracellular triglyceride estimation

Total intracellular triglyceride in HepG2 was assessed with commercial Triglyceride Quantification Kit (ab65336) from Abcam, Cambridge, UK.

Radiochemical measurement of synthesized triglyceride

HepG2 was labelled with ^{12}C-Oleate for 2-hours, then followed by total lipids extraction [28] and the resultant radiolabelled triglyceride was separated on a TLC plate with glyceryltripalmitate as standard and quantified with the scintillation counter (Beckman coulter LS6500, USA) [26] and normalized per milligram protein estimated with Bradford method [29].

Fatty acid composition in total lipids of HepG2

Fatty acid levels (µg) were normalized per milligram protein, quantified by Bradford assay [29], in HepG2 pellets. Pellets were dissolved in 0.2 mL cell lysis buffer containing 1mM phenylmethanesulfonyl fluoride [30], followed by sonication, to obtain cell lysate. Total lipids extraction [28] was achieved after adding 0.05mg pentadecanoic acid (internal standard) to cell pellets. After drying, synthesis of fatty acid methyl esters (FAME) using 3mol/l methanolic HCl (Supelco, Bellafonte, PA, EEUU) for 1-hour at 90°C, was performed. FAME were analysed by gas-chromatography [31] and fatty acid concentrations were determined in relation to peak area of internal standard.

Seahorse extracellular flux assay of mitochondrial dysfunction

Maximal respiratory capacity. Briefly, the basal oxygen consumption rate (OCR) measurement was performed in HepG2 cells in a rich substrate media (glucose-2.5mM, pyruvate-1mM, L-Glutamine-2mM, BSA-0.1%) by addition of the inhibitors Oligomycin and FCCP followed by antimycin/rotenone using Seahorse 24XF flux analyser.

Respiratory capacity in a limited-substrate (high-palmitate) supply. Then, to examine how the low B12 HepG2 function with the endogenous supply of high extracellular levels of palmitate and other limited substrate, we incubated HepG2 in a limited-substrate KHB medium (only 0.5mM L-carnitine and 1.25mM glucose), which is poorly enriched with other supplements compared with the rich-substrate KHB medium, for one-hour. After the basal OCR was measured in HepG2, the cells were either exposed to 200µM palmitate (dissolved in 33.3 µM BSA) or 33.3µM BSA only (basal control) in the substrate medium to assess how HepG2 cells efficiently uptake palmitate for ATP metabolism.

Statistical analysis

All quantitative measurements, where applicable, were obtained n=6 for standards, controls and cases for precision. Differences between either parametric groups or non-parametric groups were observed respectively by performing Student's t-test or Mann-Whitney U test. P values of <0.05 were considered statistically significant.

Results

Effect of B12 on Lipogenesis

Lipid droplets accumulation. To determine the effect of B12 on hepatic lipogenesis, we imaged lipid droplets in HepG2 cells using x40 objective of a light microscope under different conditions of B12 [500nM (control), 1000pM, 100pM and 25pM] following the initial fixing and staining of cells with ORO. We observed HepG2 cells in low B12 had high number of intensely stained lipid droplets compared to control with few lightly stained lipid droplets (Fig. 1A.i). Then the lipid content was quantified using the elution assay standardized by milligram protein concentration of HepG2, which showed significantly higher amount of lipids eluted from cells of low B12 compared with control (Fig. 1A.ii).

Total intracellular triglyceride levels. HepG2 in low B12 had significantly higher level of total intracellular triglyceride, normalized per milligram protein, compared with control cells (Fig. 1B), thus confirming the earlier evidence obtained in ORO staining and elution assays.

Triglyceride synthesis utilizing radio-labelled fatty acid. We observed a high measure of radioactivity (disintegration per minute, DPM) by scintillation count in HepG2 of low B12, normalized per milligram protein, following initial extraction and isolation of radiolabelled triglyceride. This provided a direct indication that increased levels of fatty acids were incorporated and synthesised in HepG2 treated with low B12 compared to control (Fig. 1C).

Genes regulating fatty acid synthesis. Next, we assessed the effect of B12 on gene expression of sterol regulatory element-binding protein (SREBF), which is a master regulator of biosynthesis pathways of fatty acids, triglyceride and cholesterol, and then the downstream genes regulating fatty acid synthesis: acetyl-CoA by ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), and elongation of very long-chain fatty acids (ELOVL6). We observed that gene expression of SREBF1 and the genes involved in *de novo* lipogenesis including ACLY, ACC, FASN and ELOVL6, were increased in low B12 cells compared with control (Fig. 1D).

Genes regulating triglyceride biosynthesis. Triglyceride biosynthesis occurs by conversion of saturated fatty acids to monounsaturated fatty acids by Stearoyl-Co-desaturase 1 (SCD1) and sequential esterification onto glycerol-3-phosphate (G3P) by Glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol-3-phosphateacyltransferase (AGPAT), phosphatidate phosphatase (LPIN1) and Diacylglycerol acyltransferase (DGAT) enzymes into triglyceride, which may be stored in the liver or exported into the blood as VLDL.

The mRNA expression of the genes (SCD1, GPAM, AGPAT, LPIN1 and DGAT2) were increased in low B12 cells. Therefore, these findings demonstrate that both *de novo* synthesis and retention of fatty acids in triglycerides were increased in low B12 cells compared with control (Fig. 2A).

Genes regulating cholesterol synthesis. Dysregulation in fatty acid and triglyceride metabolism could affect cholesterol biosynthesis as the latter utilizes acetyl CoA and VLDL which are derivatives of fatty acids (endogenous or exogenous) and triglycerides in the mitochondria and cytosol, respectively. We observed an increased gene expression of

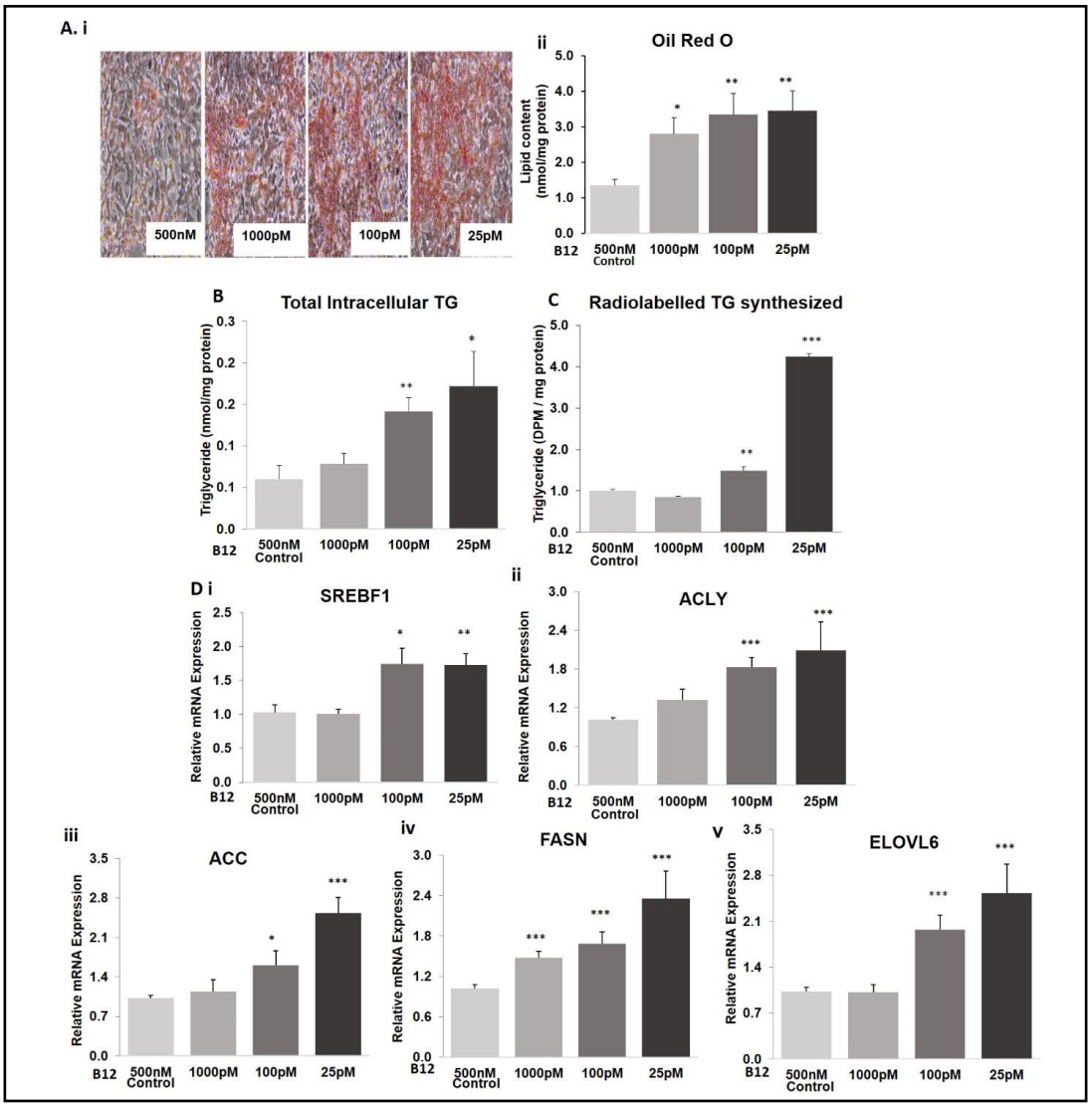

Fig. 1. Low B12 increased lipid droplets, triglyceride levels and genes regulating fatty acid synthesis in HepG2: (A) Image of lipid droplets in HepG2 cells stained with ORO and estimation of lipids eluted from hepatocytes standardized per milligram protein. (B) Total intracellular levels of triglycerides quantified in hepatocytes using the triglyceride kit and normalized per milligram protein under each B12 condition. (C) Levels of synthesized triglyceride in HepG2 was assessed radio-chemically (using ^{14}C-Oleate, L-carnitine) and normalized per milligram protein. (D) Expression of gene SREBF1 (i) and genes regulating de novo fatty acid synthesis [ACLY (ii), ACC (iii) FASN (iv) and ELOVL6 (v)], normalized to 18S rRNA. Data is mean ± SEM (n=6), and *compared to control; *p< 0.05, **p< 0.01, ***p< 0.001.

LDLR (master regulator of cholesterol synthesis) in low B12 cells. Likewise, genes including 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS1) and Isopentenyl-Diphosphate delta Isomerase 1 (IDI1) were increased in low B12 compared with control (Fig. 2B). This evidence therefore suggests that hepatocyte cell line in low B12 had higher levels of cholesterol compared with control.

Fatty acid levels. We showed evidence of low B12 induced upregulation of genes involved in pathways leading to de novo synthesis, elongation and desaturation of certain fatty acid. We further performed fatty acid quantification in HepG2 under different conditions of B12 to assess how levels of different fatty acid were affected in the hepatocyte cell line.

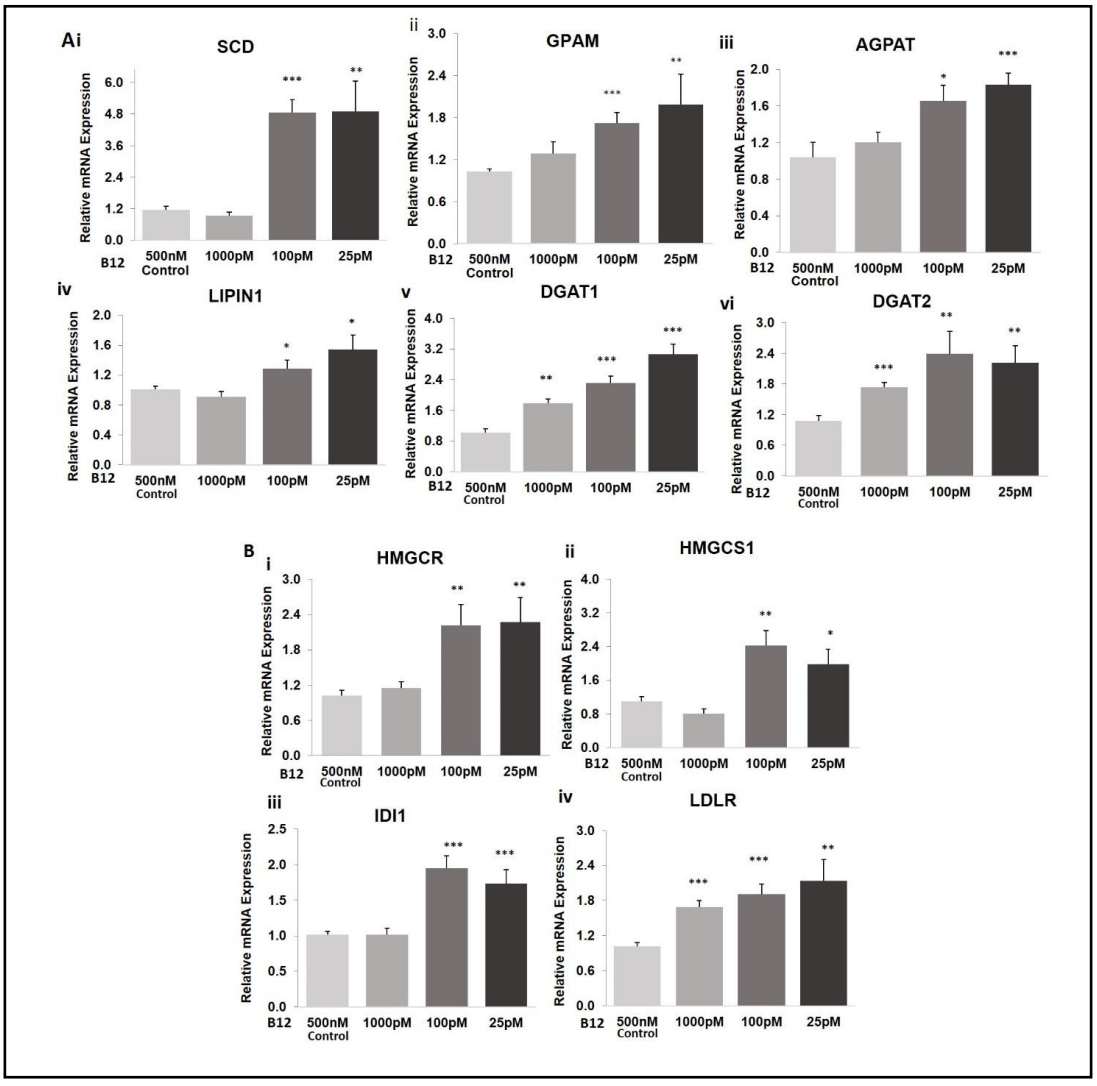

Fig. 2. Low B12 upregulates enzymes involved in triglyceride and cholesterol biosynthesis in HepG2: (A) Expression of genes regulating de novo triglyceride biosynthesis [SCD (i), GPAM (ii), AGPAT (iii), LIPIN (iv) and DGAT2 (v)] and DGAT1 (vi) normalized to 18S rRNA. (B) Expression of genes regulating cholesterol biosynthesis [HMGCR (i), HMGCS1 (ii) and IDI1 (iii) and LDLR (iv) in hepatocytes. All gene expressions were normalized to 18S rRNA. Data is mean ± SEM (n=6), and *compared to control; *p< 0.05, **p< 0.01, ***p< 0.001.

Fatty acid families that contributed to the accumulation of total fatty acid were saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), n-6 polyunsaturated fatty acid (n-6 PUFA), n-3 polyunsaturated fatty acid (n-3 PUFA), n-9 long chain polyunsaturated fatty acid (n-9 LC-PUFA) and trans fatty acid. Our data showed that the level of total fatty acids synthesized in HepG2 with low B12 (25pM) was 38% higher compared with control (Control - 254 ± 31 µg fatty acid/mg protein vs low B12 (25pM) - 367 ± 37 µg fatty acid/mg protein, P=0.006) (Fig. 3i). We observed that the concentration of the predominant fatty acid form, the SFA and MUFA were significantly higher in low B12 which accounted for the overall increase in the total fatty acid in low B12 hepatocyte-cell line compared to control (Fig. 3ii, iii). In PUFAs-total, there was no significant difference observed between low B12 and control however, sub-groups of PUFA such as n-6 PUFA, n-6 LC-PUFA, n-6/n-3 PUFA and trans-fatty acid were significantly higher in low B12 hepatocytes (Fig. 3vii-x). Moreover, n-3 PUFA and n-3 LC-PUFA concentrations showed no significant difference between low B12 and control (Fig. 3v, vi).

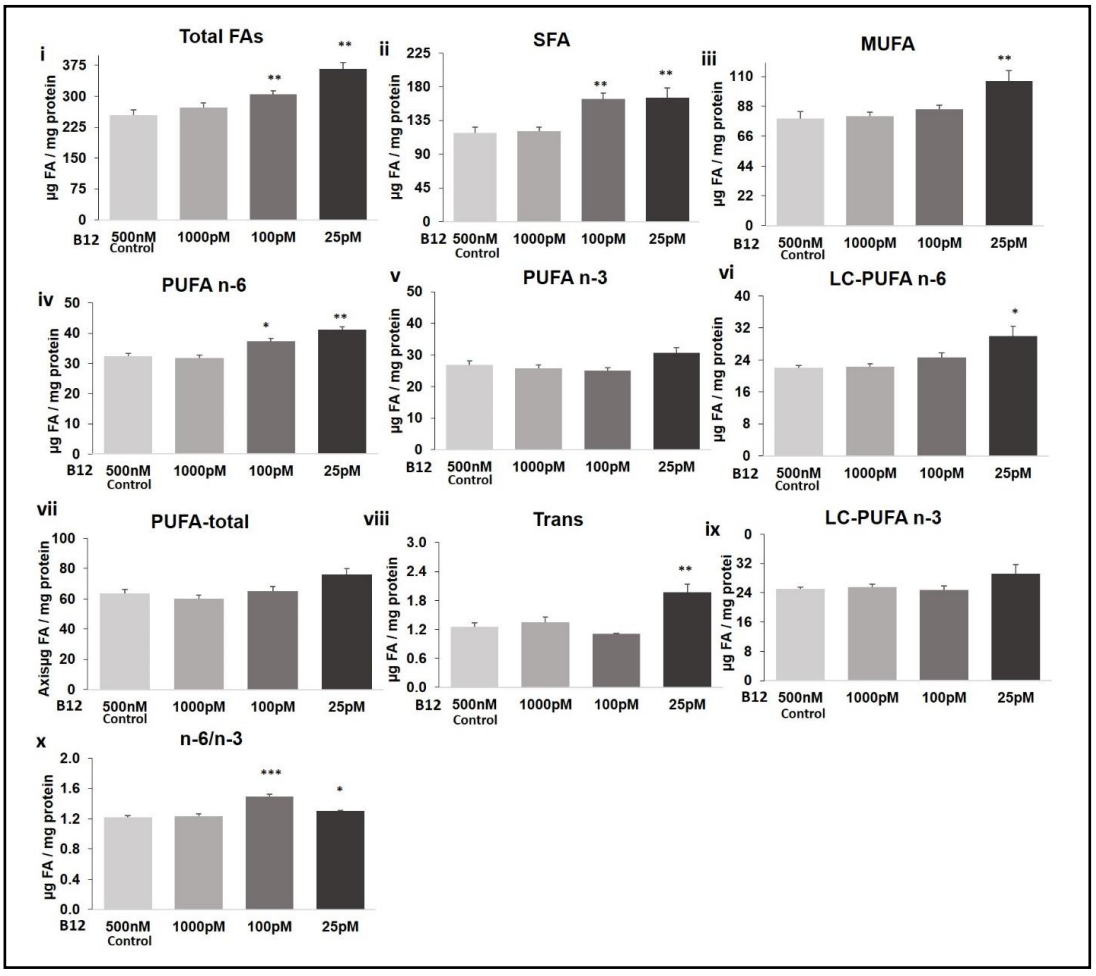

Fig. 3. Levels of various groups of fatty acids in HepG2 in different conditions of B12: Fatty acid levels in HepG2 cell line in different B12 conditions [500nM (Control), 1000pM, 100pM and 25pM] obtained in total lipids extracted from cell pellets of hepatocytes and analysed using gas chromatography. Fatty acid levels (µg) normalized per milligram protein showing various fatty acid groups such as total fatty acid (i), SFA (ii), MUFA (iii), PUFAn-6 (iv), PUFAn-3 (v), LC-PUFAn-6 (vi), PUFA-total (vii), Trans-fatty acids (viii), LC-PUFAn-3 (ix), PUFA n-6/n-3 ratio (x). Data is mean ± SEM (n=6), and *compared to control; *p< 0.05, **p< 0.01, ***p< 0.001.

Levels of individual fatty acids in different B12 conditions are listed in Table 1, which explained the differences observed in the various sub-groups of the fatty acids. While some of the saturated even chain fatty acid, odd chain fatty acid and trans-fatty acid showed higher concentrations in low B12 levels compared to control, no differences were seen in others. Similar observations were seen in PUFA subgroups (n-3, n-6 and n-9). Interestingly, all the MUFAs showed higher concentrations in low B12 levels except Gondoic acid (20:1n-9) which was significantly lower compared to control.

Effect of B12 on Fatty acid oxidation

Genes regulating fatty acid oxidation. Free long chain-fatty acids 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). This precedes dehydrogenation, hydration, oxidation and thiolytic cleavage by enzymes including acyl-CoA dehydrogenase long chain (ACADL), acyl-CoA dehydrogenase medium chain (ACADM), acyl-CoA dehydrogenase short

Table 1. Levels of individual fatty acids in HepG2 of different B12 conditions. * indicates significance compared to control; * p< 0.05, ** p< 0.01, *** p< 0.001. n. d: not detected

Fatty acid µg FA/mg protein ± S. D	500nM B12 (control) N= (6)	1000pM B12 N= (6)	100pM B12 N= (6)	25pM B12 N= (6)
Even chain				
Lauric acid (C12)	n.d	n.d	0.93 ± 0.3	0.61 ± 0.1
Myristic acid (C14)	3.73 ± 0.7	3.86 ± 0.3	4.82 ± 0.4**	5.71 ± 1.1**
Palmitic acid (C16)	75.47 ±12.3	77.65 ± 7.8	102.85 ± 11.3**	111.72 ± 15.8**
Stearic acid (C18)	34.76 ± 5.9	35.07 ± 3.7	47.53 ± 5.0**	47.74 ± 9.0*
Odd Chain				
Margaric acid (C17)	1.97 ± 0.4	2.01 ± 0.2	3.05 ± 0.5**	3.43 ± 1.2*
Heneicosylic acid (C21)	0.66 ± 0.1	0.70 ± 0.2	0.81 ± 0.2	1.45 ± 0.4**
Tricosylic acid (C23)	1.20 ± 0.2	1.29 ± 0.1	2.38 ± 0.3***	2.34 ± 0.7**
MUFA				
Hexadecanoic acid (C16:1n-9)	3.47 ± 0.6	3.70 ± 0.5	3.9 ± 0.5	5.00 ± 1.0**
Palmitoleic acid (C16:1n-7)	7.95 ± 0.7	8.36 ± 1.2	8.39 ± 0.8	10.27 ± 1.7*
Oleic acid (C18:1n-9)	47.66 ± 2.3	47.21 ± 5.3	47.20 ± 5.0	61.04 ± 10.6*
Cis-vaccenic acid (C18:1n-7)	23.01 ± 1.3	23.66 ± 2.9	23.54 ± 2.2	29.94 ± 5.2*
Gondoic acid (C20:1n-9)	1.80 ± 0.4	2.20 ± 1.0	0.55 ± 0.6**	0.53 ± 0.1***
Trans FA				
Palmitelaidic acid (C16:1t)	0.33 ± 0.1	0.47 ± 0.1	0.6 ± 0.0***	0.99 ± 0.4**
Elaidic acid (C18:1t)	0.32 ± 0.0	0.38 ± 0.1	n. d	0.54 ± 0.1**
Linoelaidic acid (all trans-9, 12) (C18:2tt)	n. d	n. d	n. d	0.17 ± 0.3
Linoelaidic acid (trans-12) (C18:2ct)	0.66 ± 0.1	0.53 ± 0.1	0.49 ± 0.01	0.63 ± 0.1
PUFA n-3				
α-Linolenic acid (C18:3n-3)	2.32 ± 0.1	2.16 ± 0.1	1.90 ± 0.2	2.93 ± 0.5
Eicosatrienoic acid (C20:3n-3)	0.43 ± 0.1	0.46 ± 0.0	0.43 ± 0.0	0.67 ± 0.4
Eicosapentaenoic acid (C20:5 n-3)	3.66 ± 0.6	3.98 ± 0.5	0.43 ± 0.0	5.04 ± 1.1*
Docosapentaenoic acid C22:5 n-3)	2.50 ± 0.5	2.97 ± 0.9	2.97 ± 0.5	3.45 ± 0.7*
Docosahexaenoic acid (C22:6 n-3)	17.41 ± 1.3	17.83 ± 1.8	17.87 ± 1.7	21.00 ± 2.9
PUFA n-6				
Linoleic acid (C18:2 n-6)	10.31 ± 1.1	10.47 ± 0.9	12.75 ± 1.5**	14.40 ± 2.4**
Dihomo-γ-linolenic acid (C20:3n-6)	3.03 ± 0.5	3.17 ± 0.4	3.24 ± 0.4	4.27 ± 1.1*
Arachidonic acid (C20:4 n-6)	17.72 ± 2.6	18.07 ± 2.0	21.17 ± 2.7*	25.09 ± 5.1**
Docosatetraenoic acid (C22:4 n-6)	n. d	n. d	n. d	0.25 ± 0.3
Docosapentaenoic acid (C22:5 n-6)	0.29 ± 0.0	0.29 ± 0.0	0.49 ± 0.04**	0.58 ± 0.2*
PUFA n-9				
Mead acid (C20:3 n-9)	2.32 ± 0.3	2.41 ± 0.3	2.88 ± 0.4*	2.86 ± 0.4*

chain (ACADS) and hydroxyacyl-CoA dehydrogenase trifunctional multi-complex subunits beta and alpha (HADHB and HADHA) in the beta-oxidation pathway.

In the mitochondria, B12 is a cofactor for the enzyme methyl malonic CoA mutase (MCM). Low B12 deficiency reduces the activity of MCM resulting in the accumulation of methyl malonic acid (MMA) through inefficient conversion of methyl malonyl-CoA to succinyl CoA during propionate metabolism. MMA was however shown to be a potent inhibitor of the rate limiting enzyme of the fatty acid oxidation pathway, CPT1 in the liver and muscles of rats [32]. Since lipid metabolism is co-ordinated by an intricate balance of fatty acid synthesis and oxidation, we assessed the effect of low B12 on CPT1α and the downstream genes involved in fatty acid oxidation in the mitochondria. The genes regulating fatty acid oxidation: CPT1α, CACT, ACSL1, ACADL, ACADM, ACADS, HADHB and HADHA were significantly decreased in HepG2 with low B12 compared to control (Fig. 4A).

Effect of B12 on mitochondrial functional integrity

Efficiency of mitochondria in utilizing a rich-substrate supply. The key metabolic pathways such as fatty acid oxidation and oxidative phosphorylation (OXPHOS) in mitochondria is the principal source of energy (ATP) in eukaryotes. It has been shown that primary deficiencies in fatty acid oxidation results in secondary OXPHOS defects, although the precise underlying mechanism is unclear [33]. We assessed the effect of B12 on mitochondrial respiration (OXPHOS), by measuring the OCR in HepG2, as an assessment of mitochondrial functional integrity under various conditions of B12.

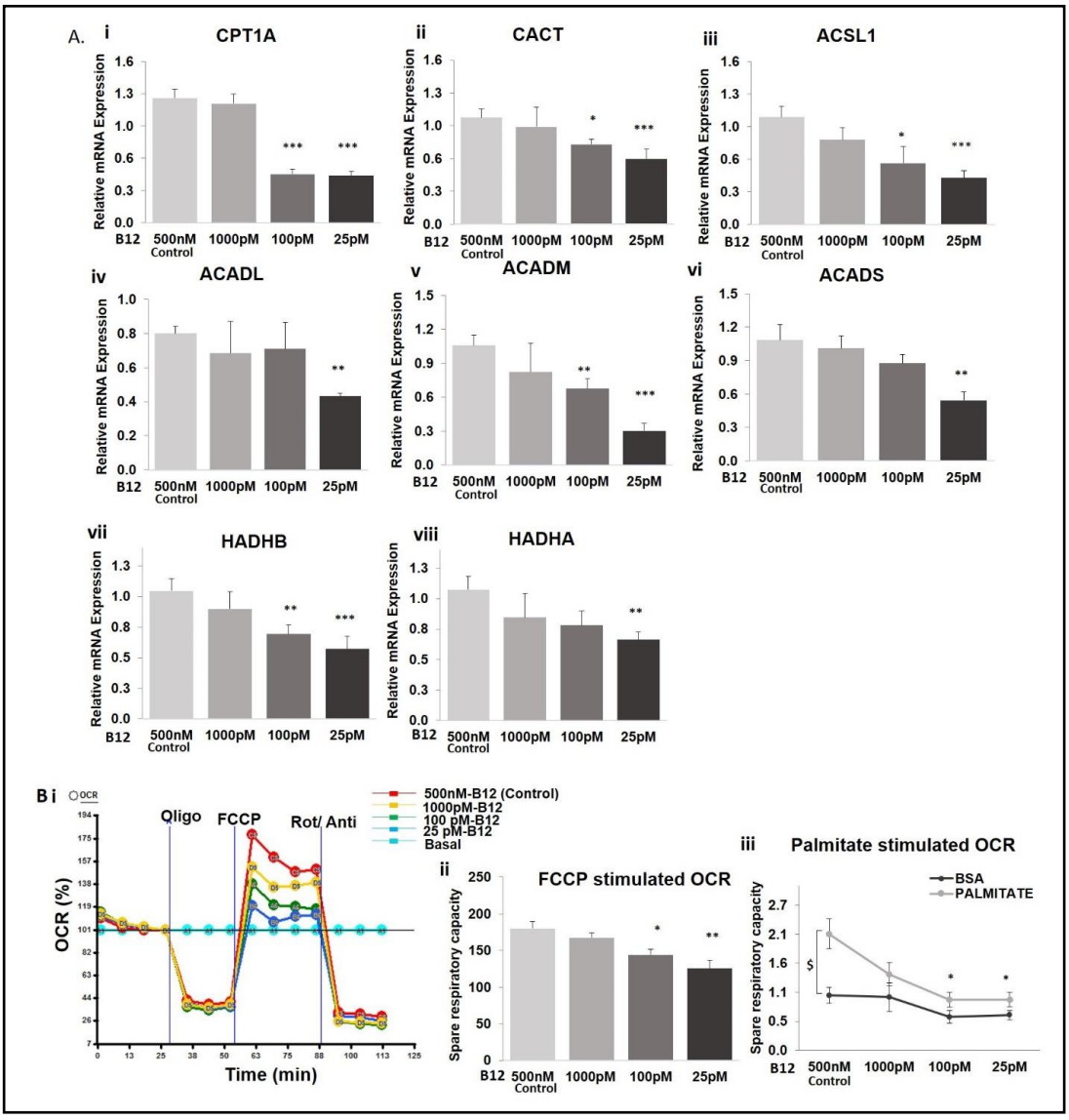

Fig. 4. Low B12 impaired fatty acid oxidation and triggered mitochondrial dysfunction in HepG2: (A) Expression of genes oxidising fatty acids - CPT1a (i), CACT (ii), ACSL1 (iii), ACADL (iv), ACADM (v), ACADS (vi), HADHB (vii) and HADHA (viii) normalized to 18S rRNA. (B) (i) OCR before and after injection of inhibitors such as oligomycin, FCCP and rotenone/antimycin-A, showing the maximal respiratory capacity in HepG2 under various B12 conditions. SRC in HepG2 of; (ii) a substrate-rich KHB substrate medium and (iii) a substrate-limited KHB medium under different conditions of B12. Data is mean ± SEM (n=6), and *compared to B12 control; *p< 0.05, **p< 0.01, ***p< 0.001. $ - represents significane compared to basal control (BSA).

In the presence of inhibitors (oligomycin, FCCP, rotenone), the maximal respiratory capacity, OCR of HepG2 in a rich-substrate medium (high glucose), was decreased in low B12 compared with control (Fig. 4B.i). Also, the spare respiratory capacity (SRC), a measure of the capacity of electron transport chain and substrate supply to respond to elevation in energy demand, was decreased in low B12 cells compared with control (Fig. 4B ii). This suggests that the efficiency of the mitochondria in utilising a rich-substrate for energy metabolism was compromised in low B12.

Efficiency of the mitochondria in utilizing limited-substrate (low glucose, high palmitate) supply. We observed that in low B12, upon exposure to high palmitate levels, the SRC of HepG2 cells was significantly lower compared with control (Fig. 4B.iii). This suggests that the capacity of the mitochondria to catabolise long chain fatty acid (palmitate) for energy metabolism was reduced in low B12, therefore, likely to accumulate in low B12.

Discussion

Here we show that low B12 in HepG2 increased gene expression of lipogenesis and decreased lipid oxidation, resulting in increased intracellular triglyceride and accumulation of lipid droplets. In addition, we also observed subclasses of fatty acids such as SFAs, MUFAs, n-3, n-6 PUFAs and trans-fatty acids were also significantly higher in low B12 condition. Our findings indicate that in low B12 conditions, increased fatty acid synthesis coupled with reduced fatty acid oxidation and catabolism of fatty acids (decreased mitochondrial respiration), may lead to higher intracellular fatty acid concentrations in HepG2 cells.

Hepatocytes demonstrate elevated level of fatty acids, which is immediately converted to triglyceride and are stored in lipid droplets. In the current study, we observed in HepG2 with low B12 that lipid droplets, total intracellular triglycerides and radiolabelled-fatty acid uptake for triglyceride synthesis were higher. This evidence was similar to the observation of elevated accumulation of lipid droplets, increased levels of triglyceride and cholesterol in adipocytes differentiated in deficient B12 condition [15]. An earlier study hypothesized that lipid droplets are much sensitive to conditions such as nutritional stress capable of inducing unique alterations in the lipidome of lipid droplets present in mice hepatocytes [34]. 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 [34]. Accumulation of lipid droplets underlies and defines the state of steatosis in the liver [35], therefore implying that our observation of low B12 induced accumulation of lipids in HepG2, may be associated with the pathogenesis of hepatic steatosis. This supports the clinical observation which showed that serum B12 negatively associated with serum triglyceride and VLDL levels in Indian subjects [17, 36]. In an animal model, severe B12 deficiency was associated with higher serum triglyceride levels which resulted in adverse pregnancy outcome [37]. Our findings extend these evidences and provide potential mechanisms for higher lipid levels in low B12 conditions.

Furthermore, we observed that gene expression of the master regulator of lipogenesis (SREBF1), cholesterol synthesis (LDLR) and downstream genes regulating synthesis of fatty acid, triglyceride and cholesterol were increased in HepG2 in low B12. 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 [38]. Higher expression of SREBF1 and LDLR were observed in human adipocytes with low B12 levels [14] and increased level of SREBP-1c in the fatty livers of ob /ob mice [39]. Similar studies have also shown increased expression of genes involved in biosynthesis of fatty acid, triglyceride and cholesterol in animals [13] and human adipocytes [15]. Three separate clinical studies involving women at (a) child-bearing age, (b) early pregnancy and (c) during delivery showed an association between low B12 and higher LDL cholesterol, total cholesterol, ratio of cholesterol-to-HDL and triglyceride [14]. In addition, adipocytes isolated from subjects with low B12 levels showed higher cholesterolgenesis and lipogenesis, which may result from epigenetic modulations *via* hypomethylation of SREBF and LDLR [14] and altered micro-RNAs targeting PPARγ and insulin resistance [15], respectively. In humans, *de novo* lipogenesis is reported to be responsible for about 26% of total triglyceride synthesis in the liver and contributes to the incidence of hepatic steatosis and NAFLD [40]. Our findings raised the possibility that an increase in hepatic *de novo* lipogenesis due to low B12 levels may increase the future risk of obesity and NAFLD and in turn the risk of developing T2D.

Although, genetically driven NAFLD causally promotes T2D and central obesity [41], our data adds that future research should address the epigenetic mechanisms of B12 driving obesity and NAFLD.

An important observation of the present study was the extensive evaluation of 27 fatty acids in hepG2 cells in four different conditions of B12. Overall, total fatty acids and subclasses of fatty acids (SFAs, MUFAs, n-6 PUFAs and trans-FAs) were higher in HepG2 cells in low B12. Recently, the Fatty Acids and Outcomes Research Consortium (FORCE) study involving 17 global cohorts (n=65, 225) reported that higher concentrations of SFA (16:0 - palmitate, 18:0 -stearate) and MUFA (16:1 n-7 – palmitoleate, 18:1 n-9 - oleate) was associated with higher incidence of T2D [42]. We found higher levels of 16:0 and 18:0, *de novo* lipogenesis related fatty acids in low B12 status. Several studies have shown association of SFAs [43], MUFAs [44] and trans-fatty acids concentrations [44] with subclinical inflammation, increased risk of cardiometabolic diseases. Chen *et al.* showed that serum SFA and MUFA were elevated in GDM women at delivery and also reported a graded trend between the severity of maternal hyperglycaemia and individual serum fatty acid concentrations [45]. Similarly, the same authors showed that percentage of saturated even chain fatty acid (14:0 - myristic acid) was inversely associated with insulin resistance and inflammation [5, 46]. This was in line with an animal study that reported increased percentages of these fatty acids in liver tissues of B12-deficient rat [47]. These data therefore highlight the potential importance of *de novo* lipogenesis and these individual fatty acids in the low B12 status which might predispose to development of T2D and other related co-morbidities. Whether B12 plays an epigenetic role in the synthesis of 16:0 and 18:0 fatty acids, similar to our previous observations on adipocytes [14, 15] and animal studies [13, 48], requires future studies.

In addition, we also observed n-3 PUFA (EPA (eicosapentaenoic acid), DPA (docosapentaenoic acid)), n-6 PUFA (LA (linoleic acid), DGLA (dihomo-γ-linolenic acid), AA (arachidonic acid), DPA) and n-6/n-3 ratio levels were significantly higher in low B12 status. Iglesia *et al.* in European adolescents found an inverse association of serum B12 with triglyceride and phospholipid fatty acid, mainly with n-3 PUFAs [44]. Similar relationship in liver tissues from offspring of B12 restricted rats was shown between B12 and n-3 and n-6 PUFA percentages [18]. Association of higher n-6 /n-3 ratio with dysregulation in hepatic metabolism of lipids [49] and the risk of developing NAFLD [50] has been studied. On the contrary, a separate FORCE consortium study (30 prospective cohorts; n=68,659) showed circulating and adipose tissue concentrations of LA were inversely associated with CVD [51]. EPIC-InterAct study (eight European cohorts; n=15,919) showed inverse association of LA and positive association of DGLA and DPA with T2D [22]. In addition, a longitudinal study of 2803 pregnant women showed an inverse association of EPA and DPA with insulin resistance markers and positive association of DGLA with GDM [52]. These large cohort studies showing differential association of these n-3, n-6 PUFA and the risk of metabolic diseases may depend upon complex interplay of other risk factors. It is known that circulating n-3 and n-6 PUFAs are a function of exogenous (diet) and endogenous (*de novo* lipogenesis) sources, but our finding clearly indicates that low B12 in HepG2 contributes to some fraction of endogenous PUFAs. Our data suggests that in low B12 status, the pathophysiological process of dyslipidaemia and insulin resistance may be linked to these *de novo* lipogenesis related fatty acids. Future investigations studying the role of complex interplay of these fatty acids in obese individuals with B12 deficiency and GDM risk are warranted.

Another significant finding in this study was low B12 accounted for impaired beta-oxidation of fatty acids which is revealed by decreased expression of the rate limiting enzyme CPT1α and the downstream genes crucial in the fatty acid oxidation pathway. In addition, we observed that mitochondrial functional integrity (OCR and SRC) was decreased in low B12 condition thereby indicating that the oxidation of long chain fatty acid (palmitate) is reduced in the mitochondria. Likewise, an animal study had shown evidence of hepatic fatty acid oxidation impairment by methyl donor (both B12 and folate) deficiency *via* hypomethylation of PGC1-α [8]. The authors however, supplemented mice with methyl donors and observed

a deficit of carnitine in the liver of subjects compared with control [8], confirming fatty acid oxidation improvement *via* CPT1α upregulation [53]. These observations indicate impairment of combined fatty acid oxidation and mitochondrial respiration in low B12 state and the importance of B12 in hepatic metabolism [54].

Conclusion

In summary, our study provided novel evidence that low B12 in hepatocytes accumulated more lipids, intracellular triglyceride and increased uptake of fatty acid under the influence of increased fatty acid synthesis and decreased fatty acid oxidation. Interestingly, we found that there are higher levels of fatty acid concentrations, especially the subclasses of SFA and MUFA. Our data also support that inefficiency to couple beta oxidation of fatty acid with mitochondrial respiration might be a possible confounder to accumulate more fatty acids in the hepatocytes in low B12 status which could result in dyslipidaemia. These findings highlight the need to recognize the distinct association between B12 and individual fatty acids and their metabolic role. Future studies elucidating the possible epigenetic mechanisms of B12 in metabolic tissues are warranted.

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Author Contributions

Conceptualization: AA and PS; Methodology: JB, AA, AG, EL and VZ.; Investigations: JB, AA, AG, EL; Writing-original draft preparation: JB and AA; Writing – Review & Editing: JB, AA, PS, AG, EL and VZ; Supervision: AA and PS. All authors contributed and approved the manuscript for submission; PS is the guarantor of this work and had full access to all the data presented in the study and takes full responsibility for the integrity and the accuracy of the data analysis.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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