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Low vitamin B12 in pregnancy is associated with adipose derived circulating miRs targeting PPARγ and insulin resistance

Antonysunil Adaikalakoteswari[1], Manu Vatish[2], Mohammad Tauqeer Alam[3], Sascha Ott[3], Sudhesh Kumar[1,4] and Ponnusamy Saravanan[1,5]*

[1]Warwick Medical School, University of Warwick, Warwick, UK, [2]Nuffield Department of Obstetrics & Gynaecology, University of Oxford, UK, [3]Department of Computer Science, University of Warwick, Warwick, UK, [4]University Hospital of Coventry and Warwickshire, Coventry, UK, [5]Academic department of Diabetes and Metabolism, George Eliot Hospital, Nuneaton, UK

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Context: Low vitamin B12 (B12) during pregnancy is associated with higher maternal obesity, insulin resistance(IR) and gestational diabetes(GDM). B12 is a key co-factor in 1-carbon metabolism.
Objective: We hypothesize that B12 plays a role in epigenetic regulation by altering circulating miRNAs(miRs) during adipocyte differentiation and results in an adverse metabolic phenotype.
Design, settings and main-outcome measure: Human pre-adipocyte cell-line(Chub-S7) were differentiated in various B12 concentrations: Control(500nM), LowB12(0.15nM) and NoB12(0nM). Maternal blood samples(n=91) and subcutaneous adipose tissue (SAT)(n=42) were collected at delivery. Serum B12, folate, lipids, plasma 1-carbon metabolites, miR profiling, miR expression and gene expression were measured.
Results: Our i*n vitro* model demonstrated that adipocytes in B12 deficient conditions accumulated more lipids, had higher triglyceride levels and increased gene expression of adipogenesis and lipogenesis. MiR array screening revealed differential expression of 133miRs involving several metabolic pathways (adjusted $p<0.05$). Altered miR expression were observed in 12miRs related to adipocyte differentiation and function in adipocytes. Validation of this data in pregnant women with low B12, confirmed increased expression of adipo/lipogenic genes and altered miRs in SAT, and altered levels of 11 of the 12miRs in circulation. After adjusting for other possible confounders, multiple regression analysis revealed an independent association of B12 with BMI (β: -0.264; 95% CI: -0.469, -0.058; $p=0.013$) and was mediated by four circulating miRs targeting PPARγ and IR.
Conclusions: Low B12 levels in pregnancy alters adipose derived circulating miRs, which may mediate an adipogenic and IR phenotype leading to obesity.

Our study showed that low B12 levels in pregnancy alters adipose derived circulating miRs, which may mediate an adipogenic and insulin resistant phenotype leading to obesity. .

Introduction

Maternal obesity is a major public health concern and its prevalence has been doubled over the past two decades. In UK(1) and USA(2), 27% of women of child-bearing age are overweight and 20-32% are obese. Maternal obesity is characterized by the presence of excessive amount of adipose tissue (AT) and has adverse effects on maternal health and the developing foetus, predisposing them to cardio-metabolic disease later in life(3). AT development involves two distinct processes: 1) adipogenesis (increased adipocyte number) and 2) lipogenesis (increased accumulation of lipids)(4). In humans, adipogenesis occurs predominantly in the prenatal/postnatal periods and is set during childhood and adolescence(5). However, during child-bearing age, the rate of adipocyte generation gradually reduces. Any dietary or environmental changes that disturb the balance between

adipogenesis and lipogenesis can result in increase in adipocyte size and accumulation of lipids, both known to increase IR(6). Therefore understanding the adipocyte biology during this period will help to understand the role of adiposity on maternal and child health.

B12 deficiency in pregnant women is increasingly common(7) and has been shown to be associated with higher BMI in many studies(8) as well as IR(9), GDM and type 2 diabetes (T2D) in later life(10). An animal study demonstrated(11) that B12 restricted diet resulted in higher adiposity, adipocytokines, dyslipidemia and adverse gestational outcomes. While biochemical plausibility has been postulated, the exact mechanisms of this link between B12 and BMI are not known(12). B12 is required for the synthesis of methionine, the precursor of S-adenosyl-methionine (SAM), a key methyl donor for DNA methylation(12). DNA methylation is involved in the functioning of genes, and depends on the supply of methyl groups by methyl-donors such as B12 from the diet(13). Evidence from two independent US cohorts demonstrated that the methylation variant of a transcription factor HIF3A (rs3826795) exhibited opposite effects on weight change in response to low and high B-vitamin intakes (14). We have shown that low B12 is associated with hypomethylation of cholesterol transcription factor, SREBF1. Our experiments with methylation inhibitor also showed that there may be other epigenetic mechanisms involved(15). Thus, it is plausible that deficiency in B12 might influence methylation patterns in the DNA as well as other epigenetic modulators such as miRs, which regulates gene expression(13).

Recently, much attention has been given to other regulators of AT development such as miRs. MiRs are epigenetic mediators that controls adipocyte differentiation which when perturbed can potentially result in unhealthy metabolic phenotype(16, 17) such as dyslipidemia, hypertension, IR and possibly elevated risk of developing T2D. Maternal diet induced obesity can program AT and modulate miRs during fat cell development(3, 13). In addition, circulating miRs have shown to be altered in gestational obesity(18) and in adults with different degrees of obesity and T2D(16, 19).

Taking these observations together, we hypothesize that low B12 levels during pregnancy may affect the AT development due to altered adipose derived circulating miRs resulting in metabolic phenotype. In this study, we aimed to investigate, 1) the effects of B12 deficiency on adipogenesis and lipogenesis in human adipocyte cell line (Chub-S7), 2) the effects of B12 deficiency on the miR profile in differentiated Chub-S7 and their secretion, and 3) validate the miRs identified in Chub-S7 with the levels of miRs in SAT and circulating miRs in serum of pregnant women with low B12 levels.

Materials and methods

Elaborate methodology of clinical data collection, *in vitro* and *in vivo* experiments are detailed in 'supplemental methods'. They are articulated in brief below.

Differentiation of human pre-adipocyte cell line (Chub-S7):

The normal culture media of adipocytes (DMEM/F12 - Cat#11039-Gibco) contains 500nM of B12. Green *et al*(20) cultured 3T3-adipocytes in B12 concentrations (0-500nM) and showed that the accumulation of odd-chain fatty acids and methylmalonic acid (MMA, tissue marker of B12 deficiency) occurs in B12 deficient conditions, which were prevented by supplementation of 500nM B12. Our previous study also showed that the methylation potential was optimal at 500nM of B12(15, 20). Hence, we chose similar conditions [Control (500nM), Low B12 (0.15nM) and NoB12 (0nM)] for our *in vitro* experiments.

Study population:

A cross-sectional study was conducted in the University Hospital Coventry and Warwickshire (UHCW), Coventry, UK. Fasting maternal blood samples (n=91) and SAT (n=42) were collected at the time of caesarean section(21).

Lipid accumulation:
In adipocytes, cellular lipid accumulation was determined by oil red O staining and triglycerides were determined in cell lysates according to manufacturer's protocol (Abcam).

Quantitative real-time PCR (q-RT-PCR) of mRNA:
RNA isolation and q-RT-PCR from Chub-S7 and human SAT were performed(15).

Locked nucleic acid (LNA) - based miR array profiling:
miR profiling of RNA from Chub-S7 differentiated in low B12 were performed using LNA miRCURY arrays (Exiqon, Denmark).

Bio-informatics analysis of miRs:
To examine which metabolic/signalling pathways were affected, we used bioinformatics prediction database - Bioconductor (R) package miRNAtap which has prediction algorithms, such as DIANA(22), MiRanda(23), PicTar(24) and TargetScan(25). To further determine functional relationships of the significant miRs, an integrated regulatory network of miR-gene-pathway was constructed using Cytoscape software.

Q-RT-PCR of miR:
miRCURY LNA miR PCR system (Exiqon) was used to assess the miRs in Chub-S7 & SAT, and circulating miRs in conditioned media & maternal serum.

Analytical determinations:
Serum B12, folate, cholesterol, triglycerides, HDL-cholesterol(21) and plasma 1-carbon metabolites (SAM, S-adenosyl-homocysteine-SAH, methionine, homocysteine, MMA) were determined by methods as described in supplement(26).

Statistical analysis:
Continuous data were reported as mean ± standard deviation(SD). *In vitro* data were presented as mean ± standard error of the mean (SEM) for at least six independent experiments to ensure reproducibility. Student's t-test was used for comparison of groups, all tests were two-sided, and p-values of <0.05 were considered to be statistically significant. Where appropriate, clinical data were log-transformed before correlation and regression analyses. All analyses were performed using SPSS Statistics v21 (IBMCorp).

Results

In vitro study of B12 deficiency on human adipocyte cell line (Chub-S7):

B12 deficiency on adipogenesis and lipogenesis:
To evaluate the effect of B12 deficiency in adipocytes and the underlying mechanism, we differentiated Chub-S7 in B12 deficient conditions. As shown in Figure 1A-E, we demonstrated increased accumulation of lipid droplets and the triglyceride content in adipocytes differentiated in B12 deficient conditions. We found that through the process of differentiation of adipogenesis, B12 deficient conditions increased the gene expression of key transcriptional regulators of adipogenic differentiation such as PPARγ (Peroxisome-proliferator-activated-receptor-gamma) within 48hrs and CEBPα (CCAAT/enhancer-binding-protein-alpha) after 6 days, and the levels remained significantly higher for the rest of the differentiation time course (14 days) (Supplemental Figure 1A,B) indicating that low B12 directly affects adipogenesis. Then we showed at day 14, in addition to gene expression of PPARγ and CEBPα, the nuclear receptor – RXRα (retinoic-acid-x-receptor-α) that hetero-dimerizes with PPARγ to regulate lipid metabolism were upregulated. Similarly, gene expression of lipogenic enzymes such as fatty acid synthase (FASN) and acetyl CoA carboxylase (ACACA) and the lipid-coating protein (perilipin) were also increased in

adipocytes with B12 deficient levels (Figure 1F,G). These findings suggest that low B12 levels in adipocytes might induce adipogenesis and lipogenesis.

B12 deficiency on epigenetic regulation:
To assess the effect of B12 on epigenetic regulation, we treated the adipocytes with B12 in the presence of a methylation inhibitor (5-aza-2-deoxycytidine). Gene expression of adipogenic regulators (PPARγ, CEBPα, RXRα), lipogenesis (FASN, ACC1) and lipid-coating protein (perilipin) were increased similar to low B12 (Supplemental Figure 2A,B). It is clear from these findings that methylation inhibition (or hypomethylation) alone may not be the only mechanism involved in these alterations, and this led us to further explore other epigenetic mechanisms such as miRs.

B12 deficiency on miR profiling in Chub-S7:
To investigate the effect of B12 on miR, expression of more than 2042 human miRs annotated in miR-Base 18.0 was assessed using miR microarrays in adipocytes differentiated with low B12. The miR profiling detected 560 mature human miRs, out of which 133 miRs (23.8%) were differentially expressed (adjusted $p<0.05$). The two-way hierarchical clustering analysis showed 97miRs were significantly down-regulated and 36miRs were up-regulated in adipocytes cultured in low B12 ($p<0.01$) (Figure 2A). These findings show that low B12 alters miR levels. We then carried out the pathway analysis involved with these aberrant miR expression.

MiR targets and biological pathways prediction:
To further identify and validate the biological roles of the aberrant miRs, analyses were carried out using Bioconductor (R) package miRNAtap for target gene prediction. Number of target genes for significantly differentially expressed miR varied from 22 target genes (miR-146a/miR-377) to 994 target genes (miR-23c), with a median number of 344 target genes (Figure 2B). The union set of all predicted target genes of 133 differentially expressed miRs was analyzed using the hypergeometric statistical test to significantly enrich pathways with pathway definitions taken from KEGG database for biological processes and Recon2 for metabolic processes. Pathway enrichment analysis resulted in significant ($p<0.01$) enrichment of genes that were related to the regulation of metabolic processes such as lipid, aminoacid, nucleotide, transport and glycan metabolism (Figure 2C). Enrichment of biological processes revealed that that these miRs were involved in signalling pathways particularly related to IR, developmental biology, immunity and inflammation (Figure 2D). Interestingly, enrichment analysis indicated that these miRs were involved in classical metabolic and adipocyte differentiation pathways, such as the insulin signalling, Wnt signalling, adipocytokine signalling, PPAR signalling, phosphotidyl-inositol signalling and triacylglycerol synthesis (Figure 2C).

B12 deficiency on miR expression in Chub-S7 and its secretion:
To confirm the differences observed in miR array screening, miRs with significant change in expression level and the putative target genes associated to adipocyte differentiation and function were selected (Figure 2E) and validated by q-RT-PCR analysis. 12miRs chosen for validation were the following: 3 targeting PPARγ (miR-27b, miR-23a, miR-130b), 1 targeting CEBPα (miR-31), 6 targeting adipocyte differentiation (miR-143, miR-145, miR-146a, miR-221, miR-222, miR-125b) and 2 involved in IR pathways (miR-103a, miR-107) (Figure 3A). RT-PCR analysis confirmed that these 12miRs were significantly altered in adipocytes and 9 miRs were significantly altered in the condition media (except miR130b, miR103a, miR107) (Figure 3B). These results show that low B12 alters adipose derived miRs related to adipocyte differentiation and function. To further confirm whether altered miRs in response to low B12 could be a putative epigenetic mechanism, we validated these 12 miRs

in adipocytes with B12 in the presence of a methylation inhibitor. We found 9 miRs in adipocytes and 8 secreted miRs in conditioned media were altered similar to low B12 (Supplemental Figure 3&4). Here it is evident that in addition to methylation inhibition, other epigenetic mechanisms such as miRs are involved in B12 deficient conditions.

Study on pregnant women with low B12 levels:

One-carbon metabolites in pregnant women:
The clinical characteristics of the study population are shown in supplemental Table 1. Reduced methylation potential was observed in women with low B12 status such as lower SAM and methionine (Supplemental Table 1). These results indicate that low B12 disturbs the 1-C metabolism and alters the levels of SAM, the methyl donor which may reduce the effect of methylation of DNA and other epigenetic regulators (miRs).

Adipo/lipogenic gene expression in human maternal SAT:
To further evaluate the tissue-specific effect of B12 on human SAT, gene expression of adipogenesis and lipogenesis were performed in SAT in pregnant women with low B12 levels compared to normal B12. We demonstrated that the gene expression of adipogenic regulators (PPARγ, CEBPα, RXRα), lipogenesis (FASN, ACC1) and lipid-coating protein (perilipin) (Figure 4) were upregulated in maternal SAT with low B12 similar to the observation seen in Chub-S7 (Figure 1F&G). These findings suggest that low B12 levels in pregnant women might enhance adipogenesis and lipogenesis.

Validation of miR expression in human maternal SAT:
Next, we attempted to validate the differential miR expression identified in Chub-S7 to human maternal SAT. Expression levels of all the 12 miRs were significantly up- or down-regulated in SAT from pregnant women with low B12, similar to the observation seen in Chub-S7 (Figure 5A).

Validation of circulating miRs in serum from pregnant women:
Similarly, we validated the adipose derived miR expression observed in conditioned media from Chub-S7 to serum from pregnant women. We observed 11 of the 12 miRs were significantly altered in the circulation, except one (miR-31, not detected) in pregnant women with low B12 levels (Figure 5B). Interestingly, we also observed that 6 of the 11 miRs from serum correlated significantly with miRs from SAT, and 9 of the 12 miRs from the conditioned media correlated significantly with the miRs from the Chub-S7 (Supplemental Table 2). These results reveal that these circulating miRs are adipose derived and are altered due to low B12.

Association of circulating B12 and circulating miRs with obesity:
To further explore the relation between circulating B12 and miRs with obesity, we performed the following correlation analyses. Circulating B12 was inversely correlated with maternal BMI (r=-0.292; p=0.007) and positively with seven circulating miRs - miR-27b (r=0.390; p=0.001), miR-103a (r=0.344; p=0.004), miR-107 (r=0.387; p=0.001), miR-125b (r=0.311; p=0.010), miR-23a (r=0.323; p=0.007), miR-221 (r=0.274; p=0.026) and miR-222 (r=0.400; p=0.001). To further investigate whether the circulating B12 and miRs independently contribute to BMI, multiple regression analysis was carried out. Circulating B12 and four circulating miRs (miR-27b, miR-23a, miR-103a, miR-107) independently associated with BMI after adjusting for likely confounders (age, parity, smoking, insulin, glucose and supplement use) (B12 - β: -0.264, p=0.013; miR-27b - β: -0.250, p=0.041; miR-23a - β: -0.271, p=0.026; miR-103a - β: -0.226, p=0.049; miR-107 - β: -0.228, p=0.041). We also confirmed that excluding the GDM subjects with low B12 on insulin/metformin therapy (n=2), the association of B12 and miRs with BMI remained the same suggesting no effect of associated therapy on BMI (data not shown). However, in multiple regression analysis, the

association of B12 with BMI became non-significant after further adjusting for these four circulating miRs, thereby highlighting a mediating role by circulating miRs between B12 and BMI (Table 1).

To further study the function of these four circulating miRs in metabolic pathways, we constructed a miR-gene-pathway network for these four miRs with their known or predicted target genes and annotated pathways. Figure 5C shows the network of these four miRs shared the targets related to five metabolic pathways related to IR (PPARγ, adipocytokine, insulin, Wnt and T2D) and the six validated genes uniquely regulated by at least one miR each (PPARγ gene as a predicted target of miR-27b and mir-23a; CEBPα as predicted target of miR-23a; FASN as predicted target of miR-107, miR-27b and miR-103a; RXRα, ACACA and PLIN as predicted targets of miR-27b). Therefore, our findings strongly implicate that these four miRs could regulate the adipogenic/lipogenic genes and may mediate an obesity and insulin resistant effect in low B12 status.

Discussion

Our study shows that B12 deficiency in human adipocytes changes tissue-specific miRs and circulating miRs, and leads to adverse metabolic phenotype. Here we have demonstrated that B12 deficiency in adipocytes: 1) caused excess accumulation of lipids, 2) increased the expression of genes that regulates adipogenesis and lipogenesis and 3) resulted in aberrant expression of miRs involved in key metabolic pathways such as PPARγ and IR. These were first demonstrated in human adipocyte cell line and then validated in human SAT. In addition, our clinical study findings supports that the association between low B12 and obesity appeared to be mediated by adipose derived circulating miRs targeting these pathways.

AT development is associated with both increasing adipocyte cell numbers and their ability to accumulate lipids (4, 6). We observed low B12, both *in vitro* (adipocytes) and *in vivo* (adipose tissue at the time of childbirth), caused increased adipogenesis (PPARγ, CEBPα, RXRα) and lipogenesis (FASN, ACACA), indicating that low B12 affects the two distinct processes of AT development. Similar findings were observed in rats where B12 deficiency resulted in differential expression of PPAR signalling pathway(27) and higher activities of hepatic FASN and ACACA(28). We have previously shown in adipocytes that low B12 conditions caused hypomethylation of cholesterol transcription factor, SREBF1 (15). It is known that SREBF1 induces PPARγ and regulates genes necessary for lipogenesis(29).

Our *in vitro* experiments showed that adipocytes in low B12 conditions displayed increased lipid accumulation. As B12 is a key micronutrient essential for functioning of most tissues, similar process may also happen in hepatocytes. If such dysregulation of lipid occurs in hepatocytes, it is plausible, this might contribute to higher circulating lipids, an observation seen in women with low B12(16), who had lower methylation potential (supplement Table 1) and in mice fed with a B12 restricted (11, 28) or methyl-deficient diet(30). Interestingly, their pups also exhibited dyslipidemic profile, an observation previously reported by us from this clinical cohort that lower maternal B12 was associated with lower HDL in the cord blood(21). Whether this is due to adverse epigenetic programming requires further longitudinal, mechanistic as well as interventional studies.

Nutrient imbalance can cause epigenetic modifications through several mechanisms including DNA methylation, histone modification, chromatin remodelling, and changes in the expressions of small and long non-coding RNAs such as miRs(13). While DNA methylation and histone modification are extensively studied, number of recent studies explored the mechanistic aspects of miRs on regulation of protein-coding genes. However, there is no study demonstrating the effects of micronutrient deficiency on miR expression, especially in

SAT. In this study, we report a comprehensive database of differential expression patterns of 133miRs in adipocytes differentiated with low B12. Pathway enrichment analysis of miR array data revealed that these miRs were involved in several metabolic pathways including adipocyte biology and IR such as insulin signalling, Wnt, adipocytokine, PPAR, phosphotidyl-inositol as well as triacylglycerol synthesis pathways. We reported here, 12 miRs related to adipocyte differentiation and function that have been associated with obesity. Our *in vitro* adipocyte experiments showed that these miRs were significantly altered in B12 deficient conditions. These findings were replicated in SAT from pregnant women with low B12 levels. Previous studies have shown that overexpression/knockdown of miR-27b(31), miR-23a(32) and miR-130b(33, 34) as important regulators of adipogenesis by targeting PPARγ and these miRs are downregulated in AT from obese subjects with or without diabetes. Studies in AT from obese women have shown that miR-31 downregulates CEBPα expression at both the transcriptional and translational level (35). In addition, miR-143(17), miR-145(36), miR-146a(37), miR-221(16, 17), miR-222(16, 17) and miR-125b(16, 17) have been shown to exhibit a role in adipocyte differentiation and were significantly altered in morbid-obese patients, pre-pubertal children and in AT of obese mouse models. Furthermore, the role of miR-103a and miR-107(17) in IR has been shown in rodent T2D models and in 3T3-L1 adipocytes. Thus, we showed that low B12 causes aberrant miR expression in SAT and their association with adipogenesis and obesity. Our findings support similar other observations of altered tissue specific miR expression in human placenta exposed to low folate(38) and 3T3-L1 adipocytes exposed to Vitamin A(39).

In addition to these tissue level changes, we observed that the secretion of the adipose derived miRs were significantly altered in adipocytes differentiated *in vitro* in B12 deficient conditions and correspondingly in the circulation in pregnant women with low B12. The tissue expressions of the miRs and circulating miRs also correlated with each other (Supplemental Table 2), indicating the primary source of these miRs could be the SAT. However, 3 of the 12miRs (miR-143, miR-145, miR-146a) and the correlation of miR-145 with human SAT were in the opposite direction. As the effects of low B12 status can be global, other tissues such as liver, muscle or placenta could also contribute to the circulating levels, which may explain this observation. It is also possible that these miRs were induced during adipogenesis but are downregulated in obese state, consistent with previous studies(17). Circulating miRs provide a possible mechanism for cross-talk between tissues. If these aberrant miRs gets transferred across the placenta, it may cause adverse epigenetic changes in the tissues of the offspring and predispose them to metabolic disorders in their later life(40). Future studies are required to prove these speculations.

Finally, multiple regression analyses revealed that the circulating B12 and 4 of the circulating miRs (miR-27b, miR-23a, miR-103a and miR-107) were independently associated with BMI, after adjusting for other possible confounders. Further regression analysis showed that the inverse association between B12 and BMI was reduced when adjusted for these 4 miRs. This suggests that the link between B12 and BMI may be partly mediated through these miRs (Table 1). Other studies(8, 10, 15, 21) have shown strong inverse association between B12 and BMI. However, this is the first description demonstrating that these miRs regulating adipogenesis may potentially play a causal role. On the contrary, whilst our clinical data showed the association of B12 with miRs targeting IR, it did not show with HOMA-IR. This might be due to the sample size or it is possible that these pregnant women will develop IR in the future, as obesity usually precedes IR. If these findings are replicated in longitudinal studies, these miRs may represent early pregnancy bio-markers for IR in women with low B12 levels.

In summary, our study identified that B12 deficiency in pregnancy is independently associated with adipose derived circulating miRs, which are known to affect PPARγ and IR

pathways. This study provides new insight that these adipose derived circulating miRs can act as a novel mode of cell signalling molecules which may predispose metabolic disorders to both mothers and offspring in later life. Thus identification of B12 induced epigenetic signatures could provide unique opportunity to study predictive miR biomarkers and future therapeutic targets for obesity.

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Corresponding author: *Prof Ponnusamy Saravanan (Address: Clinical Sciences Research Laboratories, University of Warwick, UHCW Campus, Clifford Bridge Road, Coventry CV2 2DX, UK; Tel: +44 2476968668, Fax: +44 2476968653; P.Saravanan@warwick.ac.uk)

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Author Contributions: AA and PS conceived the research question and study design. AA performed all the experiments, data collection, statistical analysis, data interpretation and drafted the manuscript. MTA and SO were involved in the bio-informatics analysis and data interpretation. MV involved in the recruitment of the pregnant women, collection of adipose tissue and blood samples. SK and PS were involved in data interpretation and reviewed the manuscript for intellectual content. All authors contributed, revised and approved the final version of the manuscript before 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.

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Figure 1: Oil red O staining of adipocytes in (A) Control (B) Low B12, (C) No B12 (lipid droplets stained as red). B12 deficient conditions increases (D) lipid accumulation and (E) triglycerides in human adipocytes. Low B12 increases gene expression of (F) adipogenic regulators and (G) lipogenesis in Chub-S7. All experiments were performed as n=6; Control, LB-Low B12, NoB-NoB12. Values are mean ± SEM. *P ≤ 0.05; **P ≤ 0.01, ***P ≤ 0.001, p-value compared to control.

Figure 2: (A) Hierarchical clustering of 133 differentially expressed miRs in adipocytes differentiated *in vitro* with low B12 levels. C-Control: n=6; LB-Low B12: n=6. Red – represents expression level below mean (down-regulated) and Green - represents expression level above mean (up-regulated); adjusted p<0.05. (B) Number of predicted target genes for 133 differentially expressed miRs that varied from 22 targets (miR-146a and miR-377) to 994 targets (miR-23c), with a median number of 344 targets. (C) Enrichment analysis for metabolic pathways using Recon2; p<0.01. (D) Enrichment analysis for signalling pathways using KEGG; p<0.01. (E) Hierarchical clustering of 12 selected miRs related to adipocyte differentiation and function; adjusted p<0.05.

Figure 3: (A) Low B12 alters miRs related to adipocyte differentiation and function in Chub-S7. (B) Low B12 alters secreted miR's related to adipocyte differentiation and function in conditioned media from Chub-S7. All experiments were performed as n=6; Control, LB-Low B12, NoB-NoB12. Values are mean ± SEM. *P ≤ 0.05; **P ≤ 0.01, ***P ≤ 0.001, p-value compared to control.

Figure 4: (A) Gene expression of adipogenic regulators and lipogenesis in human maternal SAT. Control: n=29; LB-Low B12: n=13. Values are mean ± SEM. *P ≤ 0.05; **P ≤ 0.01, ***P ≤ 0.001, p-value compared to control.

Figure 5: (A) MiR expression in a subset of human SAT. Control: n=17; LB-Low B12: n=13. (B) Circulating miRs expression in a sub-set of serum from pregnant women with low B12 levels. Control: n=38; Low B12: n=34. Values are mean ± SEM. *P ≤ 0.05; **P ≤ 0.01, ***P ≤ 0.001, p-value compared to control. (C) MiR-gene-pathway regulatory network shows the network of the five pathways related to IR and the six validated genes regulated by

at least one miR each. MiR, genes and pathways are represented by nodes such as orange diamond – miRs, green square – pathways, blue oval – genes, brown oval – validated genes regulated by at least one miR each; Edges (green arrows) represent the relationship between gene and pathway, Edges (orange arrows) represent the relationship between miR and gene predicted by at least two tools, Edges (red arrows) represent the relationship between miR and gene predicted by one tool and validated gene, Edges (blue arrows) represent the relationship between miR and gene evidenced by literature.

Table 1: Multiple regression analysis of maternal B12 with BMI

Maternal Variable* (SDS)	BMI*		
	Model 1		
	β	95% CI	p
B12	-0.264	(-0.469, -0.058)	0.013
miR-27b	-0.250	(-0.488, -0.011)	0.041
miR-23a	-0.271	(-0.508, -0.034)	0.026
miR-103a	-0.226	(-0.452, -0.001)	0.049
miR-107	-0.228	(-0.446, -0.009)	0.041
B12	Model 2		
	-0.219	(-0.523, -0.084)	0.153
B12	Model 3		
	-0.225	(-0.500, 0.049)	0.106
B12	Model 4		
	-0.217	(-0.519, -0.084)	0.154
B12	Model 5		
	-0.211	(-0.523, 0.101)	0.181

*Maternal variables (n=91) and circulating miRs (n=72, measured in a subset of pregnant women) were log transformed for statistical comparisons. β represents SDS change in the dependent variable per SDS change in the independent variable. Model 1: Maternal age, parity, folate supplement use, smoking, insulin and glucose; Model 2: Model 1 + miR-27b; Model 3: Model 1 + miR-23a; Model 4: Model 1 + miR-103a; Model 5: Model 1 + miR-107.

Fig 1

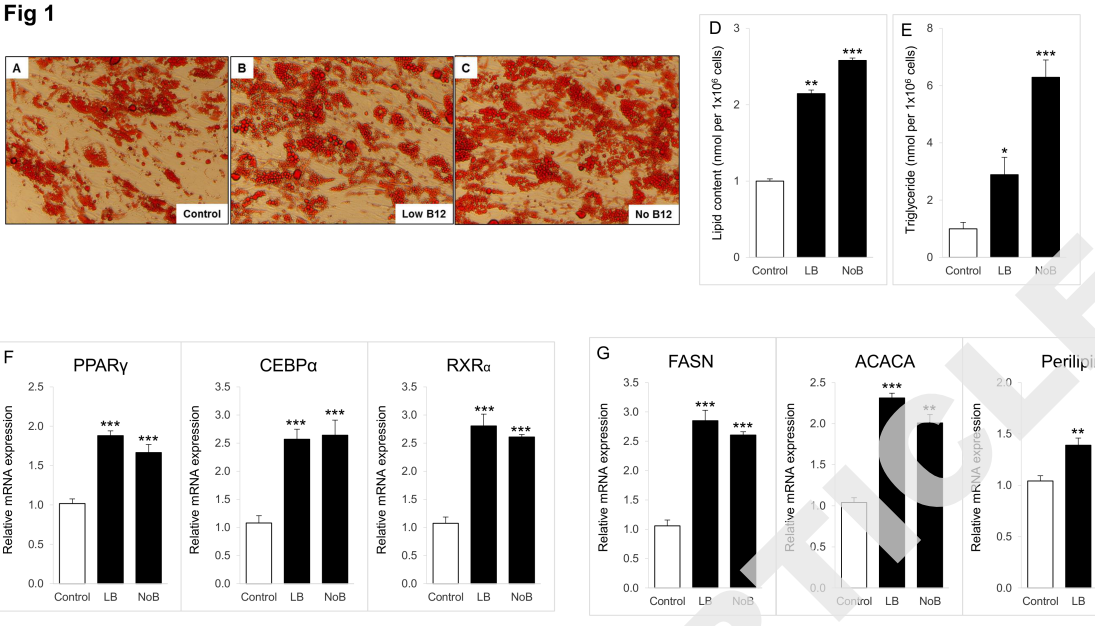

Fig 2

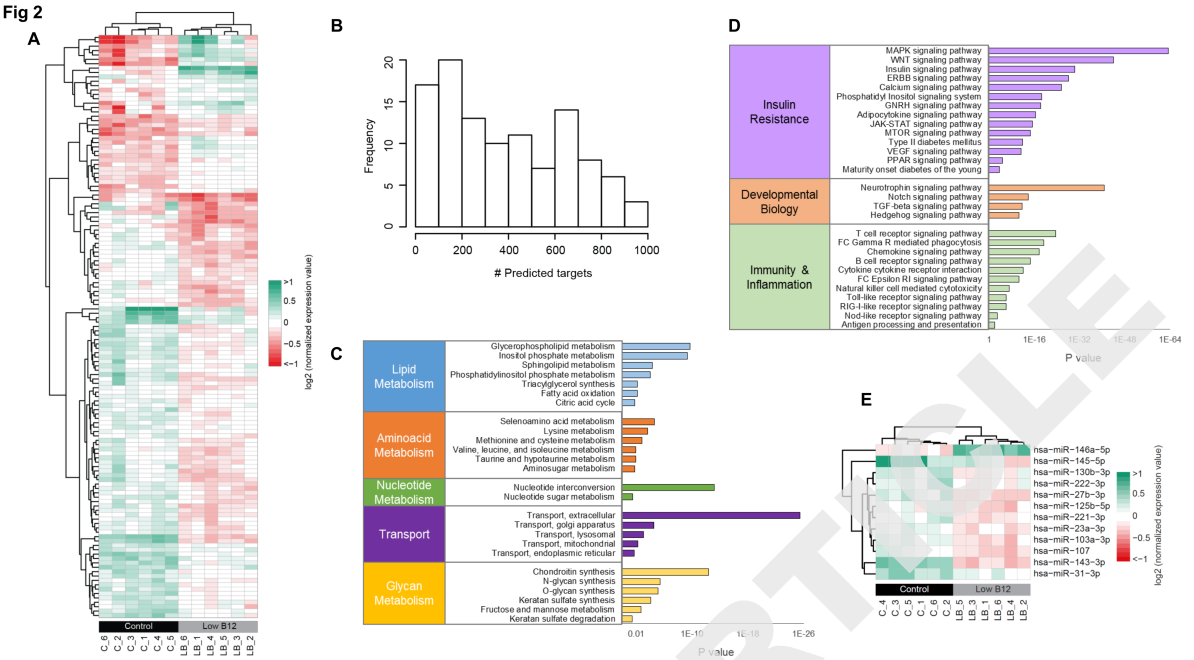

Fig 3

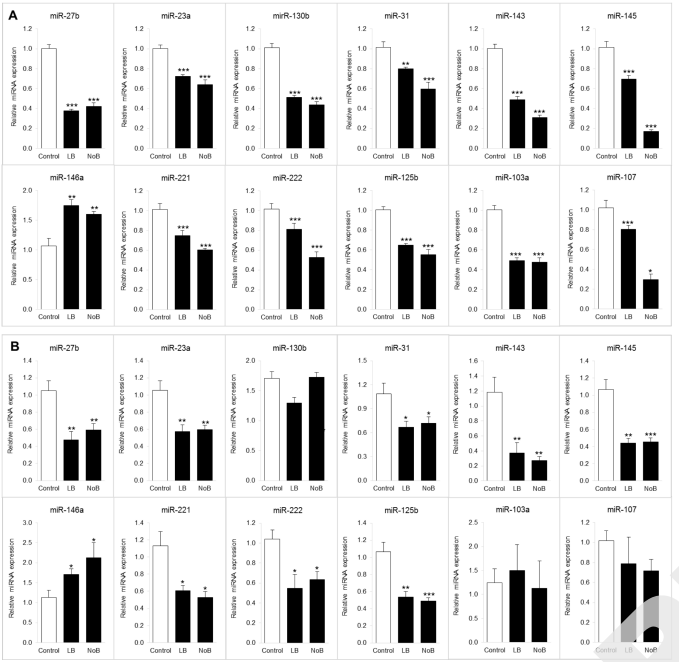

Fig 4

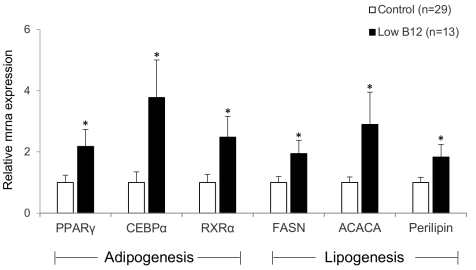

Fig 5