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The role of vitamin B12 deficiency on obesity, adipocytes and inflammation

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Table of Contents

Table of Contents	i
List of Figures.....	vi
List of tables.....	xi
Acknowledgements.....	xii
Declaration.....	xiv
Abstract.....	xv
List of Abbreviations	xvii
Chapter 1: Introduction	1
1.1 The Adipose Tissue	2
1.1.1 Types of Adipose tissue	4
1.1.2 Plasticity of the adipose tissue	6
1.2 The white adipose tissue	7
1.2.1 Anatomical distribution	8
1.3 Adipose tissue as an endocrine organ	10
1.4 Fatty acid biosynthesis, lipolysis and beta oxidation in the adipose tissue	16
1.4.1 Fatty acid uptake	17
1.4.2 Lipogenesis	17
1.4.3 Fatty acid oxidation	19
1.5 Obesity	21
1.6 Morbid obesity and current management	22
1.7 Micronutrient deficiency and Obesity	24
1.8 Vitamin B12	26
1.8.1 Definitions of B12 deficiency	31
1.8.2 B12 deficiency across the globe	31
1.8.3 Causes of vitamin B12 deficiency	31
1.8.4 Complications of B12 deficiency	33
1.8.5 B12 deficiency associated with obesity, diabetes and dyslipidemia	33
1.8.6 The role of B12 like as an antioxidant and anti-inflammatory molecule	35
1.8.7 B12 deficiency in Bariatric Surgery	35
1.8.8 The role of the paternal diet	35
1.8.9 B12 and male fertility	37
1.9 Male infertility	39
1.9.1 Epidemiology	39

1.9.2 Male hypogonadism	40
1.9.3 Reproductive parameters in obese men	41
1.9.4 Oestrogens of adipocyte origin and hypogonadism	42
1.10 Summary and gaps in evidence	43
1.11 Hypothesis and Aims	44
Chapter 2: Material & Methods	45
2.1 Materials	46
2.1.1 Cell Culture Reagents	46
2.1.2 Reagents and materials for Radioactive flux assay	47
2.1.3 Reagents and materials for seahorse flux assay	48
2.1.4 Reagents and materials for RNA, cDNA and gene expression studies	49
2.1.5 Kits	50
2.1.8 Solutions	51
2.1.9 Primers and oligonucleotides	51
2.1.9.1 Taqman primers for human genes	51
2.1.9.2 Primer sequences for human genes	52
2.1.10 Miscellaneous materials and reagents	53
2.2 Methods	54
2.2.1 Cell culture	54
2.2.1.1 Human preadipocyte ChubS7 cell line	54
2.2.1.2 Human primary preadipocytes (ScWAT)	54
2.2.1.3 Cell culture media composition	55
2.2.1.4 Harvesting of adipose primary cells and Chubs-S7 for lipid metabolism experiments	57
2.2.2 Gene expression assay	57
2.2.2.1 RNA isolation from adipose primary cells and Chubs-S7 cell line	57
2.2.2.2 Reverse transcription	58
2.2.2.3 Gene expression assay – Real-time quantitative PCR (RT-qPCR) Principle:	58
2.2.3 Radioactive flux of TAG synthesized via utilization of radiolabelled-fatty acid (14C-oleate) by Chubs-S7 and primary adipocytes	60
2.2.4 Total intra and extracellular vitamin B ₁₂ quantification by immunoassay technique	61
2.2.5 Mitochondrial dysfunction assessment using seahorse extracellular flux assay	61
2.2.5.1 Cell culture for seahorse assay	64

2.2.5.2 Seahorse inhibitors	64
2.2.5.3 Sample preparation and seahorse assay	64
2.3 Human adipose tissue study	65
2.3.1 Subjects and study design	65
2.3.2 Adipose tissue collection	65
2.3.3.1 Quantikine ELISA Human CCL2/MCP1	65
2.3.3.2 Quantikine ELISA Human IL-8/CXCL8	66
2.4 Statistical Analysis	66
Chapter 3: Vitamin B12 receptors and transporters.....	67
3.1 Introduction	68
3.2 Results	71
3.2.1 Assessment of Intracellular B12 levels in Chubs-S7 cells	71
3.2.2 Assessment of gene expression of B12 transporter TCN2 and receptor CD320 in Chubs-S7	73
3.2.3 Gene expression of B12 receptor CD320 and transporter TCN2 in human primary adipocytes with different degrees of obesity	75
3.2.4 Gene expression of B12 receptor CD320 and transporter TCN2 in human omental and subcutaneous adipose tissue	77
3.2.5 Correlation between gene expression of omental and subcutaneous TCN2 and CD320 with biochemical parameters	79
3.3 Discussion	84
Chapter 4: Vitamin B12 and <i>de novo</i> lipid synthesis	87
4.1 Introduction	88
4.2 Results	90
4.2.1 <i>In vitro</i> experiments	90
4.2.1.1 Acetyl-CoA Carboxylase Alpha (ACACA)	90
4.2.1.2 Fatty acid synthase (FASN)	92
4.2.1.3 ELOVL Family Member 6, Elongation of long chain fatty (ELOVL6)	94
4.2.1.4 Stearoyl-CoA Desaturase (SCD)	96
4.2.1.5 Glycerol-3-Phosphate Acyltransferase (GPAT2)	98
4.2.1.6 Phosphatidate Phosphatase LIPIN1	100
4.2.1.7 Diacylglycerol O-Acyltransferase 2 DGAT2	102
4.2.2 <i>In vitro</i> experiments: Flux assay	104
4.2.3: Experiments on the human AT	107
Effect of B12 on mature human adipose tissue - omental and subcutaneous adipose tissue	107

4.2.4 Correlation between relative mRNA expression of de novo lipid synthesis and biochemical values	109
4.3 Discussion	114
Chapter 5: Vitamin B12 and the fatty acid β-oxidation	119
5.1 Introduction	120
5.2 Results	121
5.2.1 <i>In vitro</i> experiments:	121
Genes involved in expression of fatty acid transporters and genes involved in fatty acid β -oxidation in Chubs-S7 and primary adipocytes	121
5.2.1.1 Malonyl-CoA decarboxylase (MCD)	122
5.2.1.2 Carnitine palmitoyltransferase-1 (CPT1 β)	124
5.2.1.3 Carnitine palmitoyltransferase-2 (CPT2)	126
5.2.1.4 Acyl-CoA Dehydrogenase Long Chain (ACADL)	128
5.2.1.5 Enoyl-CoA Hydratase, Short Chain 1 (ECHS1)	130
5.2.1.6 Acetyl-CoA Acyltransferase 2 (ACAA2)	132
5.2.2 Effect of B12 deficiency on mitochondrial bioenergetic profiling of human adipocytes	134
5.2.3 Effect of low B12 on fatty acid-driven oxygen consumption in human adipocytes	136
5.2.4 Experiments in human AT:	138
Gene expression of fatty acid transporters and genes involved in fatty acid β -oxidation in human OmWAT and ScWAT	138
5.2.5 Correlation between human OmWAT and ScWAT fatty acid β -oxidation enzymes and biochemical parameters	140
5.3 Discussion	145
Chapter 6: Vitamin B12 and inflammation.....	149
6.1 Introduction	150
6.2 Results	153
6.2.1 Effect of B12 on gene expression of inflammatory cytokines in Chubs-S7	153
6.2.2 Effect of B12 on gene expression of inflammatory cytokines in human primary pre-adipocytes derived from different BMI groups.	155
6.2.4 Experiments in human AT:	157
Effect of B12 on gene expression of inflammatory cytokines in mature human AT from different depots	157
6.2.5 Serum inflammatory cytokines levels detection in the cohort study	159

6.2.6 Association between inflammatory cytokines in AT, circulating B12 and biochemical values	160
6.3 Discussion	168
Chapter 7: Serum and seminal correlation of B12 and holotranscobalamin levels with seminal quality in morbid obesity	174
7.1 Introduction	175
7.2 Methods	176
7.2.1 Patients	176
7.2.2 Biochemical and Anthropometric Measurements	177
7.2.3 Seminal analysis	177
7.3 Results	178
7.3.1 Biochemical and anthropometric characteristics of the patients	178
7.3.2 Vitamin B12 and Holotranscobalamin measurements and correlation in serum and seminal fluid	179
7.3.3 Association between seminal parameters and seminal HoloTC and B12	183
7.4 Discussion	188
Chapter 8: General discussion	190
8.1 Discussion	191
8.2 Conclusion and future directions	198
List of references	201
Publications.....	233
List of manuscripts awaiting publication.....	248
List of conference presentations	250

List of Figures

Figure 1: Major adipose tissue depots	5
Figure 2: Schematic structure of a white fat cell	8
Figure 3: Paracrine effects of adipose tissue.	16
Figure 4: Adipose <i>de novo</i> lipogenesis	18
Figure 5: Adipose fatty acid β -oxidation	20
Figure 6: Molecular structure of vitamin B12	27
Figure 7: Coenzyme function of B12	28
Figure 8: Schematic representation of the differences between lean and obese subjects of the hypothalamic-pituitary-gonadal axis	42
Figure 9: Seahorse assay	63
Figure 10: Intracellular vitamin B12 intake is increased in low B12 treated cells	72
Figure 11: Effect of B12 deficiency on the gene expression receptor CD320 (A) and transporter transcobalamin TCN2 (B) in Chus-S7 cells	74
Figure 12: Effect of B12 deficiency on the gene expression receptor CD320 (A) and transporter transcobalamin TCN2 (B) in primary adipocytes derived from four categories of BMI	76
Figure 13: Effect of B12 deficiency on the gene expression receptor CD320 (A) and transporter transcobalamin TCN2 (B) in omental and subcutaneous adipose tissue	78
Figure 14: Positive correlation between TCN2 gene expression and LDL (A) and Cholesterol (B) in subcutaneous adipose tissue	81
Figure 15: Subcutaneous CD320 gene expression and B12, HDL and Glucose correlate with serum B12	82

Figure 16: Correlation of omental TCN2 and CD320 gene expression with BMI and B12	83
Figure 17: Catalytic action of Acetyl-CoA carboxylase	90
Figure 18: Gene expression of Acetyl-CoA Carboxylase alpha (ACACA) in human adipocytes	91
Figure 19: Catalytic action of Fatty acid synthase	92
Figure 20: Gene expression of Fatty acid synthase (FASN) is increased in B12 deficient adipocytes	93
Figure 21: Catalytic action of Fatty acid elongase	94
Figure 22: Gene expression of Fatty acid elongase ELOVL6 is increased in B12 deficient adipocytes	95
Figure 23: Catalytic action of Stearoyl-CoA Desaturase	96
Figure 24: Gene expression of Stearoyl-CoA Desaturase (SCD) is increased in B12 deficient adipocytes	97
Figure 25: Catalytic action of Glycerol-3-phosphate acyltransferase	98
Figure 26: Gene expression Glycerol-3-Phosphate Acyltransferase (GPAT2) is upregulated in B12 deficient adipocytes	99
Figure 27: Catalytic action of Phosphatidate phosphatase	100
Figure 28: Phosphatidate Phosphatase LIPIN1 gene expression is upregulated in B12 deficient adipocytes	101
Figure 29: Catalytic action of Diacylglycerol O-Acyltransferase 2	102

Figure 30: Diacylglycerol O-Acyltransferase 2 DGAT2 gene expression is significantly increased in B12 deficient adipocytes	103
Figure 31: Increased real time triglycerides biosynthesis by primary adipocytes under B12 deficiency	105
Figure 32: Amount of triglycerides synthesized in real time by primary adipocytes derived from lean and obese patients cultured under lowB12 (25pM) compared with control (500nM)	106
Figure 33: Effect of B12 deficiency on de novo fatty acid synthesis and triglyceride biosynthesis in omental and subcutaneous adipose tissue	108
Figure 34: Subcutaneous lipid synthesis relative gene expression of ACACA and FASN correlate with serum B12	112
Figure 35: Omental lipid synthesis relative mRNA expression correlate with serum B12	113
Figure 36: Catalytic action of Malonyl-CoA decarboxylase	122
Figure 37: Gene expression of Malonyl-CoA decarboxylase (MCD) is altered in B12 deficient adipocytes	123
Figure 38: Acyl-CoA transport from cytosol to the inner mitochondrial membrane	124
Figure 39: Gene expression of Carnitine palmitoyltransferase-1 (CPT1-β) is downregulated in B12 deficient adipocytes	125
Figure 40: Schematic representation of Acyl-CoA transport from cytosol to the mitochondrial matrix	126
Figure 41: Gene expression of Carnitine palmitoyltransferase-2 (CPT2) is reduced in B12 deficient adipocytes	127

Figure 42: Catalytic action of Acyl-CoA Dehydrogenase	128
Figure 43: Gene expression of Acyl-CoA Dehydrogenase Long Chain (ACADL) is altered in B12 deficient adipocytes	129
Figure 44: Catalytic action of Enoyl-CoA Hydratase	130
Figure 45: Gene expression of Enoyl-CoA Hydratase, Short Chain 1 is decreased in B12 deficient adipocytes	131
Figure 46: Catalytic action of Acetyl-CoA Acyltransferase 2	132
Figure 47: Gene expression of Enoyl-CoA Hydratase, Short Chain 1 is decreased in B12 deficient adipocytes	133
Figure 48: Low B12 compromises mitochondrial functional integrity of Chubs-S7 in rich-substrate media	135
Figure 49: Low B12 compromises mitochondrial functional integrity of primary adipocytes in rich-substrate media	135
Figure 50: B12 deficiency downregulates fatty acid oxidation of Chubs-S7 and primary adipocytes	137
Figure 51: Effect of B12 deficiency on genes involved in fatty acid oxidation in human omental and subcutaneous adipose tissue	139
Figure 52: Omental fatty acid oxidation gene expression correlate with serum B12 levels	143
Figure 53: Subcutaneous fatty acid oxidation gene expression correlate with serum B12 levels	144
Figure 54: Gene expression of inflammatory markers altered in low B12 treated cells	154
Figure 55: Effect of B12 deficiency on the gene expression of inflammatory cytokines in primary adipocytes derived from four categories of BMI	156

Figure 56 Effect of B12 deficiency on inflammatory cytokine gene expression in omental and subcutaneous adipose tissue	158
Figure 57: Inflammatory cytokines serum levels are increased in B12 deficient patients	159
Figure 58: Subcutaneous inflammatory cytokines mRNA expression correlate with serum B12	163
Figure 59: Omental inflammatory cytokines mRNA expression correlate with serum B12	164
Figure 60: Correlation between serum inflammatory cytokine MCP-1 and IL-8 and serum vitamin B12	167
Figure 61: B12 and holoTC concentrations in serum and seminal samples from morbidly obese and lean subjects	181
Figure 62: Positive correlations between CBL and holoTC concentrations in semen (A) and serum (B) in all subjects	182
Figure 63: Seminal parameters stratified for seminal holoTC and B12 in the total population of obese and lean subjects	185
Figure 64: ROC analysis of seminal holoTC and B12 potency in predicting sperm number and concentration	186
Figure 65: Potential mechanistic pathway in B12 deficient adipocyte	198

List of tables

Table 1: Intracellular and extracellular levels of B12 in Chubs-S7.	71
Table 2: Clinical and biochemical characteristics according vitamin B12 values.	80
Table 3: Subcutaneous lipid synthesis gene expression correlate with serum biochemical metabolic related values	110
Table 4: Omental lipid synthesis gene expression correlates with serum biochemical metabolic related values	111
Table 5: Subcutaneous fatty acid β -oxidation gene expression correlates with serum biochemical metabolic related values	141
Table 6: Omental fatty acid β -oxidation gene expression correlates with serum biochemical metabolic related values	142
Table 7: Subcutaneous fatty acid β -oxidation gene expression correlates with serum biochemical metabolic related values	161
Table 8: Omental fatty acid β -oxidation gene expression correlates with serum biochemical metabolic related values	162
Table 9: Clinical and biochemical characteristics according to vitamin B12 values	166
Table 10: Biochemical and anthropometric characteristics of the patients	178
Table 11: B12 and holoTC concentrations in serum and semen	180
Table 12: Seminal parameters	184
Table 13: Correlations between seminal holoTC or B12 and seminal parameters	184
Table 14: Stratification of the whole subject cohort according to seminal holoTC and B12 cut-off values identified by ROC analysis	187

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“Living away from home is not for everyone. You must have a big heart, big enough to be a suitcase for everything you leave: joys and sorrows, friends and love. This heartbeat that beats even when you touch a ground that doesn't belong to you or when you're lying on a mattress that doesn't have your shape and an uncomfortable pillow, and you look at the ceiling wondering where you're going. Friends who are not yours, a city that is not yours. You must have a big heart, so big to make room for new things. A heart that sometimes fears that others have forgotten, because the present has taken over in their lives. A big heart, but not too strong. Then that's where it stops for a moment. It stops, confuses you and no longer knows who you are. So, you lie down on the mattress which has now taken your shape, and the pillow is softer on one side and you wonder who you are becoming more than where you are going.

Because when you leave, rather than move towards a destination, you go towards a destiny, YOURS.”

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.

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Abstract

Background: Obesity, considered a pandemic disease by the World Health Organization (WHO) is constantly increasing worldwide. Its prevalence has doubled since 1980 and the biggest rise is in the developing world. The change from traditional foods to high-calorie fast food and the more sedentary lifestyle is probably the main mechanism driving this rise of obesity. However, it is not known whether there is an interaction between underlying susceptibility and lifestyle/environmental changes that (for example through epigenetic programming) exacerbates this rate of rise in obesity. The increased availability of unhealthy highly processed food with poor nutritional value can lead to micronutrient deficiency resulting in profound metabolic changes. Micronutrients play an important role in the metabolism of macronutrients and their deficiency can bring about cellular dysfunction. Vitamin B12 (B12) is an essential micronutrient required for optimal hematopoietic, neurologic and other several metabolic reactions. Indeed, in the last decade, animal and clinical studies have revealed a correlation between B12 deficiency and adverse metabolic health profiles. In particular, B12 deficiency in pregnancy has been associated with maternal obesity, development of insulin resistance and dyslipidaemia. Furthermore, studies have also explored the effect of B12 on male infertility. Given the key metabolic role of adipose tissue (AT) in obesity, we hypothesised that B12 deficiency may adversely influence lipid synthesis and metabolism by the adipocytes leading to adipose tissue inflammation. We also hypothesised that low levels of circulating B12, particularly in the presence of obesity, might influence their intracellular levels in this key 'metabolic tissue' as well as in other biological fluids such as seminal fluid. If B12 deficiency adversely affects seminal quality/function, this may also provide evidence towards adverse epigenetic programming through the paternal route. **Methods:** To test these hypotheses, the human abdominal subcutaneous pre-adipocyte cell line (Chub-S7) and human abdominal subcutaneous primary pre-adipocytes were differentiated under different B12 concentrations and Human omental white adipose tissue (OmWAT), subcutaneous white adipose tissue (ScWAT) and blood samples were collected from 106 pregnant women at delivery. Serum B12 and relevant metabolic risk factors were measured. Gene expression was performed by q-RT-PCR, *de novo* triglyceride synthesis was quantified by radioactive tracing, β -oxidation, palmitate-induced oxygen consumption rate was determined using seahorse-XF analyser, and serum cytokine levels was evaluated with ELISA technique. To test the role of B12 in seminal fluid in obese

patients, we analysed the cross-sectional data of 47-morbidly-obese and 21 lean men. Total B12 and holotranscobalamin (the active form of B12; holoTC) levels in serum and semen has been evaluated. **Results:** Adipocytes cultured in decreasing extracellular B12 media concentrations resulted in progressively increased intracellular B12 and an increased gene expression of B12 receptor CD320 and transporter TCN2 has been shown in adipocytes cultured in low B12 and in ScWAT and OmWAT of B12 deficient pregnant women. Adipocytes cultured in low-B12 conditions showed significantly increased expression ($P<0.01$) of genes involved in triglyceride biosynthesis, a significantly decreased expression ($P<0.01$) of genes involved in β -oxidation and an increased expression ($P<0.01$) of pro-inflammatory cytokines. These data were also confirmed in the AT of B12-deficient pregnant women where an increase of cytokine secretion has been shown. Additionally, real-time fatty acid flux synthesis and fatty acid oxidation (FAO) induced by palmitate were significantly altered ($P<0.05$) in B12-deficient adipocytes. Furthermore, associations between B12 and metabolic related biochemical values has been found. Moreover, both seminal and serum concentrations of holoTC and B12 were lower in morbidly obese compared to lean men, although the difference did not reach any statistical significance for serum holoTC. Seminal B12 and holoTC were significantly higher than B12 and holoTC serum levels in both groups. Significant positive correlations were observed between seminal holoTC and total sperm motility, sperm concentration, total sperm number and negative correlation with semen pH. ROC analysis supported seminal holoTC as the best predictor of sperm number. **Conclusion:** Our study highlights that the increased lipogenesis, as well as impaired FAO and mitochondrial dysfunction in low B12, induce uncontrolled lipid accumulation in the AT leading to local increased inflammation in adipocytes and AT. Our data highlights that B12 deficiency has profound effects on adipocyte dysfunction, opening new insights into the pathogenesis of maternal obesity and the relevance of micronutrient supplementation for pregnant mothers. In addition to this, our findings suggest that paternal B12 levels might also important in adverse programming of the offspring, through poor seminal quality. While this is interesting and novel, it requires further work especially in fathers.

List of Abbreviations

1,2-DAG	1,2Diacylglycerol
ABCD4	ATP-binding cassette sub-family D member 4
ACACA	Acetyl-CoA Carboxylase Alpha
ACADL	Acyl-CoA Dehydrogenase Long Chain
ACACA	Acetyl-CoA Carboxylase Alpha
ACE	Angiotensin-converting enzyme
Ado-Cbl	Adenosylcobalamin
AGPAT	Acylglycerol-3-Phosphate O-Acyltransferase
AMN	Amnionless
AMP	Adenosine monophosphate
ANP	Atrial natriuretic peptide
ADIPO Q	Adiponectin
ASP	Acylation-stimulating protein
AT	Adipose tissue
ATP	Adenosine triphosphate
B12	Vitamin B12
BAT	Brown adipose tissue
BMI	Body mass index
BSA	Bovine serum albumin
CACT	Carnitine-acylcarnitine translocase
cDNA	Complementary (copy) DNA
CPT1- β	Carnitine Palmitoyltransferase 1B

CPT2	Carnitine Palmitoyltransferase 2
CVD	Cardiovascular disease
DGAT	Diglyceride acyltransferase
DMB	Dimethylbenzimidazole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
d NTP	Deoxynucleoside triphosphate
DOHAD	Developmental origins of health and disease
ECAR	Extracellular acidification rate
ECHS1	Enoyl-CoA Hydratase, Short Chain 1
EGF	Epidermal growth factor
ELOVL6	Very Long Chain 3-Ketoacyl-CoA Synthase 6
EMEM	Eagle's Minimum Essential Medium
ER	Endoplatic reticlm
FA	Fatty acid
FAO	Fatty acid β -oxidation
FASN	Fatty acid synthase
FATP	Free fatty acid transporter protein
FBS	Foetal bovine serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FFA	Free fatty acid
FGF	Fibroblast growth factor
FGF21	Fibroblast growth factor 21

FSH	Follicle stimulating hormone
G3P	Glyceraldehyde 3-phosphate
GDM	Gestational diabetes mellitus
GnRH	Gonadotropin-releasing hormone
GPAT	Glycerol-3-phosphate acyltransferase
Hcy	Homocysteine
HDL	High-density lipoproteins
HG	Hypogonadism
Holo-TC	Holotranscobalamin
HPT	Hypothalamus, Pituitary, Testis
HSL	Hormone-sensitive lipase
hTERT	Human telomerase reverse transcriptase
IBMX	3-isobutyl-1-methylxanthine
IDF	International Diabetes Federation
IF	Intrinsic factor
IL-18	Interleukin-18
IL-1 β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IMM	Inner mitochondrial membrane
IQR	Interquartile range
IR	Insulin resistance
LDL	Low-density lipoproteins
LDLR-A	Low density lipoprotein receptor class A

LH	Luteinizing hormone
LIPIN1	Phosphatidate Phosphatase LIPIN1
LMBRD1	LMBR1 Domain Containing 1
LPA	Lysophosphatidic acid
LPL	Lipoproteinlipase
MCM	Methylmalonyl-CoA mutase
MCP-1	Monocyte chemoattractant protein 1
Me-Cbl	Metyl-Cobalamin
miR	MicroRNA
MMA	Methylmalonic acid
M-MLV	Moloney Murine Leukemia Virus Reverse Transcriptase
mRNA	MessengerRNA
MS	Metabolic syndrome
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHANES	National Health and Nutrition Examination Survey
NIH	National Institutes of Health
OCR	Oxygen consumption rate
OmWAT	Omental white adipose tissue
PAI-1	Plasminogen activator inhibitor-1
PCR	Polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid

RT-qPCR	Quantitative reverse transcription PCR
SAM-e	S-adenosyl-L-methionine
SAT	Subcutaneous adipose tissue
SCD	Stearoyl-CoA desaturase
ScWAT	Subcutaneous white adipose tissue
SD	Standard deviation
SEM	Standard error mean
T2DM	Type 2 diabetes mellitus
TAG	Tryacilglycerol
TC	Transcobalamin
TCN2	Transcobalamin 2
TGF- β	Transforming growth factor beta
TNF- α	Tumour Necrosis Factor alpha
UCP-1	Uncoupling protein
UHCW	University Hospitals Coventry and Warwickshire
VLDL	Very low-density lipoproteins
WAT	White adipose tissue
WC	Waist circumference
WHO	World health organization

Chapter 1: Introduction

1.1 The Adipose Tissue

The adipose tissue (AT) functions as a key energy reservoir of the organism in vertebrates and, in particular, in mammals. The energy is stored as triglycerides (TAG), hydrophobic molecules consisting of three esterified fatty acids (FA) and a glycerol molecule that can be stored in big quantities and have an energy yield, per unit of mass, greater than other nutrients.

During evolution, the AT has specialized not only in the trophic function, but also in playing a role of mechanical protection and support for the organism. Furthermore thanks to scientific discoveries, AT is now considered a highly dynamic endocrine organ involved in several physiological and metabolic processes far beyond the paradigm of fuel storage (Berry et al., 2013). White adipocytes secrete indeed several major hormones together with a diverse range of other signal proteins and factors characterized by paracrine, autocrine and endocrine effects. These various protein signals have been given the collective name of "adipokines". The "adipokinome", pool of molecules produced and secreted by fat cells (Trayhurn et al., 2004), is involved in several physiological processes like lipid metabolism, insulin sensitivity, blood pressure regulation and angiogenesis as well as regulation of energy balance.

AT is not only a connective tissue, lacking a particular organization, but it has precise cytological characteristics, peculiar anatomical and specific innervations and vascularisations. Despite being predominantly characterized from mature adipocyte (90%), among the cell types that compose it are elements of the immune system, endothelial cells, fibroblasts, stem cells and adipocytes at different stages of differentiation (Cinti, 2001) (Berry, Stenesen, Zeve, & Graff, 2010). Among the different cell types, adipocytes are the ones responsible of synthesis (lipogenesis), storage and mobility (lipolysis) of lipids; They have in fact, all the enzymes needed to synthesize, store and mobilize fatty acids. The adipocyte picks up FA and TAG from the blood and accumulates them in the form of intracellular lipid drops (lipogenesis) and then demolishes them through the process of beta oxidation which leads to the formation of metabolic energy in the form of ATP (lipolysis). They derive from multipotent stem cells that differentiate in a commissioned stage to preadipocytes and subsequently mature into adipocytes, through a complex process called adipogenesis. Numerous endocrine and paracrine factors regulate adipogenesis, allowing the sequential expression of key genes in adipocyte maturation, such as PPAR gamma.

Any alteration in this process results in dysfunction of the adipose organ that is associated with metabolic pathologies (Wajchenberg BL., 2000). Among the main endocrine hormones regulating adipogenesis, steroid sex hormones play a fundamental role, also leading to gender dimorphism. For example, oestrogens are positive regulators of adipogenesis, stimulating the proliferation of preadipocytes and the growth of already mature adipocytes. In contrast, the differentiation and maturation of adipocytes is negatively regulated by androgens, mainly testosterone. In fact, high androgenic levels stimulate the myogenic differentiation of multi-potent mesenchymal stem cells inhibiting adipogenesis (Karen P Phillips et al., 2010).

1.1.1 Types of Adipose tissue

Mammals present two types of AT, well distinguishable due to anatomical and functional characteristics: white adipose tissue (WAT) and brown adipose tissue (BAT). The morphological differences between the two types of tissue are relative to form, mitochondrial structure and lipid droplets (Frontini and Cinti, 2010). The BAT, characteristic of small mammals and represented in humans, mainly in early childhood, is specialized in thermogenesis, due to the presence of a protein specifically expressed in this tissue, the UCP-1 (uncoupling protein 1), which determines the uncoupling of mitochondrial oxidative phosphorylation, leading to heat production. BAT adipocytes are characterized by the presence of numerous mitochondria that release heat through the oxidation of FA and the dark color of this tissue is due to the high concentration of cytochrome oxidase at the mitochondrial level (Cannon & Nedergaard, 2004). On the contrary, WAT is considered a long-term depot of energy stored as TAG in lipid droplets that could be used during starvation. There are two main anatomical and functional sites of WAT: the visceral adipose tissue (VAT) that surrounds the organs and insert itself between the fibers of the muscles, to which it offers mechanical protection by attenuating the impacts of the movements, and the subcutaneous tissue (SAT) located below the dermis. In most of adult mammals, white adipocytes are the most prevalent type in the AT; the relationship between these and brown adipocytes varies, in fact, based on sex, age, nutritional status and environmental conditions (Frontini & Cinti, 2010).

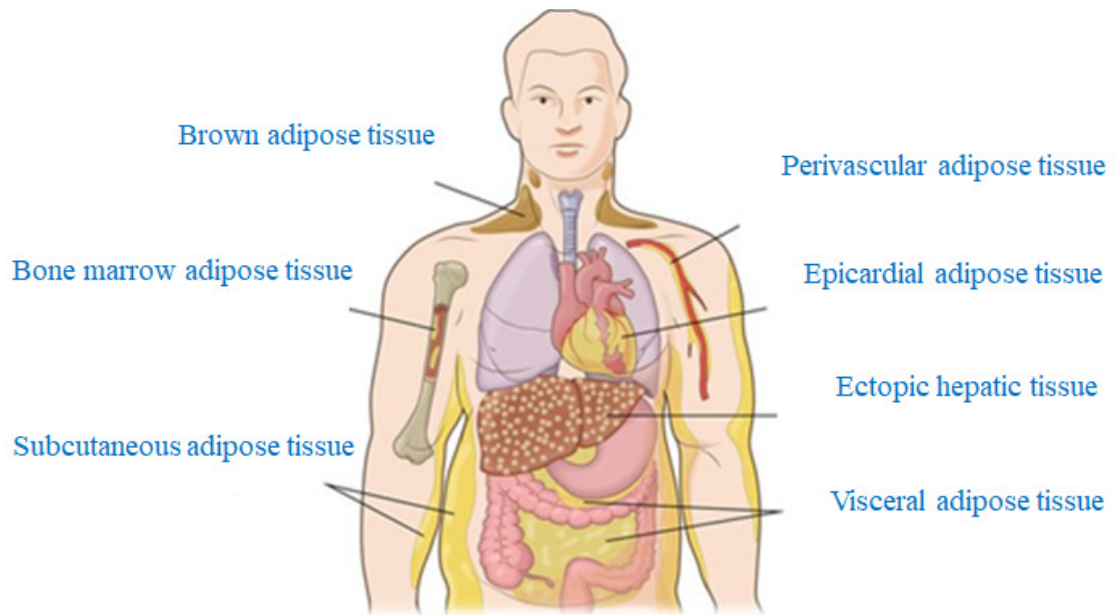


Figure 1: Major adipose tissue depots (obtained and modified from Fuster *et al.*, 2016)

Abdominal fat distribution can be assessed by measuring the waist circumference. A different distribution of fat is also observed according to gender, with a prevalence of accumulation of SAT at the level of the buttocks and hips in women (pear distribution), less associated with cardiovascular disorders, and VAT at trunk level in the male (apple distribution), most associated with cardiovascular risk. The expansion of VAT is in fact associated with an increased risk of cardiovascular and metabolic pathologies, such as T2DM, hypertension, IR (Gesta *et al.*, 2007).

1.1.2 Plasticity of the adipose tissue

A unique feature of the adipose organ is its plasticity, as it undergoes significant and rapid structural changes concerning cellular composition, vascular and nervous supply followed by different stimuli, such as changes in environmental temperature and nutritional requirements.

The number of brown and white adipocytes in the adipose organ is not fixed, but varies according to physiological and pathological conditions, such as the expansion of the BAT sector following the phenomenon of adaptation to cold (acclimatization). The balance between WAT and BAT compartment is regulated by numerous physiological stimuli [lowering of temperature, exercise, excess of lipids, secretion of some hormones such as irisine from muscle, Atrial natriuretic peptide from the heart (ANP), Fibroblast growth factor 21 (FGF21) from the liver and Bone morphogenic protein-7 (BMP7)] that determine an expansion of BAT at the expense of WAT. In particular, the expansion of the BAT compartment is stimulated within deposits of WAT (browning phenomenon). The stimulation of the transformation of excess and dysfunctioning white fat that characterizes the condition of obesity in BAT that dissipates energy in the form of heat and regulates metabolic balance, represents an innovative therapeutic approach to obesity and metabolic diseases (Harms & Seale, 2013; Villarroya & Vidal-Puig, 2013; Whittle *et al.*, 2012).

The appearance of BAT at the level of WAT type accumulations suggests that the two types of AT show a remarkable plasticity. In addition to the browning phenomenon, the opposite phenomenon of reduction of the BAT sector and expansion of the WAT (whitening) has been described. In particular, chronic exposure to cold and stimulation with beta 3 agonists leads to rapid expansion of BAT and its activation, especially in rodents, while WAT increases in obese animals (Cinti, 2001; Cinti, 2002).

However, the nature of the brown cells that form inside the white deposits seems to be different also from the classic brown adipose cell (interscapular deposits, along the neck and the vertebral column and perirenales, in humans). These cells in fact have less marked brown characteristics (less expression and UCP-1 activity, lower density of mitochondria) compared to the classic brown adipocyte and for this reason they have been called BRITE (nor BRown nor whITE) (Cannon & Nedergaard, 2004; Nedergaard *et al.*, 2010; Petrovic *et al.*, 2010) or BEIGE (for the intermediate color between white and brown) (Harms &

Seale, 2013). Furthermore, these cells present early markers different from the classical brown lineage like CD137, TMEM26, Tbx1 (Cawthorn *et al.*, 2012; Wang *et al.*, 2015)

1.2 The white adipose tissue

The white adipose tissue (WAT), also called unilocular tissue, is composed of cells in close contact, with little extracellular matrix, consisting mainly of collagen and reticular fibres (Ahima & Flier, 2000). It is widespread throughout the body, surrounding organs in the abdominal and mediastinal region, infiltrating even between the muscle fibers. In the first case, it offers mechanical protection; in the second, it favours the movement of the fibers, without however compromising the functional integrity. Moreover, it is widely distributed in the dermis and in the subcutaneous zones, with heat insulation function.

AT is responsive to hormonal stimulation, in particular, to sex hormones and glucocorticoids. Furthermore, its distribution varies with age, decreasing in the subcutaneous areas and increasing in intra-abdominal level (Gesta *et al.*, 2007). Considering the morphology, the mature adipocyte is a circular cell, of variable size, characterized by a large cytoplasmic vacuole, within which the TAGs are accumulated, and occupies 85-90% of the cell volume. To synthesize a TAG molecule, a glycerol-3-phosphate (G3P) molecule is needed, which is derived from the glycolytic pathway, and three free fatty acids (FFA). The free fatty acids, de novo synthesized or present in the circulation associated with lipoproteins, are internalized by the adipocytes due to the presence of a membrane transporter (CD36) and presented to another transporter protein, FFA transporter protein (FATP), which allows them to reach the cytoplasm where they are esterified with coenzyme A, to form the acylCoA. Then there is the formation of an ester bond with the G3P to obtain TAG, which constitute the intracytoplasmic lipid drops. This lipid drop crushes the nucleus and the other organelles towards the periphery. It is precisely the variable accumulation of TAGs in the form of a lipid droplet, which determines the great variability of cellular dimensions that can vary between 20 and 160 μm (Pond, 2000). The adipocytes have the enzyme lipoproteinlipase (LPL) on their membrane: in the vessel lumen this enzyme acts on the triglyceride component of circulating lipoproteins, especially lipoproteins rich in TAG, such as chylomicrons and VLDL (Very Low Density Lipoprotein), generating di- and mono -glycerides, FFA and intermediate density lipoproteins. Furthermore, these cells contain, within the cytoplasm, the enzyme Hormone-sensitive lipase (HSL) which, when stimulated by glucagon, noradrenaline, adrenaline and thyroxine, breaks down TAG into FA and glycerol.

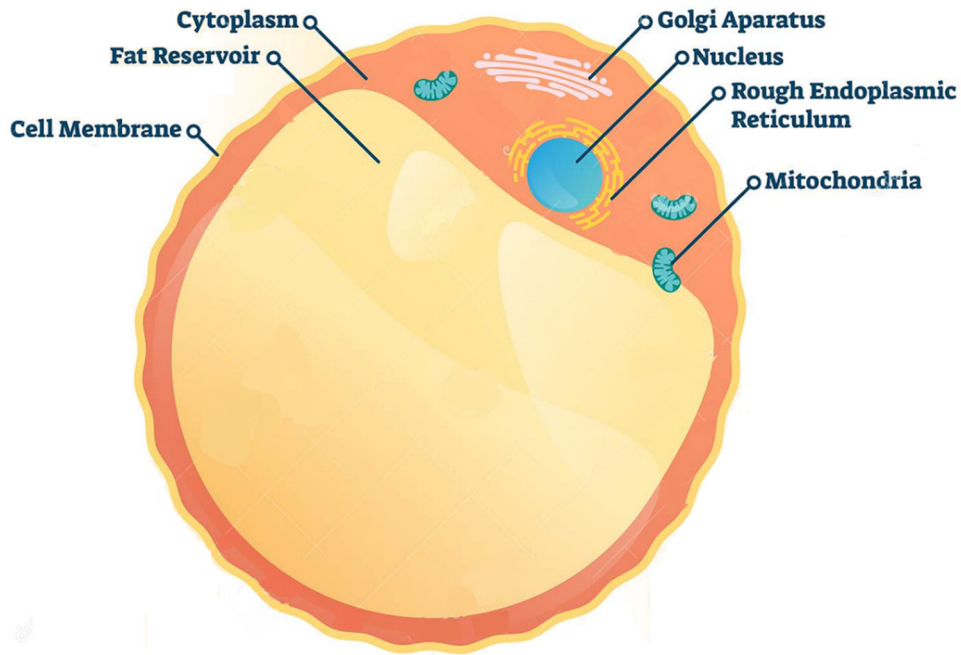


Figure 2: Schematic structure of a white fat cell. The cytoplasm is occupied almost entirely by a single lipid droplet. In the residual area, bounded by the membrane, the decentralized nucleus and the cellular organelles are visible (mitochondria, golgi apparatus and endoplasmic reticulum).

1.2.1 Anatomical distribution

The WAT has two main anatomical localization sites, to which functional differences are also correlated. A subcutaneous adipose tissue (SAT) and a visceral adipose tissue (VAT) can be distinguished.

The distribution of AT, especially the subcutaneous one, undergoes hormonal regulation, in particular from sex hormones, thus presenting a sexual dimorphism. In fact, we talk about the gynoid distribution, in women (mainly hips and buttocks) and android, in men (abdominal level, above the navel). The SAT, which is mainly located below the epidermis, in particular at the abdominal level, in the buttocks and in the femoral area, is made up of large bundles of fibers that create large spaces within which the adipocytes are found (Murano *et al.*, 2008).

Its main function is to maintain constant body temperature and to offer mechanical protection to the underlying dermis. VAT, on the other hand, lies deeper, surrounding organs and systems, offering them mechanical protection and keeping them *in situ* (Lien

& Guyton, 2008). Depending on the anatomical site in which it is located, it is possible to make a further distinction between VAT, in mesenteric, retro peritoneal and omental. Compared to the SAT, it has an abundant vascularization, a high density of nerve fibers and immune system cells (Bruun *et al.*, 2005).

The specific functions, deriving from the different distribution in the organism, are due to different regulatory mechanisms linked in part to local innervation and hormones action. This regulation involves various processes involving the mature adipocyte and the adipocyte precursor, ranging from accumulation to lipid mobilization and response to insulin stimulation, recruitment and differentiation, and adipokine secretion (Kissebah & Krakower, 1994; Wajchenberg, 2000); (Ibrahim, 2010).

The accumulation of fat occurs mainly at the subcutaneous level, as a physiological response to a diet with high energy content, not accompanied by adequate physical activity. In the case of a persistent imbalance between diet and physical inactivity, particularly in the context of a particular genetic structure predisposing to dyslipidaemic dysfunctions, the SAT may no longer be able to accumulate excess lipids, which then accumulate in the deep VAT and even in ectopic sites, such as muscle and liver. This determines an alteration of the metabolic equilibrium, leading to pathological dysfunctions. In this situation the adipose compartment is subjected to hypertrophic (increase in cell size) and hyperplastic (increase in the number of adipocytes) stimulation (Lazar, 2008). VAT, when compared with the SAT, is more sensitive to the stimulation of lipolysis by catecholamines, but also has a greater capacity for capturing FFA that it stores in TAGs (Jensen *et al.*, 2003; Yang X., 2007). Even the adipose stem cells of the VAT and the SAT present functional differences and potential differentiation, presenting the visceral precursors a reduced differentiation capacity (Baglioni *et al.*, Plos One 2012).

1.3 Adipose tissue as an endocrine organ

For many years, WAT was considered essentially as an energy reserve and as an insulating barrier. However following the discovery of Leptin and its pleiotropic actions (Zhang *et al.*, 1994) AT has been considered also as an endocrine and immune organ.

It is composed primarily by pre-adipocytes and adipocytes, and other types of cells called the stromal-vascular fraction composed by different cell types including macrophages, neutrophils, lymphocytes, fibroblast, and endothelial cells among others. The secreted molecules by the pool of cells that compose the AT, play an important role in metabolism and in the balance between pro- and anti-inflammation to maintain the homeostasis. These molecules called "Adipokines", act locally and systematically with autocrine, paracrine and endocrine functions and have a key role in controlling metabolism. These are a very heterogeneous group of proteins: some are related to the immune system (tumour necrosis factor- α , TNF- α , and interleukin-6, IL-6), others are growth factors (transforming growth factor- β , TGF- β), some are proteins of the alternative pathway of the complement system (adipsin), while others are involved in the regulation of blood pressure (angiotensinogen), in blood coagulation (plasminogen activator inhibitor-1, PAL-1), and in blood glucose homeostasis (adiponectin, leptin, resistin) (Fonseca-Alaniz *et al.*, 2007). In the AT, an overload of energy results in storage of TAGs in preexisting adipocytes leading to hypertrophy. The increased volume of adipocytes leads to abnormal adipokine production, mainly cytokine, resulting in activation of some pro-inflammatory signaling pathways. Several gene expression studies conducted on AT of obese rodents and also proved on humans, confirmed indeed an over expression of genes involved in inflammatory processes that are related to macrophage infiltration in WAT (Ha *et al.*, 2013). AT of obese subjects is indeed characterized by an alteration of the immune cell system composition with an increase of T helper 1 and CD8⁺ lymphocytes that promote pro-inflammatory macrophages infiltration. Macrophages are the responsible for the production and release of inflammatory cytokines like IL-1, IL-6 IL-8 and TNF- α in AT. An accumulation of these cells could thereby represent the cause and/or the consequence of low-grade inflammation state associated with obese status.

Excessive intake of calories as FFA and glucose can also affect intracellular oxidative stress increasing the production of mitochondrial reactive oxygen species (ROS) (Hotamisligil, 2003). Everything considered, these multiple changes in obese AT promote a low-grade inflammation.

Adiponectin

Adiponectin (Adipo Q) is a hormone that controls the energy metabolism of lipids and carbohydrates. It is mainly expressed by mature adipocytes (Savino *et al.*, 2008) and is the protein produced in the greatest quantity by AT. The Adipo Q performs numerous functions, certainly, an important role is related to the increase in insulin sensitivity, since it increases glucose uptake in skeletal muscle, favouring the oxidation of FA through the enzyme pathway adenosine mono-phosphate kinase (adenosine monophosphate, AMP - kinase), and inhibiting the production of glucose by the liver (Schondorf T., 2005). Under conditions of IR or T2DM, a decrease in plasma hormone levels is observed (Schondorf T., 2005). This hormone, therefore, has various mechanisms of action that converge in the protection against atherogenic and insulin-resistant mechanisms. Precisely for this reason, the Adipo Q is considered a "protector" against MS (Garaulet *et al.*, 2007). A further confirmation of this is the fact that there is a negative correlation between obesity and Adipo Q plasma levels: with the increase in body weight in fact its blood concentration decreases (Bacha F., 2004).

In humans, the fact that the circulating levels of Adipo Q, produced by AT, are reduced in obese individuals, which present an extensive development of the adipose compartment, is a real paradox: in reality this contradiction can be explained by the fact that there seems to be a negative feedback mechanism on the production of Adipo Q that is activated when obesity develops (Tilg & Moschen, 2006). It can therefore be assumed that the abnormal development of AT causes dysfunction in the secretion of this adipokine. Among other functions, adiponectin improves the absorption of FA by the skeletal muscle where they are oxidized: this function is very important because it reduces the contribution of the same FAs to the liver, thus reducing the hepatic production of TAGs and favouring one state of greatest insulin sensitivity.

Leptin

Leptin is a 16 kDa cytokine synthesized and released by fat cells following a change in body fat. It is encoded by the LEP gene present in the q31.3 region of chromosome 7. At the hypothalamic level, it binds to receptors that stimulate anorexigenic peptides such as proopiomelanocortin and inhibits orexigenic peptides such as the neuro peptide Y (Ahima & Flier, 2000). It reduces the levels of intracellular lipids of skeletal muscle, liver and β -pancreatic cells, thus increasing insulin sensitivity. In the muscle the insulin sensitivity is achieved through the inhibition of Malonyl-CoA, which increases the transposition of FA towards mitochondria for β -oxidation. These modifications are partially mediated by the activation of adrenergic receptors (Minokoshi *et al.*, 2002). Since mature adipocytes express receptors for leptin, it is hypothesized that the hormone has a self / paracrine role: it has in fact been shown in rodents that the autocrine role is to increase the number of mitochondria able to oxidize FA inside the adipocyte and therefore reduce the amount of intracellular lipids up to 95% (Vazquez-Vela *et al.*, 2008).

Therefore, leptin plays an important role in regulating the energy balance, inhibiting nutrient intake and increasing energy consumption. Studies on rodents have indeed shown that knock-out animal models for the *ob* gene, which therefore do not produce the protein, behave as if they were always fasting and their increased appetite leads them to obesity (Zhang *et al.*, 1994). These mice therefore represent an excellent model for the study of obesity. Environmental factors, such as exposure to cold, can also reduce gene expression. By contrast, leptin synthesis is stimulated by certain hormones such as insulin, estrogens and glucocorticoids. Among the several physiological roles related to the control of metabolism and energy homeostasis, leptin has been shown to regulate also the immune response, innate and adaptive response, both in normal and pathological conditions. The role of leptin in regulating immune response has been assessed in vitro as well as in clinical studies (Lord *et al.* 1998). It has been shown that conditions of reduced leptin production are associated with increased infection susceptibility. Conversely, immune-mediated disorders such as autoimmune diseases are associated with increased secretion of leptin and production of proinflammatory pathogenic cytokines. Thus, leptin is a mediator of the inflammatory response.

Resistin

Resistin is secreted by adipocytes in significant amounts during adipogenesis. It acts both in AT and in skeletal muscle, but neither the role nor the mechanism of action is exactly known. However, its synthesis is associated with an increase in IR and it is hypothesized that the hormone modulates the insulin-dependent signalling pathways (Hotamisligil, 2003). The function of the resistin is not to block the action of insulin when there is availability of food, but rather to make the body adapt to the moments of fasting, when the level of glucose in the blood decreases and the mobilization of the FAs occurs. High levels of resistin in the blood are however associated with atherogenic processes and related complications (Piestrzeniewicz *et al.*, 2008).

Transforming growth Factor beta (TGF- β)

It's implicated in different biological processes like cell migration and adhesion, and tissue remodeling. In the AT an increased expression of this molecule by mature adipocytes is correlated to obesity and its associated complications. A fascinating study conducted on AT of obese mice shown a higher mRNA expression of TGF- β compared the lean group (Kaidar-Person *et al.*, 2008).

Monocyte chemoattractant protein 1 (MCP-1)

Is a chemokine involved in recruiting monocytes, neutrophils and lymphocytes and is highly expressed in VAT and SAT of obese subjects. Its role is to recruit inflammatory cells into the AT causing a worsening of the metabolic phenotype. Meijer *et al* reported that adipocyte derived MCP-1 can stimulate inflammation in human AT independently of macrophages and of interest are the large cohort studies conducted in Caucasians and Chinese population that indicate an increase of CCL2/MCP-1 in T2DM subjects (Jiang *et al.*, 2016; Zietz *et al.*, 2005).

Interleukin 1 beta (IL-1 β)

Cytokine expressed mainly by SAT with an autocrine and/or paracrine role. Speaker *et al* in 2012 showed an increase of IL-1 β in non-visceral AT following acute stress or exposure causing lipid retain and hyperplasia (Speaker & Fleshner, 2012). It has also been suggested that IL-1 β could have important role in the regulation of lipogenesis in the macrophage cell membrane giving to it a central role in the link between lipid metabolism and innate immunity (Ha *et al.*, 2013).

Interleukin 6 (IL-6)

This cytokine has been subject of major interest. It's expressed by cells of the stromal vascular fraction like pre-adipocytes, endothelial cells and monocyte-macrophages and then secreted in the circulation. It is thought that 15 to 30% of circulating IL-6 levels derives from AT production without an acute inflammation. Several *in vivo* and *in vitro* studies conducted both in mice and humans have shown a high expression of this molecule in AT of obese and IR subjects and glycemia results in increased IL-6 levels as well (Bastard *et al.*, 2006; Trayhurn & Wood, 2004).

Interleukin 18 (IL-18)

It's a pro-inflammatory cytokine expressed by several cell types and in the AT has been detected in both areas: SAT and VAT. Studies conducted on mice in the past decade demonstrated a high positive correlation between circulating IL-18 levels and obesity and IR and a decrease of its levels after weight loss (Membrez *et al.*, 2009). Interesting results were also obtained by the group of Wood *et al* in 2005 in which they have assessed a high expression of IL-18 and its receptor IL-18R mostly in visceral adipocytes of morbid obese patients (Netea *et al.*, 2016; Wood *et al.*, 2005).

Tumour necrosis factor alpha (TNF- α)

In humans, TNF- α is mainly secreted by subcutaneous stromal vascular fraction cells. The expression of this cytokine take place in adipocyte precursor cells and differentiation reagents used *in vitro* such as IBMX and thiazolidinediones inhibit its secretion. Several studies conducted on mice confirmed the increase of TNF- α expression in obese models and has been proposed to be linked with IR through the inhibition of the insulin receptor (Sethi & Hotamisligil, 1999). In humans the expression of TNF- α is increased in obesity and IR, but its neutralization through TNF- α antagonist administration does not improve insulin resistance with insulin resistant subjects (Jung & Choi, 2014). It has an autocrine and paracrine influence in a range of processes and plays a pivotal role of stimulation and production of other adipokines like IL-6.

Between adipokines secreted by the AT we can also find:

Acylation-stimulating protein (ASP) which increases lipogenesis and inhibits lipolysis;

- plasminogen activator inhibitor-1 (PAI-1), which causes the formation of vein thrombosis and rupture of unstable atherogenic plaques;
- angiotensinogen, renin and angiotensin-converting enzyme (ACE), which take part in adipocyte differentiation and lipogenesis contributing substantially to the AT physiology and investing essential role in the regulation of blood volume and systemic vascular resistance;
- visfatin, which regulates glycaemic homeostasis;
- apelin, involved in the regulation of nutritional entry.

Given the complexity of the pathology of obesity and the multiple factors involved in its onset, the efforts of numerous research groups to clarify the mechanisms that develop in the adipocyte in physiological and pathological conditions are understandable. A dysregulation of adipocyte function is in fact associated with the onset of metabolic pathologies. Therefore, the adipocyte must no longer be considered a mere store of energy but a cell capable of responding to paracrine and endocrine signals, with a specific secretion of hormones and cytokines, capable of regulating numerous metabolic and immune functions (Vazquez-Vela *et al.*, 2008). Therefore, intervening on obesity in the MS means reducing the risk of the development of the disease.

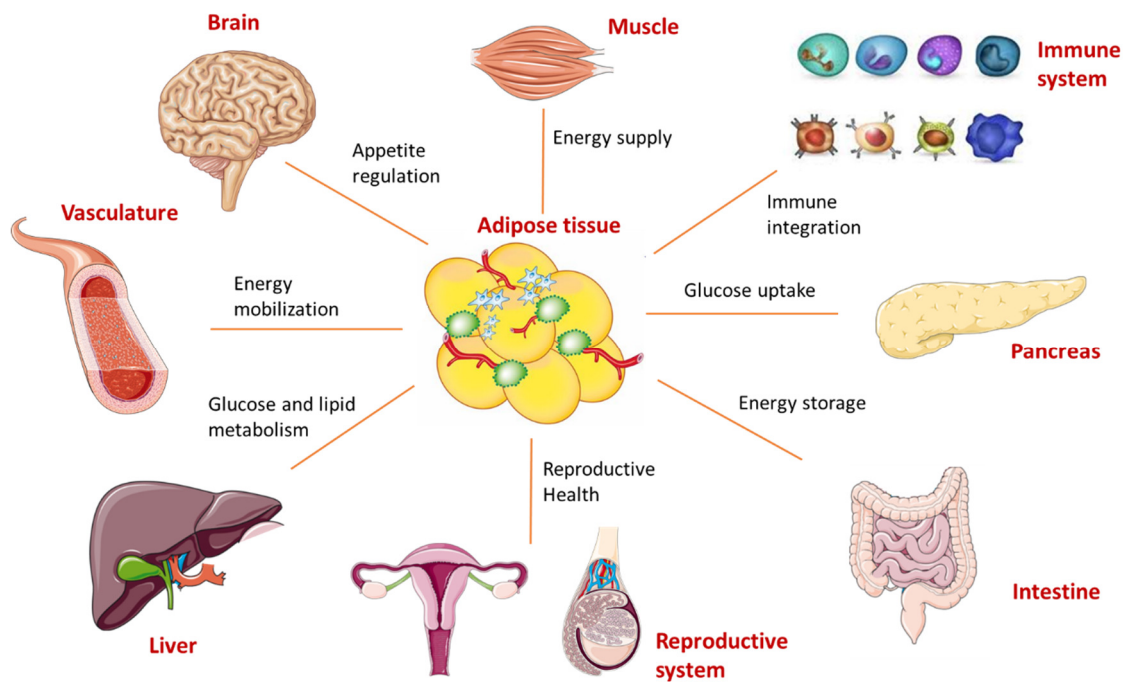


Figure 3 Paracrine effect of adipose tissue. Adipose tissue has multiple effects on several systems in the body fulfilling several critically important roles.

1.4 Fatty acid biosynthesis, lipolysis and beta oxidation in the adipose tissue

The ability to accumulate lipids can be considered as an evolutionary strategy to store energy in order to encounter relatively long periods of food insufficiency. The development of specialised fat depots in mammals implicates a high level of coordination needed to regulate food intake, lipid absorption and transport, fatty acid biosynthesis and oxidation in order to preserve the homeostatic equilibrium. Energy is stored in adipocytes in the form of TAGs and cholesterol esters in single large cytoplasmic vacuoles. Adipose cells contain the "machine" needed for lipid metabolism and since these pathways are altered during overweight and obesity a better understanding of TAG synthesis are critical for the development of strategies to treat this illness and its correlated diseases like T2DM, atherosclerosis and hypertension (Weisberg *et al.*, 2003). Moreover it has been established that FA not only show an energy function but also act as regulatory signals for the gene expression of protein involved in lipid metabolism and are also associated with inflammatory processes (Bastard *et al.*, 2006; Dasu & Jialal, 2011).

1.4.1 Fatty acid uptake

After absorption in the intestine, lipids are transported into the blood stream via the lymphatic system and are packed into lipoproteins such as chylomicrons, very-low-density lipoproteins (VLDL) to reduce hydrophobicity (Mansbach & Gorelick, 2007). In the blood stream, TAGs from the chylomicrons are hydrolysed into monoacylglycerol and FAs due to the action of the extracellular enzyme lipoprotein lipase bound to the wall of capillaries (Wang & Eckel, 2009) before being internalized by the adipocyte. FA's trafficking into adipocytes is regulated by different fatty acid transporter proteins (FATPs) and cluster of differentiation (CD) 36 (CD36) expressed on the cytoplasmic membrane (Thompson *et al.*, 2010). FAs enter then the adipocytes to be esterified with G3P and synthesize the TAG that are stored in the lipid vacuole. In this process insulin stimulates the uptake and metabolism of glucose to G3P.

1.4.2 Lipogenesis

the rate-limiting step in FA synthesis is catalysed by Acetyl-CoA Carboxylase (ACC) an enzyme which favours the carboxylation of acetyl-CoA to malonyl-CoA which is then converted into palmitate (16-carbon fatty acid) by the catalysing action of Fatty acid synthase (FASN). Palmitate is then elongated by the action of Fatty acid elongase ELOVL6, a membrane-bound enzyme located in the endoplasmic reticulum involved in the elongation of saturated and monounsaturated FAs.

FAs are then desaturated through the introduction of double bonds in determined positions of the chain by desaturase enzymes such as stearoyl-CoA desaturase (SCD). At this stage, FAs are esterified to form TAGs. The first step in TAGs synthesis that occurs in the endoplasmic reticulum and mitochondria, is the formation of lysophosphatidic acid (LPA), catalyzed by glycerol-3-phosphate acyltransferase (GPAT). In the endoplasmic reticulum LPA is further esterified and converted into phosphatidic acid (PA) in the reaction catalyzed by 1-acylglycerol-3-phosphate acyltransferase (AGPAT). PA is then converted by LIPIN1 in to the intermediate 1,2-diacylglycerol (1,2-DAG). Finally, Diacylglycerolacyltransferase (DGAT) catalyzed the acylation of 1,2-DAG to form TAGs. TAGs are then packaged into lipid droplets (Fig 3).

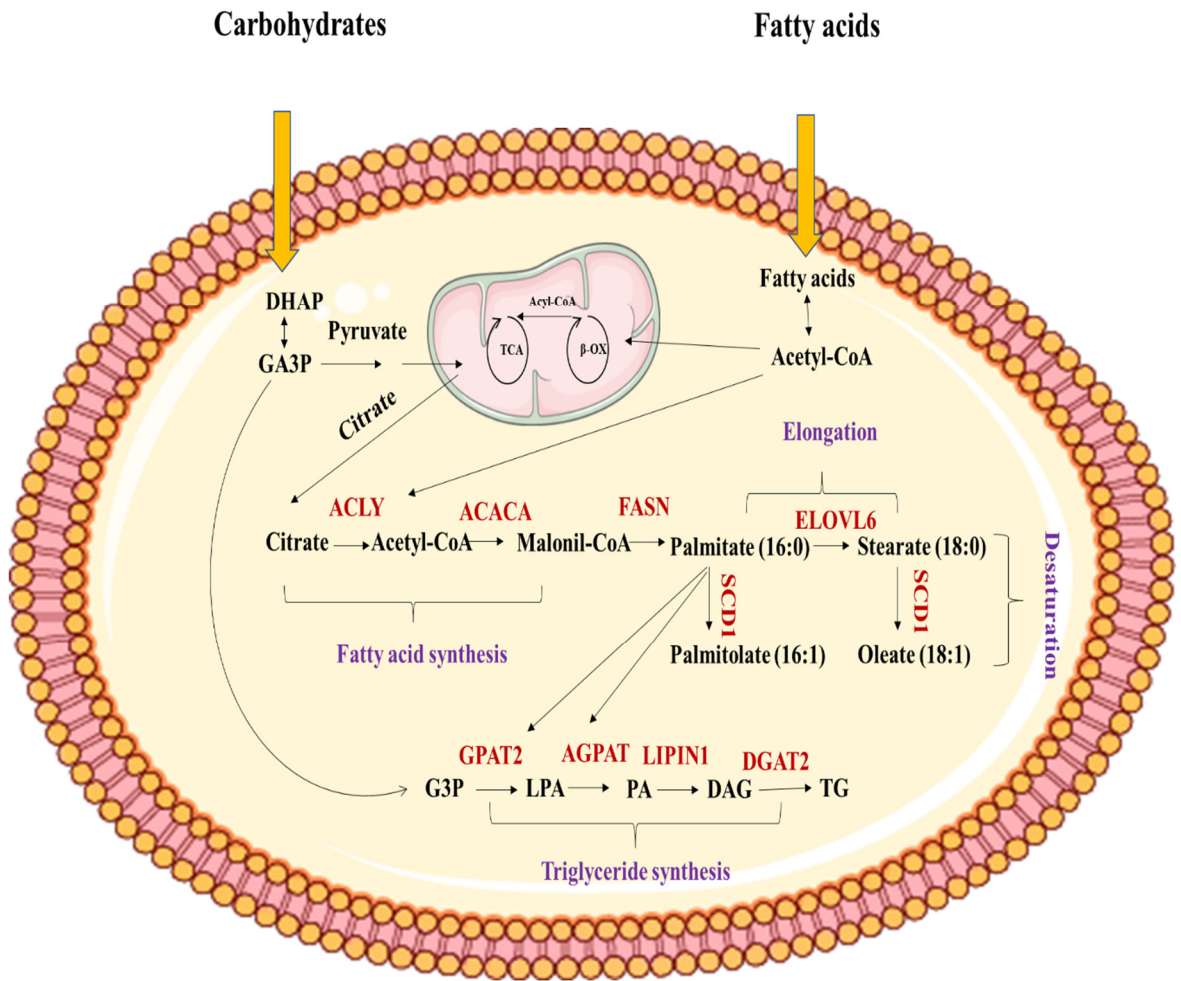


Figure 4: Adipose *de novo* lipogenesis. Dietary carbohydrates, lipids, and proteins are used as substrates for *de novo* lipogenesis. Carbohydrates are metabolized to three carbon intermediates dihydroxyacetone phosphate (DHAP) and glyceraldehyde three phosphate (GA3P) which are further metabolized to pyruvate. Pyruvate enters mitochondria to be used for energy production. When energy stores are plentiful, citrate is transported to cytoplasm where, by the action of ATP citrate lyase (ACL), it is converted to acetyl-CoA. Acetyl-CoA carboxylase (ACC) converts acetyl-CoA to malonyl-CoA. Fatty acid synthase (FASN) sequentially adds acetyl-CoA to the growing fatty acid chain to form saturated FAs, mainly palmitate. Palmitate may be further elongated to stearate or longer fatty acids by the action of elongation of very long-chain fatty acids (ELOVL6). Stearoyl-CoA desaturase 1 (SCD1) converts saturated FAs to monounsaturated FAs. Glycerol-3-phosphate acyltransferase (GPAT) adds acyl-CoA to glycerol-3 phosphate (G3P) to form lysophosphatidic acid (LPA). At this stage, fatty acids are esterified and ready to form triacylglycerides. The first step in TAG synthesis that occurs in the cytoplasm is the formation of lysophosphatidic acid (LPA) catalyzed by glycerol-3-phosphate acyltransferase (GPAT2). LPA is esterified and converted into phosphatidic acid (PA) catalyzed by 1-acylglycerol-3-phosphate acyltransferase (AGPAT). PA is then converted by LIPIN1 into the intermediate 1,2-diaclyglycerol (1,2-DAG). Finally, Diacylglycerol acyltransferase (DGAT) catalyzes the acylation of 1,2-DAG to form triacylglycerol (TAG).

1.4.3 Fatty acid oxidation

When hormones such as glucagon and noradrenaline report an energy metabolism deficit, TAGs kept in lipid droplets in the adipose tissue, are released and metabolized in order to produce energy. Fatty acid β -oxidation (or Fatty acid oxidation FAO) is a mitochondrial aerobic process of breaking down FA into acetyl-CoA units. It occurs in the mitochondrial matrix and it plays an important role in energy metabolism. Long-chain acyl-CoAs bound to carnitine enter mitochondria due to Carnitine palmitoyltransferase-1 β (CPT1 β) a carnitine-dependent enzyme located on the outer mitochondrial membrane. In the intermembrane space, the carnitine/acylcarnitine translocase (CACT) transfer the acylcarnitines to Carnitine palmitoyltransferase 2 (CPT2) which reconverts the acylcarnitine into acyl-CoA and releases it in the mitochondrial inner membrane space to be oxidized. The initial step of the FAO consisting in the introduction of trans double-bond between C2 and C3 (α,β -dehydrogenation) of the acyl-CoA thioester substrate by Acyl-CoA Dehydrogenase Long Chain (ACADL). 2-trans-enoyl-coenzyme A is then hydrated by the addition of a water molecule to the double bond with formation of L-3-hydroxyacyl-CoAs by Enoyl-CoA Hydratase (ECHS1). L-3-hydroxyacyl CoA is dehydrogenated again to create 3-ketoacyl CoA by 3-hydroxyacyl CoA dehydrogenase. The last step of the mitochondrial FAO is catalysed by Acetyl-CoA Acyltransferase 2 also known as 3-Ketoacyl-CoA Thiolase. It catalyses, using one molecule of coenzyme CoA, the thiolytic cleavage of medium- to long-chain unbranched 3-oxoacyl-CoAs into acetyl-CoA and a fatty acyl-CoA shortened therefore by two carbon atoms. The process continues until all of the carbons in the FA are turned into acetyl CoA. The FAO results in NADH and FADH generation that allow the transfer of electrons to the electron transport chain allowing therefore the creation of proton gradient by proton pumps, used to create ATP.

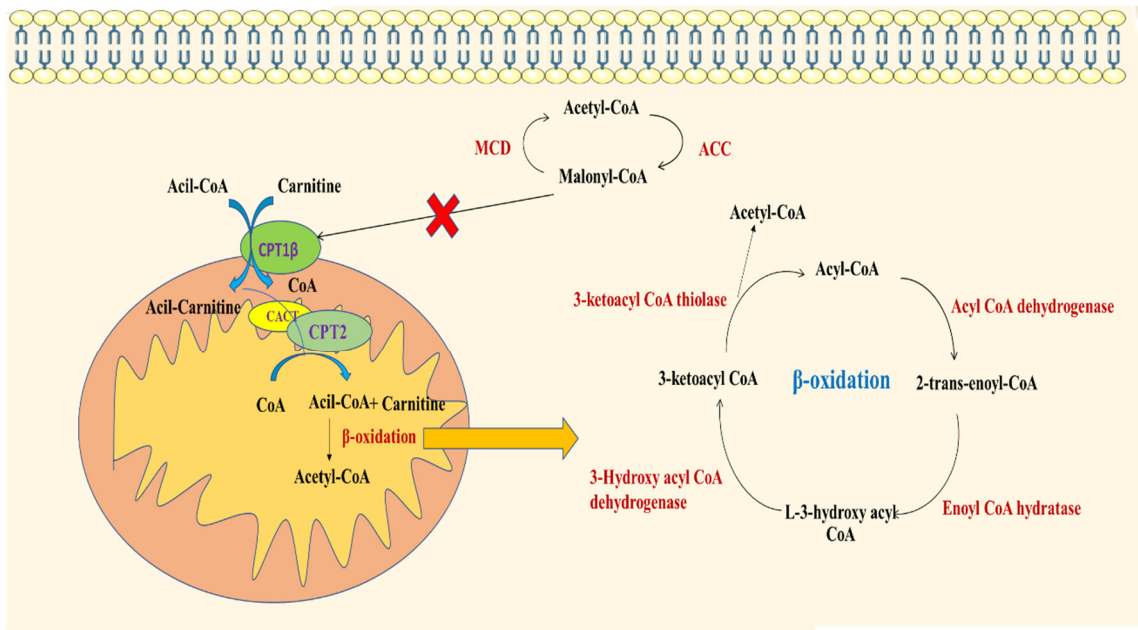


Figure 5: Adipose fatty acid β -oxidation

Fatty acids are activated to acyl-CoA, transported into the mitochondria as acylcarnitine by the enzyme carnitine palmitoyltransferase-1 (CPT1) carnitine translocase (CACT) and carnitine palmitoyltransferase-2 (CPT2), reconverted back to acyl-CoA and subject to β -oxidation. Each β -oxidation cycle of four reactions generates one acetyl-CoA and an acyl-CoA that is two carbon units shorter.

1.5 Obesity

Obesity is a chronic disease with a multifactorial aetiology, the prevalence of which is constantly increasing and considered a pandemic disease by the World Health Organization (WHO, 2000). Once considered a problem only in high-income countries, overweight and obesity are now dramatically on the rise in low- and middle-income countries. According to estimates by the WHO in 2016, overweight adults (considering a body mass index or BMI ≥ 25 kg / m²) were more than 1.9 billion adults, of these over 650 million adults were clinically obese (WHO, 2016). All this translates into an enormous social impact, as obesity and related metabolic diseases, cause a worsening of the quality of life and increasing therefore health and social costs.

Obesity is characterized by an increase in adipose tissue (AT) in various parts of the body, particularly at a visceral level and in ectopic sites. The diagnosis of obesity is made on the basis of two simple anthropometric measures which are weight and height. With these data it is possible to calculate the body mass index (BMI = weight in kilograms / square of height in meters). A BMI greater than 24.9 kg / m² is indicative of overweight, while over 30 kg / m² is obesity (WHO, 2000) . It is clear however that the problems related to overweight and obesity are continuously linked to weight gain, so even small changes in BMI can have important repercussions in terms of comorbidity for associated pathologies. The BMI also gives an indication on the possible deviation from the ideal weight but doesn't give information regarding the distribution of the adipose mass that plays an important role in the patient's prognosis. In fact, with the same BMI, there can be a predominantly peripheral distribution of fat (gynoid or subcutaneous obesity), a mainly central distribution, or a localization of fat in the abdominal level (android or visceral obesity). There are numerous techniques for the direct and indirect measurement of the quantity of body fat and its distribution, but to date the most used technique in clinical practice is represented by the measurement of the waist circumference (WC). A waist circumference ≥ 102 cm in males and ≥ 88 cm in females indicates a central distribution of fat, an abdominal obesity (WHO, 2000). Among the different types of obesity, visceral obesity seems to play a central role in the pathogenesis of the Metabolic Syndrome (MS), a hypothesis put forward for the first time by Vague in the 1950s (Bosello & Zamboni, 2000) and confirmed in the last 15 years by numerous studies.

1.6 Morbid obesity and current management

There are different approaches for the treatment of morbid obesity (BMI > 35), such as diet and exercise and drug therapies (Ricci *et al.*, 2016), but today bariatric surgery is the most effective treatment for patients with morbid obesity. There are in fact, countless works that expose the benefits of this surgical technique (Borisenko *et al.*, 2018; Lee & Cha, 2016; Nearing *et al.*, 2017). Recent studies have also shown that, in addition to inducing significant weight loss, it promotes the resolution of many of the diseases associated with obesity such as T2DM, hypertension, dyslipidaemia and sleep apnoea syndrome (Sarkhosh *et al.*, 2013; Spivak *et al.*, 2017; Stefater & Inge, 2017). It has also been shown that in the group of surgically treated patients there is a reduction in mortality, the risk of developing other associated diseases, the use of therapeutic treatments and direct and indirect health and social costs. Bariatric surgery is generally considered for patients with morbid obesity who are refractory to other weight loss interventions. In particular, it is appropriate for adult patients with a BMI equal to or greater than 40 kg / m² or with a BMI between 35 and 40 kg / m² characterized by comorbidity-dependent obesity such as T2DM, hypertension, cardiomyopathies, sleep apnoea, asthma and dyslipidaemia (WHO, 2000).

These criteria have remained unchanged since 1991, but in 2006 O'Brien and colleagues showed, through a randomized study conducted on 80 patients, that bariatric surgery can be applicable even to adults with moderate obesity, i.e. with a BMI between 30 kg / m² and 35 kg / m² (O'Brien, 2015). According to the Consensus Conference of the National Institutes of Health (NIH), the candidates for bariatric surgery are the pathologically obese subjects (BMI > 40 Kg / m² or BMI > 35 Kg / m² with comorbidity), for which diet attempts have failed, and without any significant psychological illness. Furthermore, the benefits must outweigh the risks. Surgery for morbid obesity has a low failure rate, with an average weight loss of 61.2% on the initial weight (Buchwald *et al.*, 2004). There are no age restrictions, in fact bariatric surgery is also applied to patients over the age of 60, and currently also on patients under the age of 18, due to the increase in obesity even in paediatric age.

Obesity is a metabolic pathology with a multifactorial aetiology that involves genetic, environmental, metabolic and psychological factors. In this regard, the evaluation and therapeutic approach to the obese patient must be carried out by a multidisciplinary team consisting of a surgeon, nutritionist, endocrinologist, anaesthesiologist, pulmonologist and gastroenterologist. This helps to evaluate and optimize the approach to patients before surgery and to provide care after surgery, in case it is necessary.

1.7 Micronutrient deficiency and Obesity

Besides dietary habits, multiple genetic, environmental and behavioral factors lately emerged as possible important players in the development of several diseases including obesity. The theory of foetal programming, suggesting that environmental factors surrounding the foetus during its developmental phase can influence and increase its propensity to develop certain diseases such as T2DM and obesity in later life, has in fact become widely described over the last two decades. Several epidemiological studies have in fact examined the effect of extreme nutritional deprivation (Dutch famine during the 2nd World War) on the offspring during the periconceptual and developmental period. It has been shown that progenies exposed to these conditions showed an increased risk in developing obesity and T2DM compared with their unexposed siblings (Ravelli *et al.*, 1998; Roseboom *et al.*, 2006). Analogous results were obtained also in studies conducted on babies born during the Chinese famine between 1958–1961 (Li *et al.*, 2010). Altogether these data may suggest that a combination of micronutrient deficiency and other wider deficits could be the cause of the development of metabolic related diseases, but the exact mechanism is still not explained. Nutritional-dietary factors seem to play therefore a very important role in affecting epigenetic phenomena, and alter the expression of genes at the transcriptional level. Among the various molecules, folate dependent one-carbon nutrients seem to play a special role in biological methylation reactions since they are fundamental in providing methyl groups (–CH₃) for cycle regulation and cell differentiation (Tussing-Humphreys & Nguyen, 2014) and along with recent findings, alterations in the pattern of DNA methylation that occur in-utero are capable of inducing changes in gene expression that contribute to the development of obesity by increasing AT growth and expansion. In a population study conducted in India (Pune Maternal Nutrition Study) (Yajnik *et al.*, 2008) it has been shown that low maternal levels of B12 is associated with increased adiposity and IR development in the offspring, underlying the important role that this micronutrient might play in the AT. It's a common assumption that vitamin and mineral deficiencies are rare in developed countries, however the increased availability of inexpensive, energy-dense, and low-nutrient value foods can lead to micronutrient deficiency and rise of obesity. As an example, In the UK population, B12 deficiency has been found among pregnant women and it has been associated with increased BMI and the risk of developing GDM (Adaikalakoteswari *et al.*, 2014). Similar studies have also shown a relationship between low maternal plasma

B12 and increased IR and obesity in a White British, non-diabetic population (Knight *et al.*, 2015; Sukumar *et al.*, 2016).

In vitro studies on 3T3-L1 mouse derived cell line showed that treatment of folic acid and B12 attenuates adipogenic differentiation (Bellner *et al.*, 2015), and a study conducted on Wistar rats showed that offsprings from B12 deficient mothers had an abnormal adiposity with a higher total cholesterol, TAGs and a higher expression of cytokines like IL-6 and TNF- α compared with control offsprings (Ghosh *et al.*, 2016). Similar results were also obtained by experiments conducted on human adipocytes in which it has been shown an effect of B12 deficiency on cholesterol biosynthesis (Adaikalakoteswari *et al.*, 2015; Adaikalakoteswari *et al.*, 2014). With this view micronutrients, and especially B12, seem therefore to play a very important role and an exposure to a deficiency during the fetal programming and development as well as aduring adulthood might lead to metabolic dysfunctions. Therefore, elucidating the mechanism of the effect of B12 deficiency on AT inflammation and the TAGs synthesis will help develop of a novel possible therapeutic target.

1.8 Vitamin B12

Once called “nature's most beautiful cofactor”(Stubbe, 1994), vitamin B12 is an essential micronutrient for human health and it's known to be the most chemically complex coenzyme. It's a hydro soluble molecule with a size of 1300 to 1500 Da and more than 25 enzymes are involved in its synthesis (Roth *et al.*, 1996). The core of the molecule is the corrin ring, similar to porphyrin ring found in heme, characterized by a central cobalt ion which can form between four to six bonds. In particular cobalt bonds not only the nitrogen atoms of the corrin ring surrounding but also able to create upper and lower ligands (Smith *et al.*, 2018).The upper ligand is the cyano group, while the lower ligand is represented by the nitrogen form of dimethyl benzimidazole (DMB) which results to be bound also by a side chain to the corrin ring, conferring a high specificity of the vitamin for intrinsic factor (IF) binding in the lower gastrointestinal tract (Froese & Gravel, 2010). The cyano ligand present above the corrin ring is substituted by an adenosyl group, a methyl group or water to create respectively adenosyl-cobalamin (AdoCbl), methyl-cobalamin and hydroxocobalamin. Through its active forms, methyl-cobalamin (Me-Cbl) and adenosyl-cobalamin (Ado-Cbl), is involved in essential reactions as nucleic acid synthesis, erythropoiesis in bone marrow and myelination of central nervous system. Me-Cbl is the cofactor of the enzyme methionine synthase in the metabolic homocysteine pathway (HCY) taken place in the cytosolic environment in which HCY is converted in methionine with the involvement of vitamin B6 and folate (Gherasim *et al.*, 2013). A dysfunction of this pathway, critical in the generation of the methyl donor S-adenosylmethionine, affects DNA synthesis and physiological processes that require intense cell replication. Ado-Cbl acts in the mitochondria as a coenzyme of methylmalonyl-CoA mutase in the metabolism of branched-chain amino acids and FAs with an odd number of carbon atoms. A shortage of Ado-Cbl brings to an accumulation of an intermediate molecule of this pathway, methylmalonic acid (MMA), causing neurological defects and dysfunctional nerve transmission (Rizzo *et al.*, 2016).

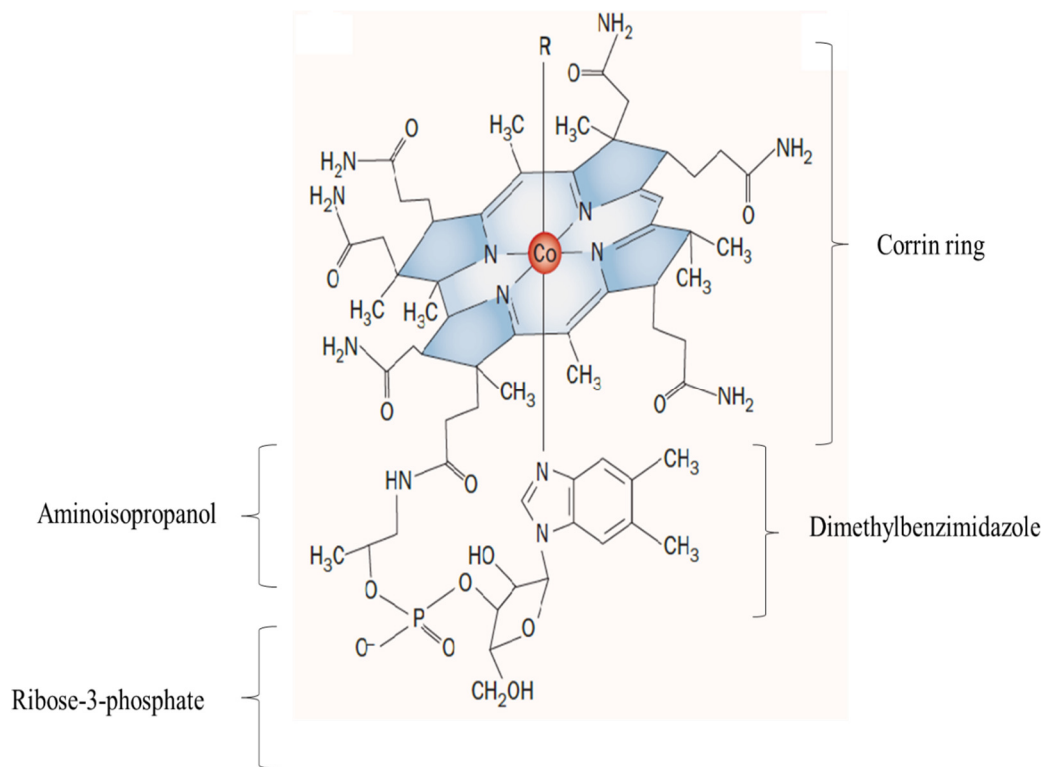


Figure 6: Structure of vitamin B12: The core of B12 is represented by a corrin ring that encloses a cobalt ion with four nitrogen atom linkages. The R-group at the β -axial (upper) position may be a methyl, 5'-deoxyadenosyl-, hydroxo- or a cyano- group whereas the α -axial (lower) ligand is a 5,6-dimethylbenzimidazole (DMB) linked through phosphoribosyl moiety to the central cobalt ion . Image adopted and modified from (Nielsen *et al.*, 2012).

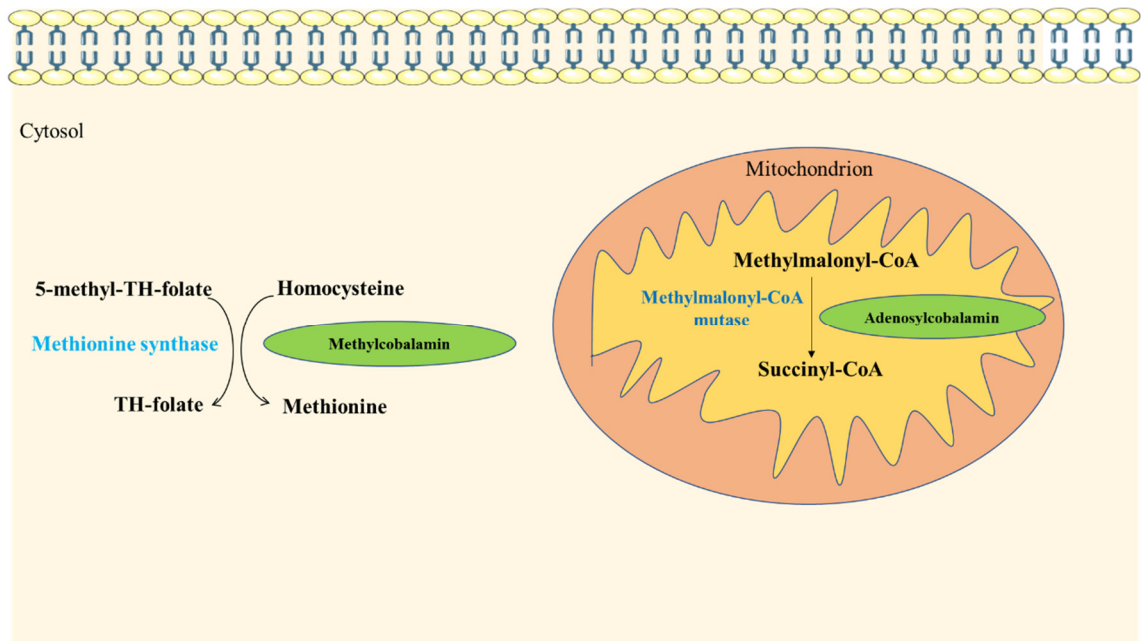


Figure 7: Coenzyme function of B12. B12 serves as a coenzyme in two distinct enzymatic processes: the conversion of homocysteine to methionine by cytosolic methionine synthase in the cytosol and the conversion of methylmalonyl-CoA to succinyl-CoA by mitochondrial methylmalonyl-CoA mutase in the mitochondria

In the plasma, Me-Cbl represents approximately 60 to 80% of total plasma vitamin B12, adenosyl-cobalamin accounts up to 20% and the remainder mainly hydroxycobalamin. Whereas in the tissue the concentrations results inverted, in fact, the major active form of B12 is Ado-Cbl (about 70% in the liver), then hydroxycobalamin represents about 25% and methyl-cobalamin less than 5% (Bender, 2003).

B12 is synthesized by certain bacteria and archaeon through aerobic and anaerobic pathways whereas other B12-utilizing organisms must modify B12 acquired following uptake from other sources (Smith *et al.*, 2018). In humans, these sources are limited to animal products, and therefore a certain proportion of the population, with low intake of animal products, such as vegans and vegetarians, is moderately vitamin B12 deficient, even though dietary requirements are only a few micrograms per day.

Studies regarding the association between B12 sources and serum levels indicate that meat dairy food and fish are rich with this micronutrient. Particularly animal meats derived from ruminants results to be higher in B12 compared to the one derived from omnivorous animals like (pork and poultry). Cattle and sheep are herbivores and are able to absorb their B12 from the enteric bacteria that live in their stomachs. The produced B12 is therefore absorbed in the intestine, transferred into the blood and stored in the liver and muscles of the animal or excreted into the milk (Watanabe & Bito, 2018).

The metabolism of B12 is a complex process that involves almost 20 genes associated in absorption, selection, transport, modification, and utilization of B12 acquired from the diet.

The absorption of dietary B12 consist in a complex gastrointestinal process. After the ingestion of animal products rich in B12, hydrochloric acid and pepsin secreted by gastric parietal cells allow the release of B12 from food proteins and bound to haptocorrin; a B12-binding protein secreted by the salivary glands which protects the vitamin against possible further hydrolysis by the acidic environment (Allen *et al.*, 1978). In the duodenum pancreatic proteases cause the proteolysis of haptocorrin-vitamin B12 complex and the released B12 binds to IF in the proximal ileum. IF is secreted by stomach parietal cells and a deficiency of it, due to genetic errors, autoimmune diseases or bariatric surgeries can cause pernicious anemia (Tanner *et al.*, 2005). The IF-B12 complex binds to IF receptors on the membrane surface of polarized epithelial enterocytes in the terminal ileum. Thereafter B12 is released to bind with plasma binding proteins haptocorrin or transcobalamin (TC) for delivery and storage in the tissues, circulation in the blood and storage in the liver. The greater proportion of B12 (around 80%) is bound to haptocorrin which is thought to transport a surplus to the liver and the remaining 20% is bound to TC for which it has a higher affinity (Rush *et al.*, 2014). B12-TC complex known as holotranscobalamin (holo-TC), considered the active B12, arrives in the blood circulation and circulates until it is taken up by the cells via a cell surface receptor.

The discovery of molecular structure of TC has been made possible due to bio-crystallography and kinetic studies conducted in 2006. In particular the crystal structure of the human TC show a two-domain architecture, an N-terminal domain made up of secondary alpha helical structure and a smaller C-terminal beta strand domain able to bind a B12 molecule through a histidine coordinated binding (Wuerges *et al.*, 2006).

The controversial structural architecture of TC receptor CD320, remained although undiscovered until 2016 when Amer Alam's group and it's collaborators conducted genetic engineering and crystallography studies that allowed the discovery of the complex molecule (Alam *et al.*, 2016). CD320 is a member of the Low-density Lipoprotein Receptor Family featuring an N-terminal, extracellular fragment characterized by two LDLR type A (LDLR-A) domains separated by an epidermal growth factor (EGF) homology domain, a single transmembrane helix and a C-terminal cytoplasmic region involved in complex internalization (Jiang *et al.*, 2013). Moreover, TC receptor CD320

results to have an exclusive affinity to TC which interact only with the α -domain (Wuerges *et al.*, 2006).

The cellular uptake is mediated by B12 receptor CD320 in a biphasic process and involve binding of the holo-TC to the cell surface initially followed by internalization of the complex (Quadros & Sequeira, 2013). In the lysosome, the B12–TC complex is digested to create free B12 and is exported in the cytosol via two membrane protein, ABCD4 (ATP-binding cassette sub-family D member 4) and LMBRD1 (limb region 1) that work in synergy. Once in the cytosol, free B12 is bounded to CblC, a protein which has the ability to dealkylate and decyanalate the various forms of B12 and therefore plays an important role for the distribution of this micronutrient in various compartments of the cell (Smith *et al.*, 2018).

1.8.1 Definitions of B12 deficiency

Haematological sign, such as raised mean corpuscular volume or megaloblastic anaemia has been traditionally used as markers for B12 deficiency. However, it was shown that other clinical conditions such as neurological pathologies, characterized by B12 deficiency appear generally also in absence of anaemia. Therefore, anaemia is just one of the possible outcomes related to B12 deficiency, and is incorrect to use it as the only marker (Green, 2017). Even if there isn't a clear value to identify an adverse B12 condition, just as for intricate pathologies risk factors such as metabolic disease and CVD in which "the clinical picture in the most important factor in assessing the significance of test results" (Devalia *et al.*, 2014), recently WHO and the US Institute of Medicine established a cut-off of 148 pmol/L (200 pg/mL) or 150 pmol/L (203 pg/mL) (Smith *et al.*, 2018).

1.8.2 B12 deficiency across the globe

The prevalence of B12 deficiency is becoming widespread among the global population. There is a high prevalence of B12 deficiency in many population groups at all ages, most particularly elderly individuals and lower income communities where a restricted lifelong animal-derived food consumption define a high proportion of the population. According to recent studies, it's been estimated that between 2.5% and 26% of the world population and approximately 6-12 % of British population under the age of 60 is characterized by a subclinical deficiency of B12 (Green *et al.*, 2017; Sukumar & Saravanan, 2019).

Notably, around 12-14% of adults in the UK and more than 70% in India have a poor B12 status (Rogne *et al.*, 2017; Sukumar *et al.*, 2016b; Yajnik *et al.*, 2008). During pregnancy, the B12 deficiency ranges between 20-30% depends on the stages of pregnancy and population across the world (Sukumar *et al.*, 2016), potentially causing harm to both the mother and child. Therefore, since B12 deficiency is related with a series of negative health outcomes, its high prevalence turns to be a matter of concern to the world public health.

1.8.3 Causes of vitamin B12 deficiency

In developed countries, pernicious anaemia results to be the primary risk factor for B12 deficiency. Population studies have confirmed that this disease has a high prevalence in Northern Europe and Scandinavia where epidemiological studies have been conducted (Clausen *et al.*, 1996).

Pernicious anaemia is an autoimmune disease that involves complex environmental and immunological factors leading to a deficit of B12 due to a deficiency of IF. The lack of B12 absorption is caused mainly by gastric autoantibodies directed to parietal cells and IF needed for the intestinal absorption of B12, leading to the development of megaloblastic anaemia and several neurological diseases (Green *et al.*, 2017). However, another cause of pernicious anaemia that brings to B12 deficiency but without the autoimmune involvement is the mutation in B12 Binding Intrinsic Factor (GIF), a gene that encodes a member of the cobalamin transport protein required for adequate absorption of B12.

A lack of B12 is also common in patients undergoing partial gastrectomy, gastric bypass and gastrointestinal surgery, which compromise the absorption of this micronutrient by reducing the gastric mucosa area, and the possibility to secrete IF (Smelt *et al.*, 2017).

B12 deficiency is also common in subjects characterized by a reduced acid secretion in the stomach associated with chronic atrophic gastritis or long-term treatment with proton pump inhibitors and *Helicobacter pylori* infection that cause malabsorption by reducing the ability to release B12 from its protein-bound form (Lam *et al.*, 2013).

Inflammatory bowel diseases such as Crohn' and Celiac diseases can cause B12 deficiency due to mucosal and villous atrophy. Parasitic infection by fish tapeworm *Diphyllobothrium latum* and protozoan *Giardia lamblia*, caused by recent trend in consuming raw fish and described mostly in Northern Europe, Scandinavia United States and Japan are able to cause B12 deficiencies by depletion from the small intestine (Miller *et al.*, 2009).

1.8.3.1 Daily recommendation and storage

B12 is an essential micronutrient required for several biological processes with a recommended daily intake of 2.4 µg that raise up 6µg to in pregnant women (Yajnik *et al.*, 2008). The body can store, mainly in the liver, between 2000-5000 µg of B12 under the form of adenosylcobalamin. This explains why in populations with short supply of

B12 sources and no malabsorptive problems, the signs of B12 deficiency appear much later.

1.8.4 Complications of B12 deficiency

B12 is involved, through its coenzyme forms (Adenosyl-B12 and Methyl-B12), in several metabolic reactions and a deficit induce impaired DNA methylation, dysregulation of metabolism of methylmalonate (a molecule derived from the catabolism of certain fatty acids and amino acids), homocysteine accumulation and other cellular disruptions leading to cellular stress and apoptosis. This therefore leads to a wide range of clinical consequences from hematological to neurological (Green *et al.*, 2017).

The haematological effect of B12 deficiency is megaloblastic anaemia, which results from a downregulation of DNA synthesis resulting in large erythrocytes (macrocytes) with overgrown cytoplasm and immature nuclei. The synthesis of other haematopoietic cells result to be affected as well with enormous granulocyte precursors in the bone marrow and multilobular neutrophils (Stabler, 2013). In addition to megaloblastic anaemia a decrease of all blood cells numbers may occur (pancytopenia).

Recent studies on genetical defects have shown that the major cause of neurological defects in B12 deficiency could be due to a lack of methyl-B12 and hyperhomocysteinaemia resulting in myeloneuropathy with demyelination of central and peripheral neurons. A deficit of B12 is also associated with hypomethylation of the promoters of the genes encoding amyloid proteins result to be associated with a deficit of B12 increasing the risk of poor memory performance and Alzheimer's Disease (Rafiee *et al.*, 2017). Normal B12 level is crucial for a healthy development of neurological functions in children and recent studies have observed a correlation between B12 deficiency during pregnancy and neuronal tube defects in the offspring confirming therefore its main role in the development (Dror & Allen, 2008).

Studies conducted in the past decade have shown that less common conditions associated with B12 deficiency are psychiatric manifestation, thyroid malfunction, increased thrombotic events and infertility (Lindenbaum *et al.*, 1988; Stabler, 2013).

1.8.5 B12 deficiency associated with obesity, diabetes and dyslipidemia

In the last decade clinical evidence of a correlation between B12 deficiency and adverse metabolic health profiles triggered many scientists to study the role of this micronutrient in the development of obesity. It has been proved that B12 deficiency affects the mitochondrial methyl malonylCoA mutase reaction leading to an increase of levels of MMA and consequently enhancing increased lipogenesis (Watanabe *et al.*, 2003).

Recently a population study involving more than 9000 Danish people showed a negative correlation between B12, BMI waist circumference and body fat percentage (Allin *et al.*, 2017). A clinical study conducted in Israel on three hundred ninety-two children and adolescents revealed a low B12 concentration in obese children compared with normal weight group (Pinhas-Hamiel *et al.*, 2006). Similar results were observed in adult patients with morbid obesity (Li *et al.*, 2018).

Another study involving mice fed with a low B12 diet showed an increased body fat composition, altered lipid profile and elevated stress and adipokines markers. Moreover mother deficient in B12 gave birth to offspring with lower body weight (Ghosh *et al.*, 2016) and our group published recently a systematic meta-analysis showing the negative effect of B12 deficiency during pregnancy on offspring birth weight (Sukumar *et al.*, 2016b).

An interesting study conducted by our research group on human adipose cells, cultured in low or no B12, showed increased gene expression of genes involved in cholesterol biosynthesis. We have also reported that maternal low B12 is associated with adverse HDL levels. These data suggest that B12 can influence lipid metabolism in human adipocytes and may have an important role in obesity development (Adaikalakoteswari *et al.*, 2015).

It has been shown in Europeans and Indians with T2DM that B12 deficiency is associated with adverse lipid profile suggesting that deficiency of B12 plays an important role in development of T2DM (Adaikalakoteswari *et al.*, 2015).

Interesting studies conducted on pregnant women characterized by B12 deficiency confirmed the correlation between this condition and increase risk of developing IR, GDM and obesity (Knight *et al.*, 2015) that can contribute to childhood obesity in the new birth (Yajnik, 2014). Moreover, a recent study conducted on patients with diabetes, vegetarians and omnivores showed a correlation between high levels of B12 with lower levels of fasting glucose and a significant correlation between low B12 levels high oxidative stress and inflammatory markers. These results were reinforced in the vegetarian population (Lee *et al.*, 2016).

1.8.6 The role of B12 like as an antioxidant and anti-inflammatory molecule

Several recent studies have indicated that B12 act as an antioxidant and anti-inflammatory molecule having a function in the immune system. Me-Cbl is a cofactor of methioninesynthase an essential enzyme for the synthesis of nucleotides. This reaction results to be important especially in fast-dividing immune cells. Studies, conducted in both human and animals showing a positive correlation between low number of lymphocytes and impaired NK cells activity, suggests a possible role in acute and chronic inflammation (Partearroyo *et al.*, 2013; Tamura *et al.*, 1999). Although these recent findings led many scientist to investigate the role of B12 in several inflammatory diseases such as CVD, T2DM and obesity (Al-Daghri *et al.*, 2016; Ghosh *et al.*, 2016; Lee *et al.*, 2016), the AT specific role of this micronutrient has not well studied. Since obesity is characterized by an “inflamed” AT and obesity is associated with low B12, result to be fundamental to explore the specific effect of this micronutrient on AT.

1.8.7 B12 deficiency in Bariatric Surgery

Most of the patients experience nutritional deficiencies after any bariatric surgery procedure. Deficiency of B12 is particularly well reported. An interesting study conducted on more than 20.000 patients found an incidence rate of B12 deficiency of 20% 12 months after gastric bypass surgery (Gudzune *et al.*, 2013). The explanation of this deficiency in RYGB is due to the stomach reduction that causes a decreased nutrient absorption and decreased acidity pepsin and IF. These evidence are confirmed by a study conducted on patients undergoing gastric banding and gastric bypass: the prevalence of B12 deficiency was higher among patients submitted to gastric bypass (Silva *et al.*, 2016).

Another fascinating study conducted on morbid obese females who underwent RYGB shows a gene expression modification in TC cubilin and IF expression in stomach duodenum, jejunum and ileum tract, suggesting a genetic reprogramming after this bariatric procedure (Sala *et al.*, 2017).

1.8.8 The role of the paternal diet

DNA methylation is the most widely studied form of epigenetic modification and occurs within the one-carbon metabolism pathway, which is dependent upon several enzymes in the presence of dietary micronutrients as cofactors. According to recent evidence from both epidemiological studies in humans and in vitro and intervention studies in animals, alterations in the pattern of DNA methylation that occur in utero are capable of inducing changes in gene expression that contribute to the development of obesity by increasing AT growth and expansion. Methylation is greatly dependent on availability of SAM-e that are synthesized with the aid of methyl donors such as B12 and folate that play major roles in one-carbon metabolism. This suggest a central role B12 in epigenetic mechanisms and how a deficiency of this micronutrient could contribute to metabolic dysfunction. In support to this, Sinclair and colleagues demonstrated in an animal study conducted on sheep that a reduction of B12, folate and methionine availability from the maternal diet around the time of conception is associated with increased adiposity, IR and altered immune function (Sinclair *et al.*, 2007). Whilst many studies support the concept of the maternal influence on offspring health outcome, not much work been done to understand the underlying mechanisms linking paternal diet with litter health, specifically, its role on spermatogenesis, function and its epigenetic mechanisms. However, there is growing evidence that paternal diet would affect not only the sperm quality but also have an influence in the post-fertilization development, making it a new emerging focus for the Developmental Origins of Health and Disease (DOHAD) hypothesis. It is well known that high BMI is linked with reduced serum testosterone (Luconi *et al.*, 2013) and increased sperm fragmentation (Samavat *et al.*, 2018) and also animal models of paternal high-fat diet and paternal undernutrition revealed an increase in sperm fragmentation and impact on embryonic metabolism and fetal growth (Bakos *et al.*, 2011; Carone *et al.*, 2010; Mitchell *et al.*, 2011). Interestingly, in a recent study conducted on male mice fed with low-protein diet it has been shown that hypomethylated sperm is related with offspring' elevated growth programming, increased adiposity and disrupted cardiovascular health (Watkins *et al.*, 2018). These observations suggest therefore that not only the maternal diet but also the paternal nutrition could play a role in litter health through epigenetic mechanisms partly also due to B12 deficiency.

1.8.9.1 Male hypogonadism and Bariatric Surgery

Obesity and its associated comorbidities have been exponentially increasing during the last 40 years, representing a medical and socio-economic burden also in developing countries (Farzadfar *et al.*, 2011). Alterations in sex steroid hormones are often associated with male obesity. In particular, low circulating levels of total (TT) and free (FT) testosterone in the presence of reduced gonadotropins, characterized by central secondary hypogonadism (HG), with elevated estrogens, have been described in male MS. Obesity and HG are linked in a vicious cycle, in which low testosterone favors weight gain, and adiposity induces HG. Several studies demonstrated that weight loss and reduction in waist circumference (WC) induce an increase in testosterone levels (Corona *et al.*, 2011; Tajar *et al.*, 2010). Although a clear association between parameters defining obesity (BMI, waist circumference, weight) and reduced fertility in men is still controversial, several recent studies confirmed a general amelioration not only in sex hormones with an increase of androgens in hypogonadal individuals (Samavat *et al.*, 2014), but also in sperm quality and function in subjects undergoing bariatric surgery (Samavat *et al.*, 2014). From a clinical point of view, these data suggest that bariatric surgery results beneficial not only for the treatment of T2DM and CVD but also in HG recovery in morbid obesity.

1.8.9 B12 and male fertility

In the past 4 decades various studies have studied the effect of B12 on semen quality. It has been found that B12 is transferred from the blood to the male reproductive organs which gives a clear idea of the role of this micronutrient on spermatogenesis (Banihani, 2017). Several studies have shown an amelioration in sperm parameters after 8-24 weeks of B12 treatment (Boxmeer *et al.*, 2007; Watson, 1962). In particular, total sperm count, sperm concentration and motility were statistically increased. Moreover studies conducted on patients characterized by oligozoospermia and normozoospermia under long term treatment with Me-Cbl showed an increased sperm motility (Isoyama *et al.*, 1986; Watanabe *et al.*, 2003). Interestingly a recent study showed that infertile men with varicocele supplemented with B12 for three months had a decreased of sperm DNA fragmentation by about 22% (Gual-Frau *et al.*, 2015).

The positive effects of B12 on semen quality may be due to a decreased accumulation of ROS and a decreased inflammation-induced semen impairment. Thus, the results published so far confirm the important role of B12 in male fertility. No research about the role of B12 in sperm quality in bariatric patients has been conducted so far. The aim of my research will be therefore to clarify the role of B12 in sperm quality of morbid obese patients and how this can be effective on male fertility.

1.9 Male infertility

1.9.1 Epidemiology

In 1987 the WHO defined couple infertility as the inability to conceive after a year of unprotected sex intercourse. Epidemiological data show that the prevalence of this condition in the Western world is around 15%: of these values, about 38% of cases are due to female causes, 20% to male causes, 27% involve both partners, while in 15% of cases the gender aetiology is not clear (Vander Borgh & Wyns, 2018). Thus, about 50% of cases of couple infertility the "male factor" is at stake. The aetiology of male infertility can be of different nature and not necessarily identified with a defect in spermatogenesis. The levels of alteration can be central and peripheral, thus affecting the central control of gamete production and transport, or directly at the peripheral level. In humans three different causes of infertility can be distinguished, classified according to the level of onset of dysfunction. The dysfunction can, in fact, be determined by:

- **Pre-testicular causes (10%)**: they refer to the alteration of the hypothalamic-pituitary-gonadal axis which results in a reduced or altered production of GnRH, and consequently of FSH (hormone that acts on Sertoli cells at an intratubular level, favouring the maturation of spermatozoa) and of LH (a hormone that acts at an interstitial level by stimulating the production of testosterone from Leydig cells). This central alteration causes hypogonadotropic (or central) hypogonadism and can also develop as a consequence of anabolic steroids intake. This category also includes coitus disorders such as erection and / or ejaculation dysfunctions.

- **Post-testicular causes (15%)**: are relative to the transport of spermatozoa from the testicle through the male reproductive system and can be linked to congenital or acquired obstructive forms such as those caused by inflammations or infections of the accessory glands and epididymis. They can also involve erection and ejaculation dysfunctions at the local level.

Testicular causes (75%): relative to alterations at the testis, the site of production of spermatozoa and testosterone. It may be associated with congenital disorders that alter testicular function leading to altered sperm production and function. These alterations may derive from congenital defects, such as anorchia or cryptorchidism, from genetic disorders that concern numerical and structural abnormalities of sex chromosomes but

also from autosomes, from mechanical disorders such as varicocele, orchitis or testicular torsion.

1.9.2 Male hypogonadism

Male hypogonadism defined as reduced gonadal function and androgen action has a multifactorial aetiology that includes genetic mutations, anatomical alterations, lesions, infections and cancer. However, still a good part of the causes remains idiopathic (Basaria, 2014). This disorder has been described in 20% of elderly males over the age of 70 and in 4% of the postpubescent male population (Brunton & Sadovsky, 2010). Recent studies have also shown that 30% of subjects diagnosed with T2DM and with a BMI > 30 Kg / m² are characterized by low serum testosterone levels (Zheng *et al.*, 2016). In these subjects, however, the cause of the decrease in androgen levels is not yet clear.

HG is a disease characterized by the presence of poorly functioning gonads and can be distinguished in hypergonadotropic if associated with high levels of secretion of FSH and LH gonadotropins or hypogonadotropic if these levels are low. Furthermore, hypogonadism is also observed in subjects with hyperfunctioning gonad where the dysfunction is due to the lack of action of androgens caused by peripheral resistance to testosterone. In this case, both gonadotropin levels and testosterone levels are high.

The clinical symptoms of hypogonadism depend mainly on the severity and type of the disorder. In adults the symptoms can range from a decrease in libido, bone mineral density, infertility, hair loss and incomplete sexual development. The diagnosis is made through physical examination and by measuring the serum testosterone levels. According to the second international consultation on erectile dysfunction of the WHO (Lue *et al.*, 2004), the blood sampling, for the serum determination of testosterone, must be carried out from 8.00 to 11.00 am, or when the levels of this hormone reach a peak in healthy young males. The blood testosterone value to be considered as a cut-off to diagnose HG is still debated and therefore the diagnosis varies accordingly. In general, the threshold values are considered 8 nM, 10 nM, 12 nM, thus leaving a defined "gray" area between 8nM and 12nM. However, to distinguish between primary and secondary hypogonadism, measurement of FSH and LH levels is necessary.

1.9.3 Reproductive parameters in obese men

Recent studies have suggested overweight and obesity, is associated with changes in sexual male parameters. Research carried out on sperm parameters has shown in fact, a negative association between seminal quality and BMI (Hammoud *et al.*, 2008).

Obesity is characterized by an alteration of the distribution of fat in the visceral area. As is known, adipocytes are the main cells of the AT and play an important role in the regulation of TAG and FFA levels. Oestrogens, are positive regulators of adipogenesis, promoting preadipocyte proliferation and growth of mature adipocytes (Price *et al.*, 1998). On the contrary, adipocyte differentiation and maturation is negatively regulated by androgens, as high androgenic levels favour multipotent cell differentiation towards myogenesis and also inhibit adipogenic differentiation of existing preadipocytes (Mårin *et al.*, 1995). In the AT the local production of oestrogen from the conversion of testosterone is led by the enzyme aromatase.

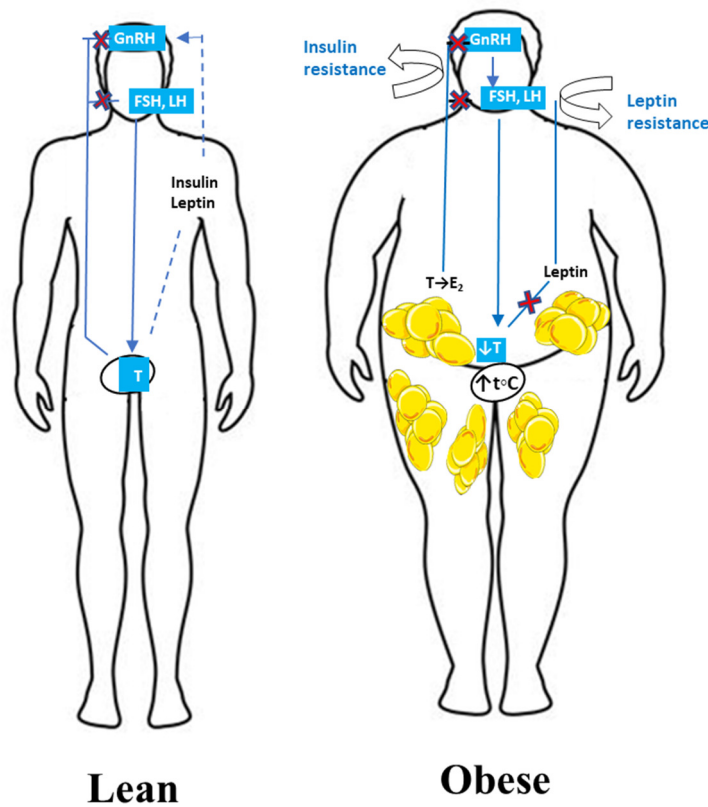


Figure 8: Schematic representation of the differences between normal weight and obese subjects of the hypothalamic-pituitary-gonadal axis

1.9.4 Oestrogens of adipocyte origin and hypogonadism

Oestrogens, derived from the aromatization process in the AT of obese men, cause a negative feedback action at the level of the hypothalamic-pituitary-gonad axis (Hypothalamus, Pituitary, Testis HPT) (Couse *et al.*, 1997). This therefore causes a vicious circle of "hypogonadal obesity", in which an increase in AT leads to an alteration of hormone production at a systemic level. Increased AT results to be a significant peripheral resource of oestrogens which in turn suppress testosterone production with a negative feed-back on the HPT axis. The consequent reduction of circulating testosterone together with the increase in oestrogen favours the increase of VAT. This imbalance in sex hormone levels (assessed by the ratio of blood T / E2 levels) has consequently also an effect on the fertility of the obese male (Mårin *et al.*, 1995).

1.10 Summary and gaps in evidence

In developed countries, the increased availability of unhealthy highly processed food rich in carbohydrates but with poor nutritional value can lead to micronutrient deficiency. The so called “high-calorie malnutrition” could lead to profound metabolic changes by the inability to utilize energy effectively and thus promote increased fat synthesis resulting in weight gain. Micronutrients play an important role in metabolism and a deficiency can bring about cellular dysfunction. In the last decades animal and clinical studies have revealed a correlation between B12 deficiency and adverse metabolic health profiles. In particular, B12 deficiency in pregnancy has been associated with maternal obesity, development of IR and dyslipidaemia. Studies shown that babies born to B12 deficient mothers show a high accumulation of fats at birth with higher risk of developing CVD later in life. Moreover, obesity and morbid obesity is associated with sub-fertility in men, reduced semen quality and impaired sperm acrosome reaction and clinical studies have found correlations between seminal B12 and sperm concentrations

According to that, there is no evidence so far whether

(1) dyslipidaemia and increased adiposity due to B12 deficiency are directly linked with regulation in the adipose metabolism of lipids and

(2) if there is a relation between B12/semen quality in morbid obese subjects.

Therefore, given the key metabolic role of AT and the importance of seminal liquid in male fertility, investigating the effect B12 deficiency on *de novo* lipid synthesis and lipid metabolism leading to AT inflammation, will help develop novel biomarkers for the diagnosis of obesity as well as enhanced development of possible therapeutic targets.

1.11 Hypothesis and Aims

The AT functions as a key energy reservoir of the organism in vertebrates and, in particular, in mammals. B12 plays a crucial role as a cofactor in two key metabolic pathways such as the methionine synthase and the methylmalonyl-CoA mutase pathways. Studies have shown that B12 deficiency would lead to disruption of these pathways leading to accumulation of metabolites such as homocysteine and MMA which are known to be associated with metabolic risk including CVD.

AT plays a dominant role in the regulation of lipid metabolism including *de novo* fatty acids (FA), TAG and cholesterol biosynthesis, as well as FAO. An accumulation of dysfunctional fat that occurs during obesity, leads to an expansion of visceral and ectopic fat increasing the risk of cardiovascular and metabolic pathologies and infertility.

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 TAGs and cholesterol. Recent studies have also found a correlation between B12 deficiency and poor seminal quality. We, therefore, hypothesize that B12 deficiency may affect AT metabolism of lipids accounting for dysregulation in the lipogenesis (FA, TAG), FAO pathways inducing a local inflammation. Furthermore, we hypothesize the possible relationship between holoTC/B12 status and semen quality.

In order to investigate this, we aimed:

1. To assess the AT regulation of B12 uptake and storage in various circulatory B12 concentrations ranging from deficient to high levels;
2. To assess whether B12 deficiency affects adipose tissue *de novo* lipogenesis;
3. To assess whether deficiency in B12 affects the adipose tissue FAO pathway;
4. To assess whether the effect of B12 deficiency leads to inflammation in AT;
5. To define the relationship between serum and seminal levels of B12 and seminal quality in morbid obesity.

Chapter 2: Material & Methods

2.1 Materials

2.1.1 Cell Culture Reagents

DMEM/F12 (Gibco, Fisher Scientific, Loughborough, UK)

Advanced DMEM (Gibco, Fisher Scientific, Loughborough, UK)

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

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

Heat Inactivated Foetal Bovine Serum (Fisher Scientific, Loughborough, UK)

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

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

Bovine Serum Albumin (Sigma-Aldrich, Haverhill, UK)

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

Vitamin B12 (Fisher Scientific, Loughborough, UK)

Folic acid $\geq 97\%$ (Sigma-Aldrich, Haverhill, UK)

Fibroblast growth factor-basic (FGF-basic), recombinant human 5 ng/mL (Fisher Scientific #VXPHG0026)

Transferrin, human 5 mg/mL (Fisher Scientific #VX0030124SA)

Preadipocyte Differentiation Medium (Promocell Heidelberg, Germany)

Preadipocyte Nutrition Medium (Promocell Heidelberg, Germany)

2.1.2 Reagents and materials for Radioactive flux assay

Glass TLC Plate (Thermo scientific, Loughborough, UK)

^{14}C 50 μCi (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 tripalmitate (Sigma-Aldrich, Gillingham, Dorset, UK) in chloroform (500 μl)

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)

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

Diethyl ether (Sigma-Aldrich, Gillingham, Dorset, UK) 46

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

Chloroform

Methanol

2.1.3 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)

2.1.4 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)

Eukaryotic L19 rRNA Endogenous Control (Fisher Scientific, Loughborough, UK)

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

Random Hexamers (Invitrogen, Fisher Scientific, Loughborough, UK)

JumpStart SYBR Green I Mix (Sigma-Aldrich, Haverhill, UK)

MMLV Reverse Transcriptase (Sigma-Aldrich, Haverhill, UK)

RNAse OUT Ribonuclease Inhibitor (Invitrogen, Fisher Scientific, Loughborough, UK)

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

100x ROX reference dye (Sigma-Aldrich, Haverhill, 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)

2.1.5 Kits

Quantikine Elisa Human CCL2/MCP-1 (R&D Systems-Biotechne, Abingdon, UK)

Quantikine Elisa Human IL-8/CXCL8 (R&D Systems-Biotechne, Abingdon, UK)

2.1.6 Media formulations

DMEM/F12 supplemented with 10% v/v FBS and 1% (v/v) Penicillin/Streptomycin

Advanced DMEM/F12 with phenol red supplemented with 3% (v/v) FBS, 1% (v/v)

Penicillin/Streptomycin and 1% (v/v) L-Glutamine.

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.

Seahorse Media for Oxygen Consumption assay (Agilent Technologies, Stockport, UK)

Seahorse XF base media, Glucose (25mM), Sodium Pyruvate (1mM), pH 7.4

2.1.7 Buffers

10x DNase I Reaction buffer (Sigma-Aldrich, Haverhill, UK) 200 mM Tris-HCl, pH 8.3, 20 mM MgCl₂

DNase Stop solution (Sigma-Aldrich, Haverhill, UK) 50 mM EDTA

MMLV 10x reaction buffer (Sigma-Aldrich, Haverhill, UK) 500 mM Tris-HCl, pH 8.3, with 500 mM KCl, 30 mM MgCl₂, 50 mM DTT

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°C.

2.1.8 Solutions

Seahorse Calibrant Solution (Agilent Technologies, Stockport, UK)

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

2.1.9 Primers and oligonucleotides

2.1.9.1 Taqman primers for human genes

CD320- [HS00213164](#) (Applied Biosystems, Warrington, UK)

TCN2- [Hs01033097_m1](#) (Applied Biosystems, Warrington, UK)

SCD – [Hs01682761_m1](#) (Applied Biosystems, Warrington, UK)

DGAT2 - [Hs01045913_m1](#) (Applied Biosystems, Warrington, UK)

2.1.9.2 Primer sequences for human genes

Gene Name	Forward primer (5'-3')	Reverse primer (5'-3')
<i>TCN2</i>	AGGCTGGTCTCACAGCTCAA	TGGTAGTAGCTAGTGTGGGGG
<i>CD320</i>	CTTCCACCCCGACCTCTG	CACTGGAACTTGGTGGGTGG
<i>FASN</i>	CTGCTGCTGGAAGTCACCTA	AGTGTGTGTTCCCTCGGAGTG
<i>ACACA</i>	AGAGGGAACATCCCTACGCT	CATGGGTCATGCCATAGTGGT
<i>ELOLV6</i>	TGCTAAGCAAAGCACCCGAA	ACAGGAGCACAGTGATGTGG
<i>SCD</i>	CCACCTCTTCGGATATCGTCC	TGGTGGTAGTTGTGGAAGCC
<i>GPAM</i>	CGAGTGGATTTTGCACAGCC	CGCTTGCTCCAGGGAAAGTA
<i>LIPIN1</i>	TTTCCACGTCCGCTTTGGG	GTGGCCAGGTGCATAGGG
<i>DGAT2</i>	GTCGCGCGAGGGGTCTG	AGCTGCTTTTCCACCTTGGAC
<i>ACADL</i>	TGCAATAGCAATGACAGAGCC	CGCAACTACAATCACAACATCAC
<i>CPT-1β</i>	AGGAGCCCTTCATGGTGAA	TGGCGTGGATGATGTTTCCC
<i>CPT2</i>	GCTGACCAAAGAAGCAGCAAT	GCAAGATGATCCCTTTGGCTG
<i>MCD</i> (<i>MLYCD</i>)	GGTTACCTGGCATTACCGT	GCGCTTCATGTCCATCCAGT
<i>ECHS1</i>	GCGATGGCCAAAGAATCAGTG	TTCCGGTCATCAGTGGCAAA
<i>IL-1 β</i>	CGCCAGTGAAATGATGGCT	GAGGGCAGAGGTCCAGGTC
<i>IL-6</i>	AGTAGTGAGGAACAAGCCAGA	GTCAGGGGTGGTTATTGCATC
<i>IL-8</i>	GAAGGTGCAGTTTTGCCAAGG	TGGGGTGGAAAGGTTTGGAGTA
<i>TNF-α</i>	GCTGCACTTTGGAGTGATCG	GTCACTCGGGGTTTCGAGAAG
<i>TGF-β</i>	CAGTGGTTGAGCCGTGGAG	TGAACCCGTTGATGTCCACTTG

2.1.10 Miscellaneous materials and reagents

Syringe filters (0.22 µm) (MicroScience)

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

Cell culture plates (Fisher Scientific)

OptiSeal 32.4 ml tubes system (Beckman Coulter)

RNase-free tubes (Sigma-Aldrich, Haverhill, UK)

Elisa Strip Plate, F16, highbinding (Greiner Bio One)

Optically clear adhesive seals (Appleton Woods)

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)

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 description of principal methods performed in the study will be outlined, however, brief description with specific requirements in each method will be stated accordingly in the respective chapters.

2.2.1 Cell culture

2.2.1.1 Human preadipocyte ChubS7 cell line

The ChubS7 cell line (Nestlé Research Centre, Lausanne, Switzerland) was derived from a preadipocyte extracted from Abd SAT of an obese female subject with a BMI of 54 kg/m² by co-expression of human telomerase reverse transcriptase (hTERT) and papillomavirus E7 oncoprotein (HPV-E7) genes (Darimont, *et al.*, 2003). ChubS7 cells retain the ability to undergo adipogenesis and have been used to study adipocyte metabolic function including glucose uptake (Gathercole, *et al.*, 2007) following a previously described glucose uptake assay method (Liu, *et al.*, 2001). Proliferating ChubS7 cells were cultured in growth media (DMEM/Ham's F-12 phenol-free medium 500 mL, 10% fetal bovine serum 1% glutamine and 1% pen/strep) in a T75 flask until 90% confluence. At this stage the cells were seeded onto 6 well at a density of 30,000 cells per well grown to confluence (day 0), differentiated in differentiation media (DMEM/Ham's F-12 phenol free medium 500 ml, 3% FBS and Promocell preadipocyte differentiation medium supplement mix: 0.5 µg/ml human insulin, 400 ng/ml dexamethasone, 8 µg/ml d-biotin, 44 µg/ml IBMX, 9 ng/ml L-Thyroxine, 3 µg/ml ciaglitazone) for one week and maintained in nutrition media for the next 7 days (day 14). To analyze B12 deficiency effects, customized media with different B12 concentrations (25pM, 100pM, 200pM, 500pM, 1nM and 500nM) were used. On day 14, the conditioned media was collected, and the cells were harvested for RNA and protein analysis and stored at -80°C until used.

2.2.1.2 Human primary preadipocytes (ScWAT)

Stromal cell isolation from subcutaneous adipose tissue was collected from non-smoking, non-diabetic, premenopausal, Caucasian women with different BMI (lean overweight, obese and morbidly obese subjects) undergoing elective caesarean section at University Hospitals Coventry and Warwickshire (UHCW). Ranges of age and BMI were 25 to 38

years and 21.7 to 41 kg/m², respectively. Primary pre-adipocytes were treated as previously detailed and gene expression detection was performed by RT-qPCR.

The WAT biopsy was digested in collagenase at 37 °C in a shaking water bath. The resulting cell suspension was then centrifuged at 2000 rpm for 5 minutes. The obtained stromal vascular fraction was re-suspended in primary adipocyte growth media (DMEM/Ham's F-12 phenol-free medium 500 ml, 10% fetal bovine serum (FBS), 1% glutamine, fibroblast growth factor-basic (FGF-basic) 5 ng/mL, transferrin 5 µg/ml) and transferred to a 75 cm² tissue culture flask. The flask was kept in a 37°C, 5% CO₂ humidified incubator and the growth media was changed every 2 days until 90% confluency was reached. At this stage the cells were seeded into 6-well plates at a density of 30,000 cells per well and grown to confluence (day 0), differentiated in differentiation media (DMEM/Ham's F-12 phenol free medium 500 ml, 3% FBS and Promocell preadipocyte differentiation medium supplement mix: 0.5 µg/ml human insulin, 400 ng/ml dexamethasone, 8 µg/ml d-biotin, 44 µg/ml IBMX, 9 ng/ml L-Thyroxine, 3 µg/ml ciaglitazone) for one week and maintained in nutrition media for the next 7 days (day 14). To analyze B12 deficiency effects, customized media with different concentrations of B12 were used. On day 14, the conditioned media were collected, and the cells were harvested for RNA and protein analysis and stored at -80°C until used.

2.2.1.3 Cell culture media composition

ChubS7 growth media

DMEM/Ham's F-12 phenol-free medium 500 ml (Invitrogen #11039047)

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

Penicillin/Streptomycin (100X) 5 ml (Fisher Scientific, Loughborough, UK)

L-Glutamine (100x) 5 ml (Invitrogen, Fisher Scientific, Loughborough, UK)

Fetal bovine serum 50 ml

Vitamin B12 at different concentrations (500 nM, 100 nM, 10 nM, 1 nM, 100pM, 25 Pm)**Primary adipocyte growth media**

DMEM/Ham's F-12 phenol-free medium 500 ml (Invitrogen #11039047)

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

Penicillin/Streptomycin (100X) 5 ml (Fisher Scientific, Loughborough, UK)

L-Glutamine (100x) 5 ml (Invitrogen, Fisher Scientific, Loughborough, UK)

Foetal bovine serum 50 ml

Fibroblast growth factor-basic (FGF-basic), recombinant human 5 ng/ml (Fisher Scientific #VXPHG0026)

Transferrin, human 5 µg/ml (Fisher Scientific #VX0030124SA)

Differentiation media

DMEM/Ham's F-12 phenol-free medium 500 ml (Invitrogen #11039047)

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

Penicillin/Streptomycin (100X) 5 ml (Fisher Scientific, Loughborough, UK)

L-Glutamine (100x) 5 ml (Invitrogen, Fisher Scientific, Loughborough, UK)

Foetal bovine serum, 15 ml (3 %)

Preadipocyte differentiation supplement pack x1 (Promocell #C39436)

Nutrition media

DMEM/Ham's F-12 phenol-free medium 500 ml (Invitrogen #11039047)

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

Penicillin/Streptomycin (100X) 5 ml (Fisher Scientific, Loughborough, UK)

L-Glutamine (100x) 5 ml (Invitrogen, Fisher Scientific, Loughborough, UK)

Adipocyte nutrition supplement pack x1 (Promocell #C39439)

2.2.1.4 Harvesting of adipose primary cells and Chubs-S7 for lipid metabolism experiments

Adipose primary cells and Chubs-S7 were scraped at 100% confluency using 500µl of lysis buffer per well and then transferred into 1.5ml centrifuge tubes and stored at -20°C. 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 °C in aliquots. 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. Protein quantification – Bradford technique 1:4 dilution of the Bradford reagent (Bio-Rad, UK) and standard BSA of 1mg/ml were prepared. Standard concentration of BSA (1-7µg/µl) were prepared. To 200µl 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.

2.2.2 Gene expression assay

2.2.2.1 RNA isolation from adipose primary cells and Chubs-S7 cell line

To isolate total RNA from primary adipocytes and Chubs-S7 the cells were washed 2x with PBS and harvested with 500µl Qiazol (Sigma, UK) transferred into 1.5ml centrifuge tubes and stored at -20 °C. RNA extraction was then carried out by incubating cells in Qiazol suspension with 200µl chloroform for 2-3 minutes following initial vigorous shaking for 15 seconds. The tubes were centrifuged at 12,000xg at 4 °C for 10 minutes leading to phase separation. The upper aqueous phase was carefully transferred into clean RNase-free tubes and the RNA was precipitated and washed with 300µl of 99% isopropyl alcohol and 800µl of 75% ethanol, respectively. After completely drying the ethanol, the RNA was re-dissolved in 25µl of nuclease-free water and used for the DNase step with DNase I amplification grade (Sigma). The DNase-free RNA was quantified spectrophotometrically (NANODrop, Labtech, UK) and 300ng was used for cDNA synthesis.

2.2.2.2 Reverse transcription

cDNA was synthesized from single stranded RNA by reverse transcribing the RNA in a solution containing a primer and nucleotides (dNTPs), using RNA-dependent DNA polymerase (reverse transcriptase) enzyme. In this assay, 300ng of DNase-free RNA was incubated with 1µl of random hexamer primer (Thermo scientific, UK) and DNase-free water (Sigma, UK) in RNase-free PCR tubes at 65 °C for 5 minutes. A cocktail comprising of 5x Buffer (Thermo scientific, UK), RibolockRNase Inhibitor (Thermo scientific, UK), nucleotides (dNTPs) (Thermo scientific, UK) and Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Thermo scientific, UK) was prepared. A volume of 7.5µl of the cocktail was added to the content of the PCR tubes, mixed and placed in the iCycler (Bio-Rad, UK). The assay was run following the manufacturer's protocol by programming the equipment to incubate samples at sequential temperatures of 25°C for 10 minutes, 42 °C for 60 minutes and 70 °C for 10 minutes. Synthesized cDNA samples were cooled on ice and stored at -20 °C.

2.2.2.3 Gene expression assay – Real-time quantitative PCR (RT-qPCR) Principle:

TaqMan technique

DNA is amplified and quantified at the exponential phase of the reaction which provides an accurate estimation of amplicons as proportional to the initial quantity of cDNA indicated by the amount of fluorescent emission produced. The probe provided by TaqMan uses the principle of 5'-3' exonuclease action of the enzyme Taq polymerase to cleave the reporter-quencher-labelled probe hybridized complementarily to its target in order to produce fluorescence for detection. In this method, 1µl of cDNA in a 96-well PCR plate (Gene Flow, UK), 24µl cocktail including universal PCR master mix (Applied Biosystems, UK), eukaryotic L19 rRNA endogenous control (Applied Biosystems, UK) and RNase-free water was added. The mixture was vortex mixed and centrifuged at 10,000 rpm for 15 seconds. The PCR was run using a standard TaqMan-RT PCR analyser (Applied Biosystems, UK) for 90 minutes following the manufacturer's protocol. The results were analyzed using the quantitative comparative threshold cycle method (qCCT). RNA expression was normalized to the housekeeping gene L19rRNA. Data were obtained as cycle threshold (Ct) values and used to determine Δ Ct values (Δ Ct = Ct of gene of interest - Ct of L19). Measurements were carried out on at least three occasions

for each sample. To exclude potential bias due to averaging, data was transformed through the equation $2^{-\Delta\Delta Ct}$.

Sybr Green I PCR

Primer design:

Primers for mRNAs of specific genes were designed using the Ensemble Genome Browser Primers tool (<http://www.ensembl.org/index.html>). Their characteristics were evaluated through USCS Genome In-silico PCR (<http://genome.uscs.edu/cgi-bin/hgPcr>). These include size of product between 70 and 150 nucleotides and melting temperature around 60 °C. Primers were ordered from Sigma Aldrich and 100 µM stocks were prepared by dissolving the dried nucleic acids in nuclease-free water. Primers were stored at -20 °C.

Sybr Green I PCR

In this study, Real-Time PCR assays were also performed using Sybr Green I. This fluorophore is an asymmetrical cyanine dye able to bind the DNA. DNA-dye-complex absorbs blue light with a maximum wavelength (λ) of 497 nm and emits green light (λ_{max} = 520 nm). Sybr Green molecules preferentially bind to double stranded DNA intercalating in its minor grooves; hence, fluorescence intensity increases with PCR product accumulation at every cycle. Two microliters of each cDNA sample or nuclease-free water (non-template control) were mixed in a 96 well plate with 18 µl of a master mix previously made and aliquoted in the wells. Master mixes were prepared in different tubes for every target and reference gene with a volume for n+1 sample. Each one contained the following reagents at the following final concentrations in 20 µl final volume: 1X Jumpstart SYBR Green I mix, 300 nM forward and reverse primer for protein-encoding genes and microRNAs, respectively, ROX reference dye 0.1 X, nuclease free-water up to the 20 µl final volume. Every cDNA and non-template sample was tested in duplicate or triplicate. PCR reactions were run in a 7500 Real-Time PCR machine (Applied Biosystem) and thermocycle conditions consisted of an initial denaturation at 95 °C for 2 min followed by 40 cycles at 94 °C for 34 s, 60 °C for 34 s and a final step at 72 °C for 30 seconds at which data were collected. Then a step for melting curves was added to evaluate the presence of single or multiple products.

2.2.3 Radioactive flux of TAG synthesized via utilization of radiolabelled-fatty acid (¹⁴C-oleate) by Chubs-S7 and primary adipocytes

The amount of de novo TAG synthesized by cells in an interval of time can be quantified by initially exposing cells to radiolabeled fatty acid (¹⁴C-oleate) in the presence of fatty acid co-transporter (L-Carnitine). Preparation of BSA and oleate cocktail: 497μl each of 70mM oleate and 0.25% BSA was prepared in a glass tube. A volume equal to 32μl of the radioactive compound ¹⁴C-labelled-0.75mM oleate at 2000 dpm/nmol stored at -20 °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°C and the cocktail was gently transferred dropwise into the glass tube and left on the shaker for 1 hour. Finally, labelled media was prepared including 1mM L-carnitine, 0.75mM cold glycerol and 0.75mM ¹⁴C Oleate in DMEM media. Experimental procedure: at 100% confluency, the conditioned media in each well of primary adipocytes was removed and replaced with 1 ml of the labelled media and incubated at 37 °C for 2 hours and for background correction control cells are incubated for 5 minutes. After the incubation, the labelled media was removed and then the cells were harvested in 2 ml of methanol. The extraction of total lipids from the adipocyte cells was performed using a mixture of chloroform and methanol (2:1 v/v). The resulting layer of chloroform containing the TAG fraction was then transferred into a glass tube and completely dried under nitrogen gas. The dried content in the glass tube was then reconstituted in 500μl of chloroform and finally transferred gradually, 15μl at a time, onto a plate of silica gel 60-coated-TLC plate. The resultant radiolabeled TAG is 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 TAG from the total lipids, 10 nMol of glyceryl tripalmitate (tripalmitin), a TAG standard was run alongside the test and included in each sample. Visualization of the TAG bands was aided by use of iodine vapor. The bands were then scraped into scintillation vials and the radioactivity of the TAG fraction was quantified by the scintillation counter. Finally, the TAG concentration was calculated by normalizing with the protein concentration in the chloroform extract by the Bradford method.

2.2.4 Total intra and extracellular vitamin B₁₂ quantification by immunoassay technique

B12 was measured using Roche Elecsys immunoassay analyzer, Cobas e 411 (Roche Diagnostics GmbH, Mannheim, Germany) at the George Eliot Hospital, Nuneaton that employs the test principle of competitive electrochemiluminescence immunoassay. The assay has a linearity range of 30–2000 pg/ml. The assay involves binding of B12 in the sample to the ruthenium labeled antibody. Streptavidin coated microparticles (solid phase), binds the biotin labeled B12 and ruthenium labeled antibody. A further wash period leaves the solid phase magnetic microparticles bound with B12-biotin and ruthenium labeled antibody, which generates chemiluminiscent emission signal that is inversely proportional to the concentration of B12 present in the patient sample. The inter- and intra-assay coefficients of variation (CV) for B12 were 3.0% and 3.7%, respectively.

2.2.5 Mitochondrial dysfunction assessment using seahorse extracellular flux assay

This technique measures the oxygen consumption rate (OCR) as well as extracellular acidification rate (ECAR) as an assessment of the mitochondrial respiratory and glycolytic mechanisms respectively. OCR and ECAR results in generation of measurable alteration in the amount of dissolved oxygen as well as liberated protons which could easily be estimated using sensor probes positioned at approximately 200 microns above the monolayer of cells, and finally calculates the respective OCR and ECAR. In the mitochondria of healthy individuals, efficient electron transfer is usually required for energy generation to enhance the proton pumping effect via the inner mitochondrial membrane (IMM) to establish an electrochemical gradient necessary for ATP formation and low amounts of reactive oxygen species (ROS) are produced. Elevated ROS can react with biomolecules such as DNA, proteins and lipids that consequently inhibit biogenesis of the mitochondria as well as increasing the rate of inflammation and or mutagenesis. Studying the OCR relative to the baseline of each treatment also provides information on how the cells respond to particular pharmacological inhibitors as part of a stress test. These metabolic events are detected by the Seahorse XF Analyzer monitoring the changes in oxygen and proton concentrations in the extracellular media. It consists of a 24 well cell culture plate where cells are seeded and a sensor cartridge that is placed on

top of the plate. The cartridge, preincubated overnight or for a minimum of 4 hours with the calibrant solution at 37 °C without CO₂, is inserted first in the instrument to allow the calibration of the probes. At the end of the calibration the cell culture plate is inserted as well after 30 minutes to 1 hour of incubation at 37 °C without CO₂. The cartridge has an individual sensor for each well. Two different fluorophore probes are embedded in polymers at the bottom of each sensor, one quenchable by oxygen and the other one by protons (H⁺). Inside the instrument, the sensor is lowered to 200 µm above the cell monolayer, creating a micro chamber, which allows the small changes in oxygen and proton concentrations to be detected almost immediately. The detection, based on the quenching chemistry, occurs upon stimulation of the fluorophores embedded in polymers at the bottom of each sensor by a light diffused by optic fibres that are inserted into each sensor sleeve during the measurements. The measurements can be repeated several times and the oxygen consumption rate (OCR) detected and calculated.

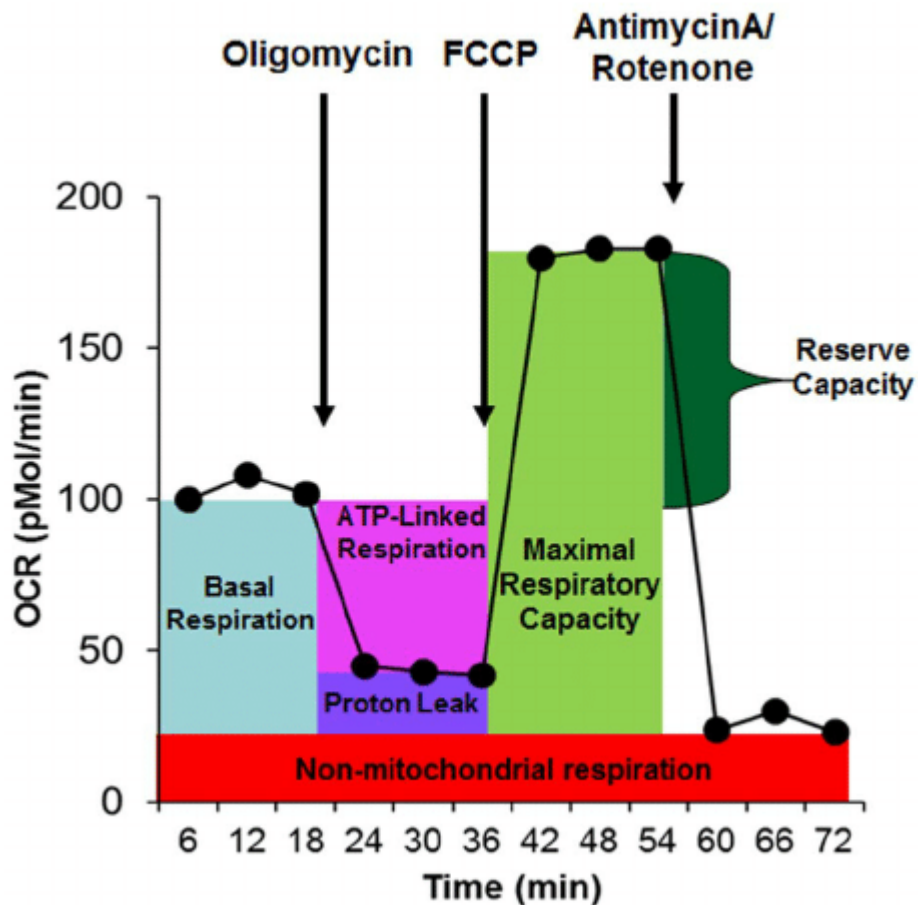


Figure 9: Seahorse assay: The oxygen consumption rate (OCR) is measured in cells undergoing respiration prior to and/or after addition of inhibitors. OCR measurement is taken every 8 minutes. To ensure reliability of measurements, an average is obtained from about 3-4 readings. The first measurement represents the basal respiration which records the cellular OCR prior addition of inhibitors. Then, Oligomycin, an inhibitor of complex V or ATP synthase is added in order to obtain OCR linked to ATP respiration (Baseline OCR – Oligomycin rate) or respiration due to proton leak (Oligomycin rate – non mitochondrial respiration). Next, the addition of FCCP is undertaken 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, antimycin (inhibitor of complex III) and rotenone (inhibitor of complex I) are added 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.

2.2.5.1 Cell culture for seahorse assay

Chubs-S7 and primary adipocytes were first cultured by seeding 75 000 cells in T-75 flasks. After confluence was reached, cells were trypsinized and seeded in 24-well (XF-24) seahorse plates at a cell density of 10,000 cells/well 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 and placed under 37°C incubation with 5% CO₂ saturation. The respective B12-media were changed every 48-hours until reaching almost 90-100%.

2.2.5.2 Seahorse inhibitors

After optimisation for 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, the optimum 10x concentrations of 4.0 µM, 7.5 µM and 4.0 µM for oligomycin, FCCP and rotenone/antimycin-A respectively were chosen. 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 injection into seahorse XF-24 plates. The inhibitors were loaded into the allocated ports of the seahorse cartridge which was initially hydrated in 1ml calibrant for 24 hours at 37°C without CO₂ saturation.

2.2.5.3 Sample preparation and seahorse assay

On the day of the Seahorse XF experiment, Chubs-S7 and primary adipocytes were washed in KHB buffer by removing 200 µl EMEM media from the cells in seahorse plates, followed by addition of 1 ml of KHB. Then, 950 µl of the KHB in 24-well seahorse plates was removed, followed by a final addition of another 675 µl of KHB to the wells of the plate. The plates were then incubated at 37°C for up to one hour without CO₂ saturation. To run the seahorse assay, the cartridge and the seahorse XF24 plate were loaded into the seahorse analyser for measurement of oxygen consumption rates (OCRs) as previously described.

2.3 Human adipose tissue study

2.3.1 Subjects and study design

106 Caucasian pregnant women undergoing elective caesarean section at the University Hospital Coventry and Warwickshire (UHCW) were enrolled for this study. Inclusion criteria: non-smoking, non-diabetic, premenopausal. Ranges of age and BMI were 25 to 38 years and 21.7 to 41 kg/m², respectively. The Ethics Committee of UHCW provided ethical approval for the collection human adipose tissue and issued the following approval number: SK06/9309. All the participants provided written and informed consent in accordance with the Declaration of Helsinki. Anthropometric measures: Height and weight, were measured in each subject and blood was drawn before the caesarean section. Biochemical values were measured by immunoassay at George Eliot Hospital, Nuneaton.

2.3.2 Adipose tissue collection

WAT was collected exclusively from non-smoking, non-diabetic, premenopausal, Caucasian women undergoing elective caesarean section at University Hospitals Coventry and Warwickshire (UHCW). Paired abdominal ScWAT and OmWAT was collected. Lean was considered pre-pregnant body mass index (BMI) of less than 25.0 kg/m² and obese was considered pre-pregnant BMI over 30.0 kg/m².

2.3.3 Serum cytokine detection with ELISA technique.

2.3.3.1 Quantikine ELISA Human CCL2/MCP1

MCP-1 serum levels of 106 women enrolled for this study was measured by immunosorbent assay (ELISA, R&D system, Minneapolis, USA) according to the manufacturer's instructions. Standard, control and sample (200 µl) were added to each well in duplicate and incubated for 2 hours at room temperature. After the incubation each well was washed 3 times with 400 µl of washing buffer. Subsequently, 200 µl of human MCP-1 conjugate was added in each well and incubated for 2 hours at room temperature. Washing procedure was repeated for 3 times and 200 µl of substrate solution was added in each well. An incubation of 30 minutes protected by light was followed. Finally, 50 µl of stop solution was added and the optical density of each well was determined using a microplate reader at 450 nm.

2.3.3.2 Quantikine ELISA Human IL-8/CXCL8

IL-8 serum levels of 106 women enrolled for this study was measured by immunosorbent assay (ELISA, R&D system, Minneapolis, USA) according to the manufacturer's instructions. Standard, control and sample (50 µl) were added to each well in duplicate and incubated for 2 hours at room temperature. After the incubation each well was washed 3 times with 400 µl of washing buffer. Subsequently, 100 µl of human IL-8 conjugate was added in each well and incubated for 1 hour at room temperature. The washing procedure was repeated for 3 times and 200 µl of substrate solution was added in each well. An incubation of 30 minutes protected by light was followed. Finally, 50 µl of stop solution was added and the optical density of each well was determined using a microplate reader at 450 nm.

2.4 Statistical Analysis

Data on adipocytes and adipose tissue were expressed as mean + standard error of mean (SEM). Whereas, data for seminal fluid analysis, was expressed as mean \pm SD and as median (interquartile range, IQR) for normally and non-normally distributed parameters, respectively. All statistical analyses were performed on SPSS 24.0 for Windows (Statistical Package for the Social Sciences, Chicago, USA) software or Prism 8 (GraphPad, San Diego, USA). Kolmogoroff–Smirnov's and Shapiro-Wilk test were used to determine the parametric distribution of data. Data obtained from the scintillation count of the radioactive flux assay were normalized with the total protein concentration (mg) in each sample (n) of Chubs-S7 and adipocytes to alleviate possible variations that might misrepresent the data. B12 and holoTC data and most of the seminal parameters had a non-normal distribution, while BMI, weight, and waist circumference were normally distributed. The Mann–Whitney U test was used for comparing two groups of non-normally distributed data, while the two-tailed Student's t-test was applied for comparison of normally distributed data. Correlations were assessed using Spearman's method for non-normally distributed parameters. ROC analysis was performed to evaluate accuracy, as well as sensitivity and specificity of the assessment of semen and serum holoTC and CBL concentrations in predicting semen quality. A $p < 0.05$ value was used for statistical significance.

Chapter 3: Vitamin B12 receptors and transporters

3.1 Introduction

The absorption, transport and metabolism of B12 involve a complex gastrointestinal and cellular process that require several proteins and cellular receptors. Defects at any stage of this process have been found to lead to B12 deficiency in patients and severe developmental defects (Alam *et al.*, 2016a). Cellular uptake of B12 via endocytosis is facilitated by a complex process involving transcobalamin II (TC), a plasma protein that binds B12, and a cell surface receptor, CD320, that specifically binds TC saturated with B12 (Quadros & Sequeira, 2013). CD320 presents a high-affinity for the complex TC-B12 and internalize it by endocytosis with the involvement of vesicles. In the endosome the TC-B12 complex is unbound from the receptor CD320 which is then recycled on the cell surface. Subsequently, the endosomes carrying the complex TC-B12 fuse to form lysosomes where TC is degraded by proteolysis, and B12 is exported into the cytoplasm of the cell (Jacobsen & Glushchenko, 2009).

Several studies explored the expression of CD320 and transporter (TCN2) that may regulate cellular uptake and storage of B12 in different tissues (Abuyaman *et al.*, 2017; Bernard *et al.*, 2018). The presence of TC/CD320 complex is highly expressed in kidney and liver acting as storage organs (Yamada *et al.*, 1991). Animal and human studies conducted on the cerebrovascular system showed a close relationship between B12 deficiency and increase risk in ischemic stroke events (Casas *et al.*, 2005; Spence, 2016) and it can be associated with neurodevelopmental and neuropsychiatric disorders such autism and schizophrenia (Zhang *et al.*, 2016). A high CD320 expression has been observed in the placenta allowing therefore to compensate any possible lack of B12 in the foetus crossing the placental barrier (Bose *et al.*, 1995). On the other hand, low maternal B12 status in early pregnancy is associated with major foetus complications such a neural tube defects, (Tang *et al.*, 2015) low birth weight (Sukumar *et al.*, 2016a) and preterm birth (Rogne *et al.*, 2017). The presence of both TC and its receptor has been widely reported to be expressed in highly proliferating cells and upregulated in many forms of cancers (Alam *et al.*, 2016a; Cho *et al.*, 2008). Animal and clinical studies have been shown in fact, that mammalian carcinoma cells show an up-regulation in B12 metabolic pathway and increased cellular uptake (Collins, 2019; Woolley, 1953). These results were confirmed also by experiments conducted on adenocarcinoma cells Caco-2 (Ramanujam *et al.*, 1991).

Population studies have shown that maternal B12 deficiency induces major health complications in their offspring (Bellows *et al.*, 2017; Lee *et al.*, 2018; Mittal *et al.*, 2017). In the Pune Maternal Nutrition Study, mothers with low B12 and high folate are associated with risk factors for the development of obesity and IR in the children at the age of 6 (Yajnik *et al.*, 2008), suggesting an important role of B12 in foetal programming. Moreover animal studies on Wistar rats showed that offspring from B12 deficient mothers had higher adiposity and altered lipid metabolism and hypothesised that this could be due to dysfunctional adipocytes (Kumar *et al.*, 2013). More recently, *in vitro* studies on human pre-adipocyte cell line showed that B12 deficiency lead to increased expression of genes involved in adipogenesis and lipogenesis and an aberrant expression of miRs involved in IR (Adaikalakoteswari *et al.*, 2017), showing therefore the potential epigenetic role of B12 in the AT metabolism.

Given therefore the possible role of B12 involvement in the adipose metabolism and in the development of metabolic diseases, the investigation of the expression of B12 transporter TCN2 and B12 receptor CD320 and intracellular B12 levels in human adipocytes is required.

As discussed in the introduction chapter, B12 appears to have tissue specific actions, especially with predilection towards red blood cells (RBCs) and neuronal cells. Previous studies have shown that rapidly dividing cells such as RBCs and neurons are more sensitive to drop in B12 levels resulting in anaemia and neuronal dysfunction (Mollin & Ross, 1953; Nuru *et al.*, 2018). However, not many studies have looked in detail of B12's tissue specific actions in other tissues such as adipocytes, hepatocytes and muscle cells. Similarly, although extreme B12 deficiency has also been associated with sub-fertility (Najafipour *et al.*, 2017), the exact mechanisms are not been studied.

Our group's previous studies show that adipocyte B12 potentially plays a major role in metabolic functions (Adaikalakoteswari *et al.*, 2017). Tissue levels rely on circulating levels of B12. However, data from the maternal-cord blood levels show that there might be active transport of B12 from the mother to the offspring through the placenta. These studies consistently show that cord blood B12 levels are higher than maternal levels (Molloy *et al.*, 2008). Whether such process happens in the adipocytes is not known. It is also not known what happens to the B12 receptors and transporters in the membrane of the AT. There is no data that has elucidated the role of the CD320 and TCN2 with intracellular levels of B12 in adipocytes.

- (1) Therefore, the aim of this study is to investigate and understand the relationship between the circulating B12 levels, the intracellular B12 levels and the gene expression of CD320 and TCN2 in *in vitro* human adipocyte cell line
- (2) Endorse these findings in cultures of human primary pre-adipocytes taken from subjects with different degrees of obesity and
- (3) Endorse these findings in mature AT from different depots of human subjects such as omental and subcutaneous.

3.2 Results

3.2.1 Assessment of Intracellular B12 levels in Chubs-S7 cells

Methods

In order to discover the cellular B12 uptake efficiency, Chub-S7 cell line pre-adipocytes were seeded at low density (30,000 cells/well in 6-well plate) and treated with six different concentration of B12 as described in session 2.2.1.1. On day 14 the conditioned media were collected, and cells were harvested with 250 μ l PBS after a double wash with cold PBS. Intracellular and fresh media B12 was then measured in George Eliot Hospital, Nuneaton using a competitive electro-chemiluminescence immunoassay.

Results

As seen in table 1 and fig 10 adipocytes cultured in low B12 (25pM and 100pM) showed 167-271% higher intracellular levels of B12 concentrations. Increasing concentrations of B12 (1nM, 10nM, 100nM, and 500nM) resulted in progressive reduction of intracellular uptake of B12 to 2.5%, 3.9%, 1.6%, and 1.3%, respectively. However, the intracellular levels are still higher than the normal physiological levels seen in humans.

B12 concentrations (pM)	Fresh media (pM) (Mean\pm SD)	Intracellular B12 (pM) (Mean\pm SD)	% intake
25	65 \pm 1.9	177 \pm 12.4	271
100	135 \pm 6.0	225 \pm 68.7	167
1000	7383 \pm 353.2	189 \pm 39.5	2.5
10000	19099 \pm 73	747 \pm 116.3	3.9
100000	95394 \pm 2349.2	1548 \pm 249.6	1.6
500000	1233198 \pm 4135	16305 \pm 1615.7	1.3

Table 1: Intracellular and extracellular levels of B12 in Chubs-S7

Cells are treated with six different concentrations of B12 as reported in the table. Fresh media and intracellular B12 concentrations are represented in pM.

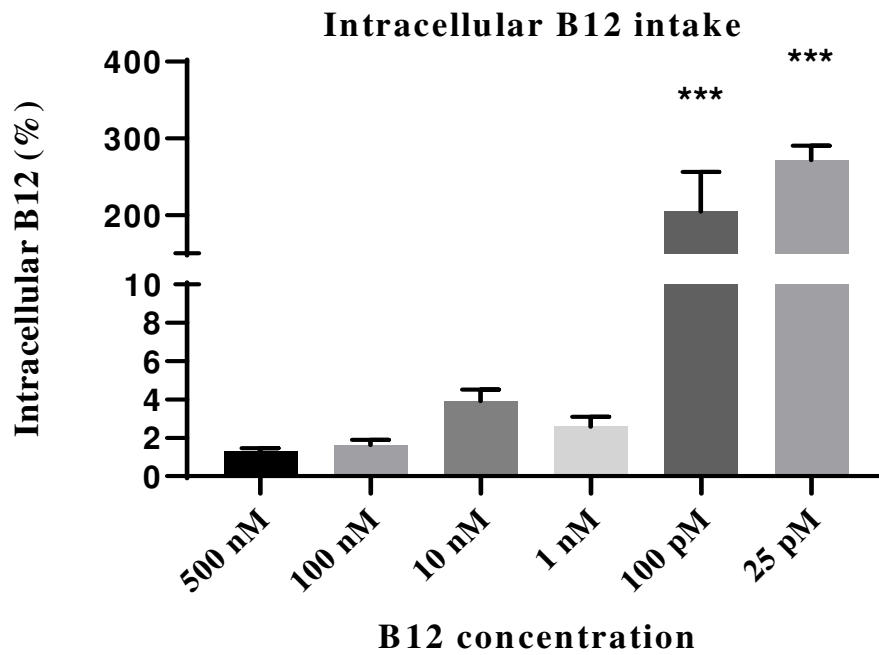


Figure 10: Intracellular vitamin B12 intake is increased in low B12 treated cells: Chus-S7 cells were treated with six different concentrations of B12 from seeding to complete differentiation, and intracellular level of B12 was measured with a competitive electro-chemiluminescence immunoassay. Data are expressed as mean \pm SD and significance levels are indicated as follows; ***P < 0.0001 according to multiple comparison one-way ANOVA comparing against the control (500 nM).

3.2.2 Assessment of gene expression of B12 transporter TCN2 and receptor CD320 in Chubs-S7

Methods

In order to assess the effect of B12 deficiency on the gene expression of CD320 receptor and B12 transporter (TCN2) in Chubs-S7 cells, RT-qPCR assay was performed. Adipogenesis was induced in Chub-S7 preadipocytes as described in session 2.2.1.1 and gene expression was determined by RT-qPCR.

Results

As seen in figure 11 we observed a significant increase in the mRNA expression of CD320 in adipocytes treated with low (25 pM, 1nM) B12 conditions ($p < 0.01$) compared with the control. An overall trend towards increase has been observed in other concentrations of B12 but not significant. Similarly, mRNA expression of TCN2 was increased in low B12 adipocytes compared to the control (figure 11B).

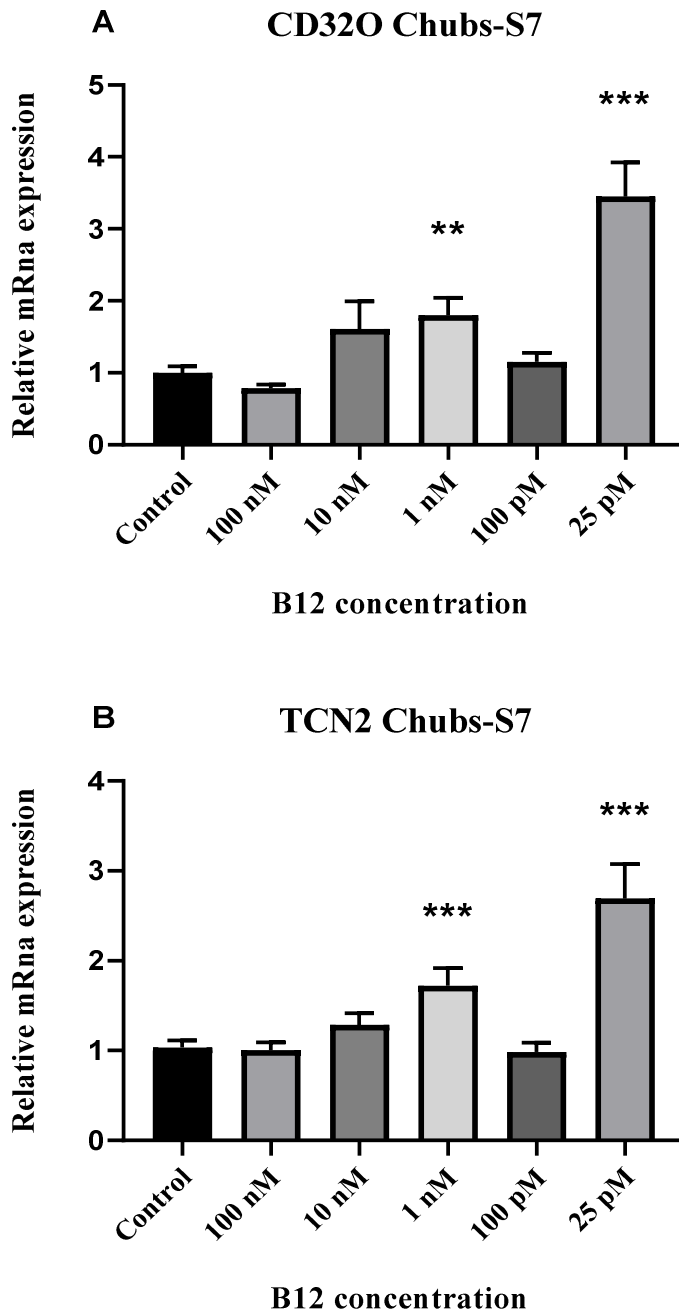


Figure 11: Effect of B12 deficiency on the gene expression receptor CD320 (A) and transporter transcobalamin TCN2 (B) in Chus-S7 cells.

Chus-S7 adipocytes were seeded and differentiated with 6 different concentrations of B12 and mRNA expression was measured by RT-qPCR. Control= 500nM. Data are expressed as mean \pm SEM and significance levels are indicated as follows; **P<0.01, ***P <0.001 compared to control, according to Mann-Whitney U' test.

3.2.3 Gene expression of B12 receptor CD320 and transporter TCN2 in human primary adipocytes with different degrees of obesity

Methods

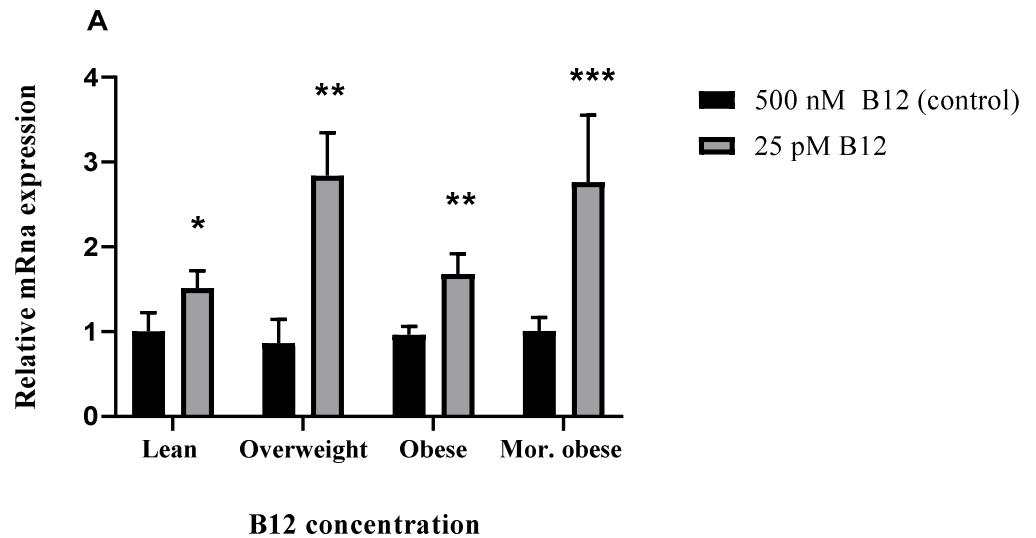
To understand the effect of B12 deficiency in different categories of BMI, human pre-adipocytes were derived from lean, overweight, obese and morbidly obese subjects (three cultures per BMI group) and cultured in control and low B12 levels. For details please refer to chapter 2.2.1.2.

Results

Our findings revealed, as shown in figure 12 A, that the relative mRNA expression of receptor CD320 significantly increased in adipocytes derived from all the categories of BMI in low concentrations of B12 (25pM) compared to control cells (500 nM treatment).

A similar trend was observed for the mRNA expression of TCN2 transporter, showing a significant increase in B12 deficient adipocytes from overweight, obese and morbidly obese patients (figure 12 B). Lean B12 deficient adipocytes showed a nonsignificant increase. These results are in line with previous findings in Chub-S7 adipocytes.

CD320 in human pre-adipocytes cultured in control and low b12



TCN2 in human pre-adipocytes cultured in control and low b12

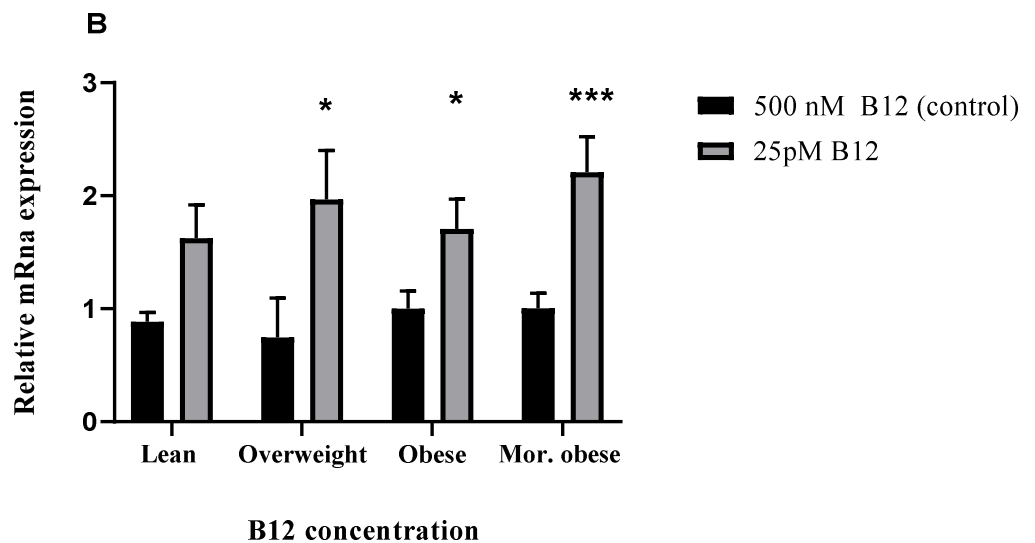


Figure 12: Effect of B12 deficiency on the gene expression receptor CD320 (A) and transporter transcobalamin TCN2 (B) in primary adipocytes derived from four categories of BMI.

Primary adipocytes derived for lean, overweight, obese and morbidly obese patients, where seeded and differentiated with 2 different concentrations of B12 (three cultures per BMI group) and mRNA expression was measured by RT-qPCR. Control= 500 nM. Data are expressed as mean \pm SEM and significance levels are indicated as follows; * $P < 0.05$, *** $p < 0.001$ compared to control (500nm), according to Mann-Whitney test.

3.2.4 Gene expression of B12 receptor CD320 and transporter TCN2 in human omental and subcutaneous adipose tissue

Methods

In order to further validate the extent of the data obtained in Chubs-S7 cell line and human primary adipocytes with mature human AT, we assessed the gene expression of CD320 and TCN2 on OmWAT and ScWAT from 106 pregnant women who underwent c-section. After a blood analysis test, patient's AT were divided into two groups according to their B12 values (<200pg/mL and >200pg/mL) and gene expression assay was undertaken.

Results

Our findings revealed that, as shown also in Chub-S7 and primary adipocytes, the relative mRNA expression of both CD320 receptor and TCN2 transporter were significantly increased ($p<0.05$) in both OmWAT and ScWAT of B12 deficient patients compared with patients with adequate levels of B12 (figure 13).

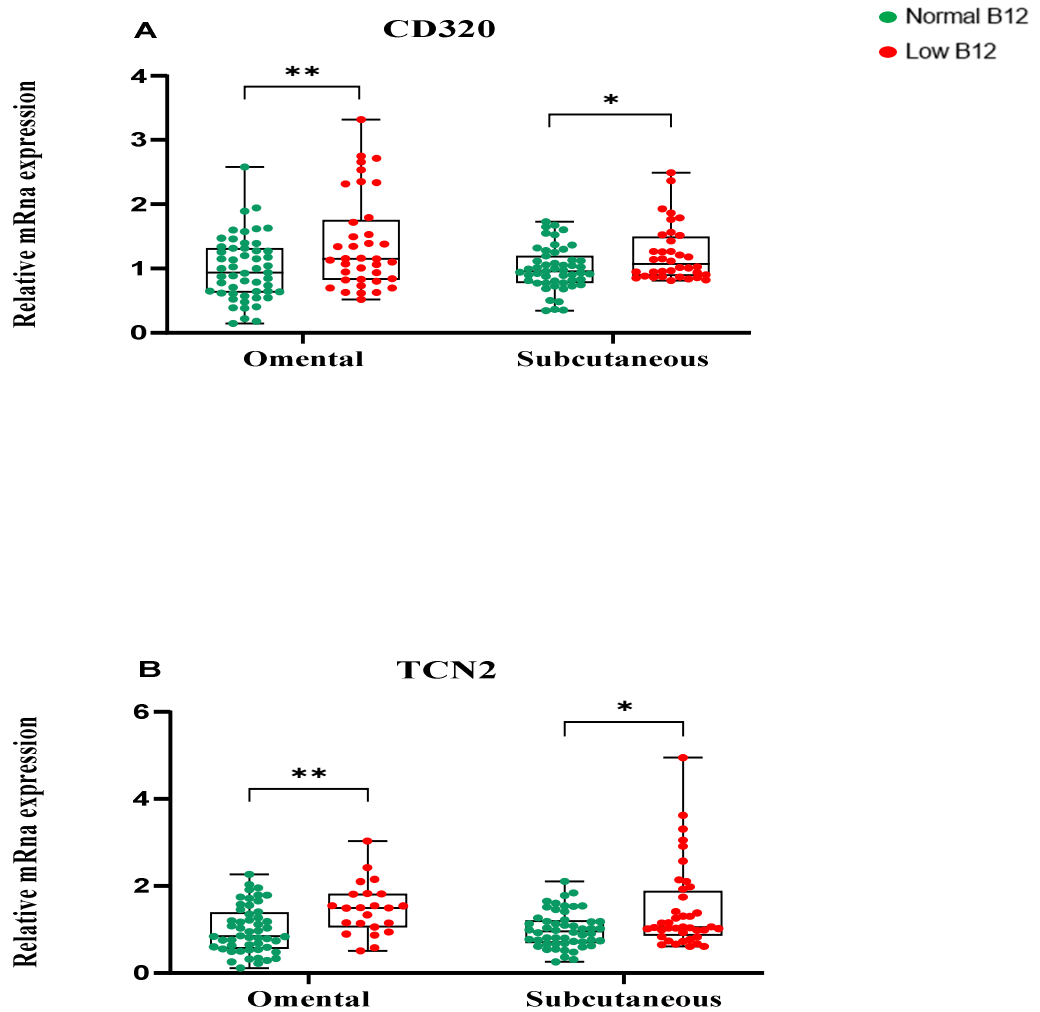


Figure 13: Effect of B12 deficiency on the gene expression receptor CD320 (A) and transporter transcobalamin TCN2 (B) in omental and subcutaneous adipose tissue.

OmWAT and ScWAT obtained from 106 pregnant women were used to assess gene expression of both CD320 and TCN2 through RT-qPCR. In order to understand the effect of B12 deficiency on these two genes, patients were divided into 2 groups according to their vitamin B12 levels [Normal B12=60 (green dots) and Low B12=46 (red dots)] and gene expression assay was assessed. Data are not-normally distributed and reported as box charts for TCN2 (A) and CD320 (B); p derived from Mann-Whitney U-test is indicated. Normal B12 (200pg/ml) low B12 (<200pg/ml).

3.2.5 Correlation between gene expression of omental and subcutaneous TCN2 and CD320 with biochemical parameters

Table 2 shows the clinical characteristics of the cohort based on the vitamin B12. There are no significant differences in mean age and weight between the two groups but a significant difference has been shown in BMI ($p= 0.041$). HDL-cholesterol was lower in the B12 deficient group ($p=0.0002$) and TAGs were higher ($p=0.0094$) compared with the non-deficient group. Serum folate was significantly lower in the B12 deficient group ($p=0.0009$).

The correlation analysis revealed the relationship between biochemical values and B12 transporter TCN2 and receptor CD320 gene expression. In the ScWAT, the correlation analysis showed a significant and positive association between TCN2 gene expression and LDL ($r= 0.208$, $p= 0.041$) and cholesterol ($r= 0.205$, $p= 0.039$) serum levels (figure 14). ScWAT B12 receptor CD320 gene expression showed a negative correlation with B12 ($r=-0.210$, $p=0.033$) serum levels and negative correlation with HDL ($r= 0.209$, $p= 0.036$) and positive correlation with glucose ($r= 0.205$, $p= 0.040$) (figure 15).

Moreover, in the OmWAT, as shown in figure 16, TCN2 positively correlated with BMI ($r=0.209$, $p=0.027$) and B12 receptor CD320 gene expression negatively correlated with B12 ($r= -0.268$, $p= 0.004$).

Variables	B12 non-deficient group (>200pg/ml) n=63	B12 deficient group (<200pg/ml) n=52	P-value
Age (yrs) [mean±SD]	32.1±5.7	31.5±6.5	0.296
Weight (kg) [mean±SD]	73.2±14.6	75.6±19.4	0.708
BMI (kg/m ²) [mean±SD]	26.5±3.9	28.5±6.37	0.041
Glucose (mmol/l) [mean±SD]	3.68±0.6	3.71±0.6	0.787
Cholesterol (mmol/l) [mean±SD]	6.6±1.4	6.7±1.5	0.947
HDL (mmol/l) [mean±SD]	1.7±0.4	1.4±0.2	0.0002
LDL (mmol/l) [mean±SD]	4.3±1.2	4.8±1.6	0.722
Triglycerides (mmol/l) [mean±SD]	2.9±0.9	3.5±1.0	0.0094
Folate (ng/ml) [mean±SD]	9.9±5.4	6.6±4.3	0.0009

Table 2 Clinical and biochemical characteristics according vitamin B12 values.

P-value from Mann-Whitney U-test. Abbreviations: SD, standard deviation; BMI, body mass index; HDL, high density lipoprotein; LDL, low-density lipoprotein;

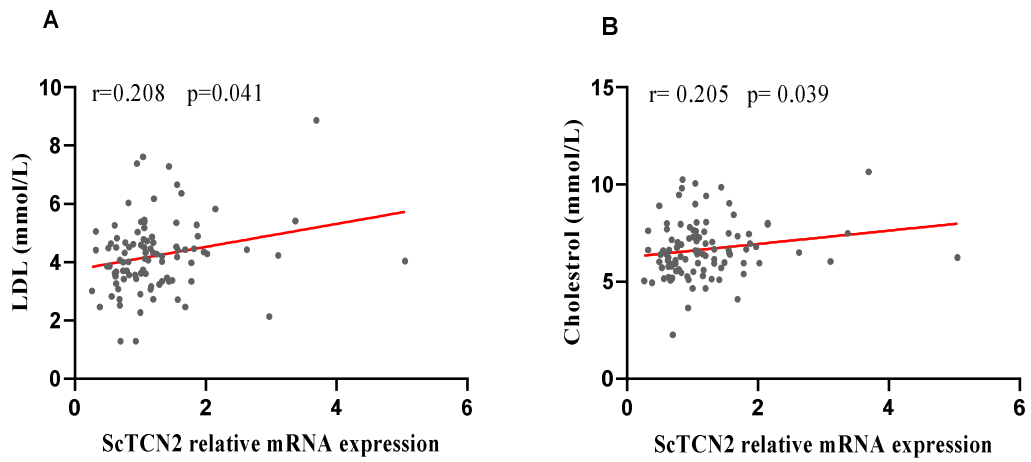


Figure 14: Positive correlation between TCN2 gene expression and LDL (A) and Cholesterol (B) in subcutaneous adipose tissue. Correlation analysis for all subjects was undertaken between the gene expression of ScTCN2 and metabolic-related biochemical values. ScTCN2 and LDL (A), scTCN2 and cholesterol (B). Correlations were evaluated by Spearman's test for not-normally distributed data in univariate analysis: r and p from linear regression are indicated.

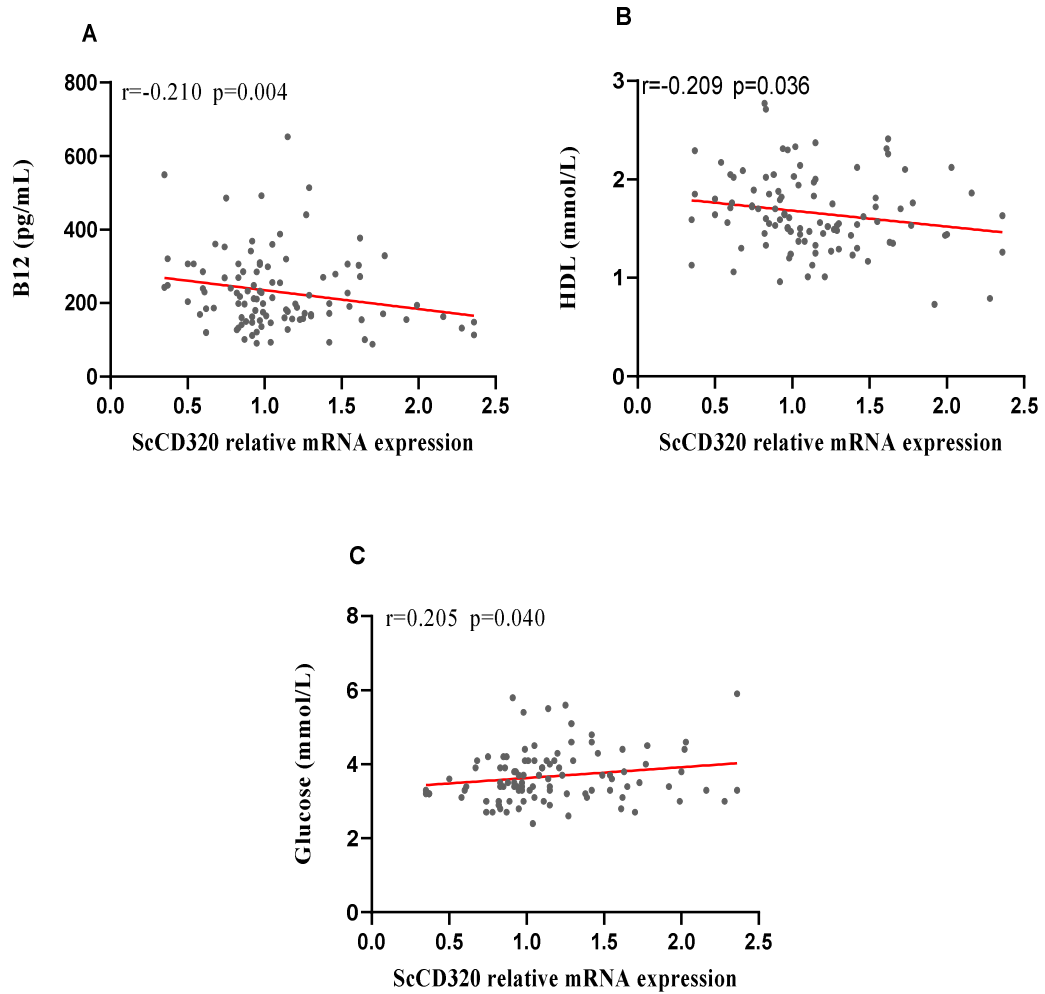


Figure 15: Subcutaneous AT CD320 gene expression and B12, HDL and Glucose correlate with serum B12 Correlation between ScCD320 and serum vitamin B12, HDL and Glucose. Dots depict individual study subjects and data are presented as Spearman's R values: r and p from linear regression are indicated. HDL: High density lipoprotein.

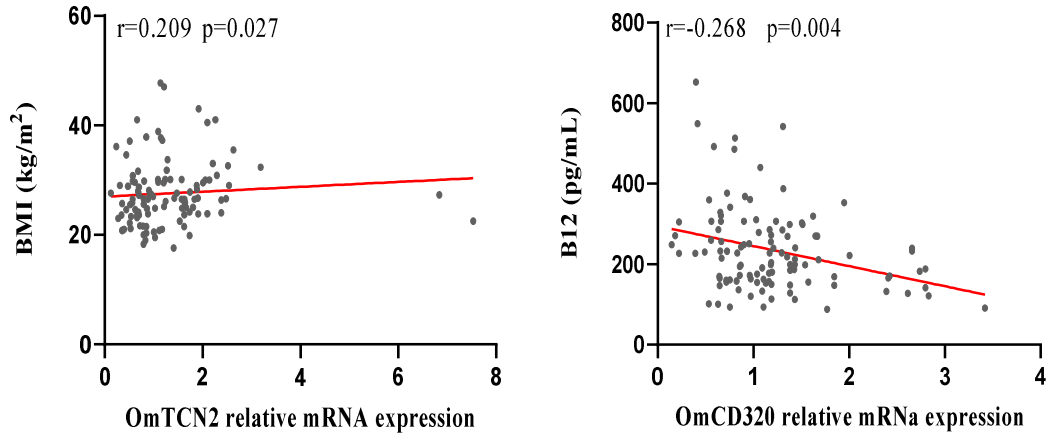


Figure 16: Correlation of omental AT TCN2 and CD320 gene expression with BMI and B12
 Correlation of Om TCN2 and OmCD320 and BMI serum vitamin B12, HDL and Glucose. Dots depict individual study subjects and data are presented as Spearman's R values: r and p from linear regression are indicated.

3.3 Discussion

The purpose of this study was to examine the relationship between the circulating B12 levels, the intracellular B12 levels and the gene expression of CD320 and TCN2 in *in vitro* human cell line adipocytes, in human primary adipocyte derived from different metabolic phenotypes cell and in mature AT of human subjects obtained from different depots such as OmWAT and ScWAT. In this study, 1) we found that human pre-adipocytes (Chubs-S7) in decreasing extracellular B12 media concentrations resulted in progressively increased intracellular B12 uptake; 2) Gene expression of B12 receptor CD320 and transporter TCN2 increased in decreasing extracellular B12 media concentrations. Human adipocytes from different degrees of obesity when cultured in low levels of B12 and mature human AT from different depots such as OmWAT and ScWAT from B12 deficient subjects demonstrated increased levels of gene expression of CD320 and TCN2. 3) B12 negatively associated with ScCD320 and OmCD320 gene expression, which in turn could be associated with metabolic risk variables such as HDL, LDL, cholesterol and glucose.

The cellular uptake of B12 bound to TC endocytosis is mediated by the transcobalamin receptor CD320 (Bloch *et al.*, 2017; Seetharam & Li, 2000). Recent studies on knockout mice and on human placenta proposed endocytosis, tranicytosis and exocytosis as important alternative mechanisms for the transport and transfer of B12 from the mother to the foetus (Arora *et al.*, 2017; Schneider & Miller, 2010). In this study, we measured the uptake of B12 in Chubs-S7 cultured in six different B12 media. Adipocytes cultured in B12 deficient media such as 100 pM- 25 pM showed 150-300% increase in intracellular uptake, whereas increasing concentrations of B12 media to 100-500 nM resulted in a progressive reduction of intracellular uptake to 1.6 -1.3%. Our result is in line with a recent research study on rats showing an increase in B12 endogenous ratio (tissue/plasma) in several tissues, although AT was not studied (Kornerup *et al.*, 2018). Similarly, a study conducted on spinal cord astrocytes and oligodendrocytes derived from B12 deficient rat showed a decreased B12 content in the medium after 24 h of incubation and increased B12 cellular uptake (Buccellato *et al.*, 2004; Quadros & Sequeira, 2013). Another study showed higher TC levels in cord blood compared with maternal blood suggesting the increasing demand of the micronutrient for the foetus (Obeid *et al.*, 2006). These evidences therefore support our findings indicating that increased intracellular accumulation in B12 deficient adipocytes may be due to active transport of B12 from the

circulation through these receptors and transporters leading to intracellular accumulation within adipocytes.

Furthermore, we investigated the gene expression of both B12 receptor and transporter under different B12 conditions. Surprisingly, in this study, increased gene expression of both TCN2 and CD320 has been observed in both cell lines and primary adipocytes under low circulating B12 concentrations (100 pM, 25 pM). A similar result was obtained in primary adipocytes derived from all categories of BMI and, in the same way, AT of B12 deficient patients showed significantly increased levels of gene expression compared to non-deficient patients. These results may suggest that the increased gene expression of both B12 transporter and receptor in adipocytes under low circulating B12 levels and in AT derived from B12 deficient patients may be an attempt to facilitate the cellular uptake of B12 as a consequence of B12 deficiency. The increased demand of B12 by the foetus growth during pregnancy could lead to a development of B12 deficiency in the mother. In that regard, a recent study showed in fact, a progressive increase in serum levels of CD320 receptor up to 35 weeks of gestation (Abuyaman *et al.*, 2013), in agreement with our results. High serum CD320 receptors in pregnancy might be derived from the placenta where higher expression of the receptor has been shown (Quadros *et al.*, 2009). 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 (Pannerec *et al.*, 2018), were increased in aged humans presenting B12 deficiency (Pannerec *et al.*, 2018). In the same study, a negative association was observed between serum AMN and B12 levels in rats (Pannerec *et al.*, 2018). In support to our findings earlier studies in hepatocytes showed that alterations in the number of cellular receptors rather than affinity is the result of the cellular uptake of B12 (Hall *et al.*, 1987). An increase expression of TCN2 has been observed also in xenograft tumour tissues (Sysel *et al.*, 2013) and in maternal cord blood suggesting a potential role in vitamin B12 transport to the foetus (Layden *et al.*, 2016). Similarly, a clinical study shown that the level of total serum transcobalamin (TC) following B12 supplementation was observed to be higher in low B12 patients (Arendt, 2012). Our results are supported by studies on human and rat tissue where the expression of transcobalamin has been detected (Li *et al.*, 1994). In support to these studies, 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.

Studies in the last decade have confirmed the importance of B12 for the foetal health. Maternal B12 deficiency can lead in fact to an increased risk of development of a series of neurological and metabolic pathologies. Recently genetic studies on B12 status have suggested its deficiency as a multifactorial trait, characterized by single nucleotide polymorphisms in genes involved in its metabolic pathway (Surendran *et al.*, 2018) and a study conducted on Sri Lankan population have suggested an impact of genetically lowered B12 on central obesity (Surendran *et al.*, 2019a). To the best of our knowledge no study so far has investigated the relationship between B12 transporter and receptor and metabolic related biochemical values and in that regard, and in line with these notions, we evaluated the association of serum laboratory values with vitamin B12 transporter and receptor gene expression in our cohort study .

Correlation analysis showed a negative association between Sc320 and B12 levels and same trend has been seen also in the Omental compartment (negative association between OmCD320 and B12 levels), in accordance with the studies (Pannerec *et al.*, 2018)

Interestingly, the gene expression of both receptor and transporter are associated with an increase in lipid levels and glucose, in accordance with results obtained on B12 deficient diabetic patients (Adaikalakoteswari *et al.*, 2014a). Our results are also supported by a recent study on non-diabetic patients in which a negative correlation between B12 and LDL and a positive correlation with HDL has been found (Yılmaz Sezgin¹, 2018) although no correlation with genetic expression of receptor and transporter has been evaluated.

Our study thus provides novel evidence that when extracellular B12 levels are low (25 pM-100pM), the intracellular B12 levels and the gene expression of receptor and transporter are higher, whereas opposite effects are seen at higher extracellular B12 levels (1-500nM). This suggests active transport of B12 in adipocytes at low concentrations. In addition, there might be a threshold for the B12 entry at the membrane level in higher concentrations of B12. Our findings support that optimal physiological levels of B12 are required. This will open new avenues into the pathogenesis of maternal obesity and the relevance of micronutrient supplementation and strategies for therapeutics for pregnant mothers

Chapter 4: Vitamin B12 and *de novo* lipid synthesis

4.1 Introduction

White adipose tissue (WAT) is considered a long-term depot of energy storage in the form of TAGs. It is able to expand and contract during adult life providing the energy requirements of the body. TAG have the highest energy content among the reserve substances, more than 38 kJ/g. They are produced from two precursors (acyl-CoA and L-glycerol-3-phosphate) and are stored in WAT in single large cytoplasmic lipid droplets. In obesity, the energy input surpasses energy output, with most of the additional calories converted into TAGs and stored in the AT. When WAT capacity is exceeded there is ectopic fat accumulation which contribute to obesity-associated IR. Moreover, obesity is also attributed to adipocyte hypertrophy occurring when TAGs synthesis (esterification), exceeds TAG breakdown (lipolysis) resulting in dyslipidaemia (Smith & Kahn, 2016). The synthesis of FAs and TAGs is a coordinated process that involves multiple enzymes. Adipocytes contain the "machine" needed for lipid metabolism which is altered during overweight and obesity leading to development of diseases like T2DM, atherosclerosis and hypertension (Ahmadian *et al.*, 2007). It has been established that FAs not only function as energy but also act as regulatory signals of protein gene expression involved in lipid metabolism and are associated with inflammatory processes (Duplus *et al.*, 2000; Sheehan & Jensen, 2000).

Human longitudinal studies and animal models show that maternal B12 deficiency is associated with the maternal obesity, development of IR and MS phenotype. Studies show independent association of TAGs with B12 in Indian and Europeans patients with T2DM (Adaikalakoteswari *et al.*, 2014). A study conducted by Khaire *et al.* showed increased cholesterol synthesis in B12 deficient wistar rats (Khaire *et al.*, 2015). According to Spence, B12 deficiency was considered a contributor to stroke by increasing homocysteine levels and increasing the risk of carotid plaque formation (Spence, 2016). These studies suggest a potential role of this micronutrient in lipid metabolism which may lead to adipocyte dysfunction and development of metabolic diseases (Kumar *et al.*, 2013; Sukumar *et al.*, 2016b). These are association studies and does not prove causality. However, if this is true and the mechanisms are understood, it offers a potential way of reducing the burden of metabolic diseases by a simple intervention. We therefore hypothesise that B12 deficiency may alter *de novo* lipogenesis in adipocytes adversely to cause adipocyte dysfunction.

Therefore, the aim of this study was to investigate and understand:

- (1) The relationship between B12 and the processes involved in fatty acid synthesis in *in vitro* human adipocytes;
- (2) Endorse these findings in cultures of human primary pre-adipocytes;
- (3) Determine the effect of adipocytes taken from humans with different degrees of obesity and
- (4) Endorse these findings in mature AT from different depots of human subjects such as OmWAT and ScWAT.

4.2 Results

4.2.1 *In vitro* experiments

Effect of B12 on fatty acid biosynthesis genes in human Chubs-S7 and human primary adipocytes with different degrees of obesity

Methods

To investigate the expression of genes required for *de novo* lipogenesis under B12 deficiency conditions, Chubs-S7 and primary adipocytes were seeded at low density (30.000 cells/well in 6 well plates) and treated with different concentrations of B12 as mentioned in section 2.2.1.1.

4.2.1.1 Acetyl-CoA Carboxylase Alpha (ACACA)

Acetyl-CoA Carboxylase (ACC) is an enzyme which catalyses the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in FA synthesis (Fig. 17). There are two ACC forms, alpha and beta, encoded by two different genes. ACC-alpha is highly enriched in lipogenic tissues such as AT.

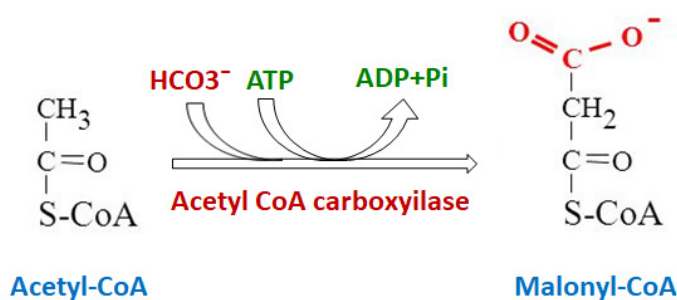


Figure 17: Catalytic action of Acetyl-CoA carboxylase

Figure 18 shows B12 deficiency has induced a significant upregulation of ACACA in Chub-S7 adipocytes compared to control. An overall increased ACACA expression was observed in B12 deficient adipocytes from primary cultures derived from different metabolic phenotypes, reaching statistical significance in morbid obese derived adipocytes.

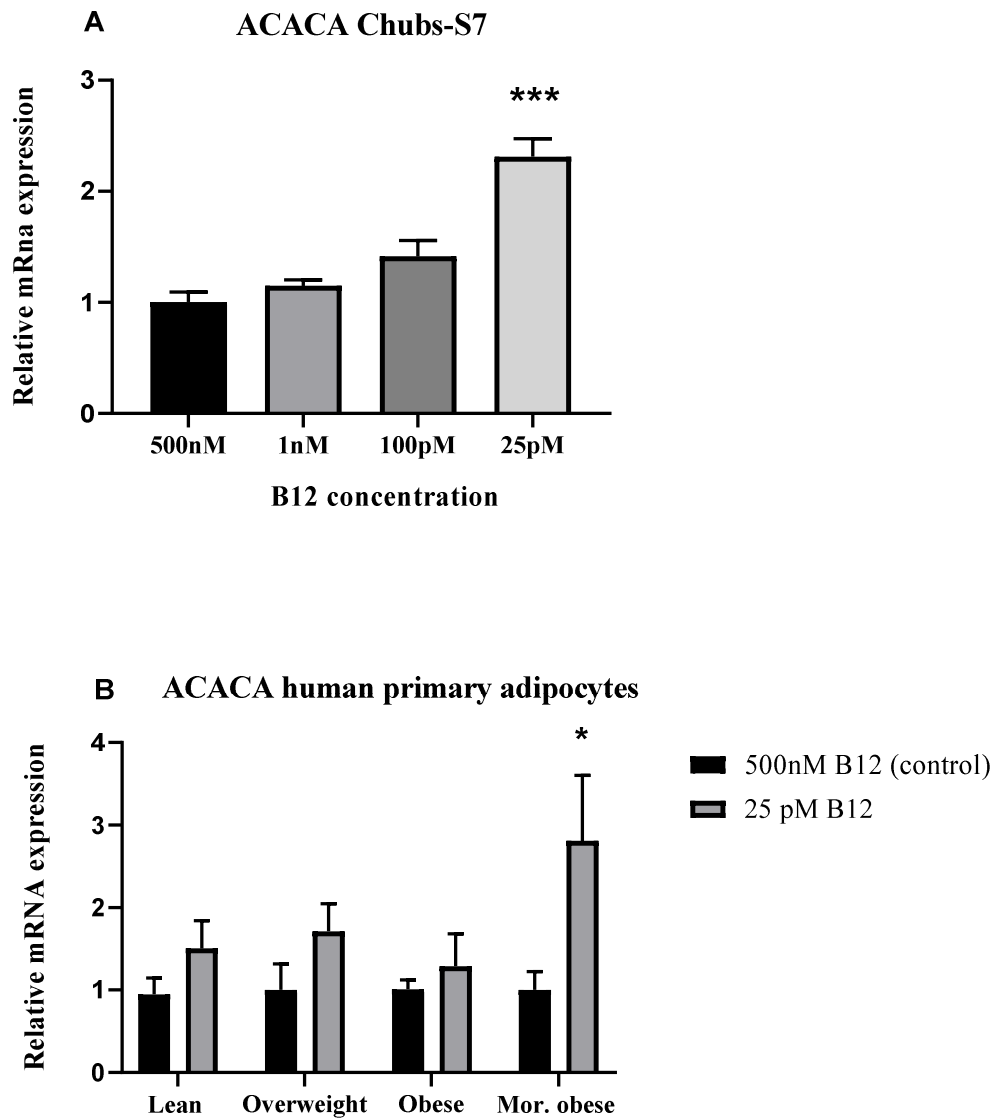


Figure 18: Gene expression of Acetyl-CoA Carboxylase alpha (ACACA) in human adipocytes:

The graphs show mRNA expression of ACACA in Chubs-S7 and primary adipocytes (three cultures per BMI group) derived from subcutaneous depots. The adipocytes were seeded and differentiated with different concentrations of B12 as indicated, and mRNA expression was measured by RT-qPCR. The mRNA levels were normalized to L19. Data are expressed as mean \pm SEM and significance levels are indicated as follows; * $p < 0.05$, *** $p < 0.001$ compared to control (500nM) according to Mann-Whitney U' test.

4.2.1.2 Fatty acid synthase (FASN)

Fatty acid synthase (FASN), is a multienzyme catalysing the synthesis of palmitate (16-carbon fatty acid) from acetyl-CoA and malonyl-CoA, in the presence of NADPH, (figure 19). FASN plays a role in the pathophysiology of human obesity and its related metabolic alterations.

As represented in figure 20 Chubs-S7 adipocytes cultured in low B12 condition (25 pM) showed a significant increase in the gene expression of FASN along with an overall trend towards increase with low concentrations of B12 (figure 20A). A general trend towards increase was found in B12 deficient primary adipocytes derived from different metabolic phenotypes and a statistical significance was reached in lean, overweight and morbid obese derived adipocytes (figure 20B).

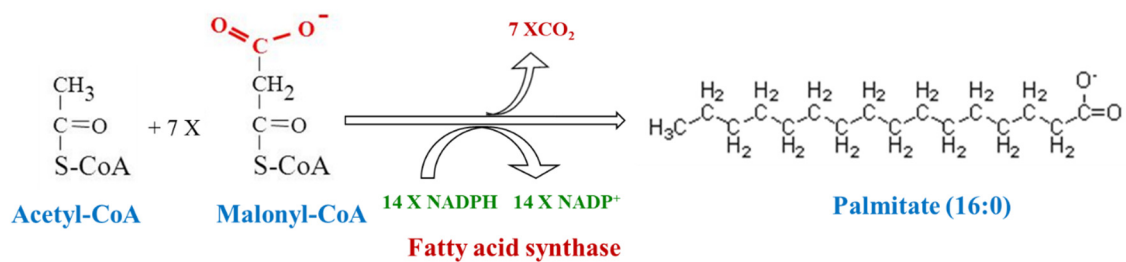


Figure 19: Catalytic action of Fatty acid synthase

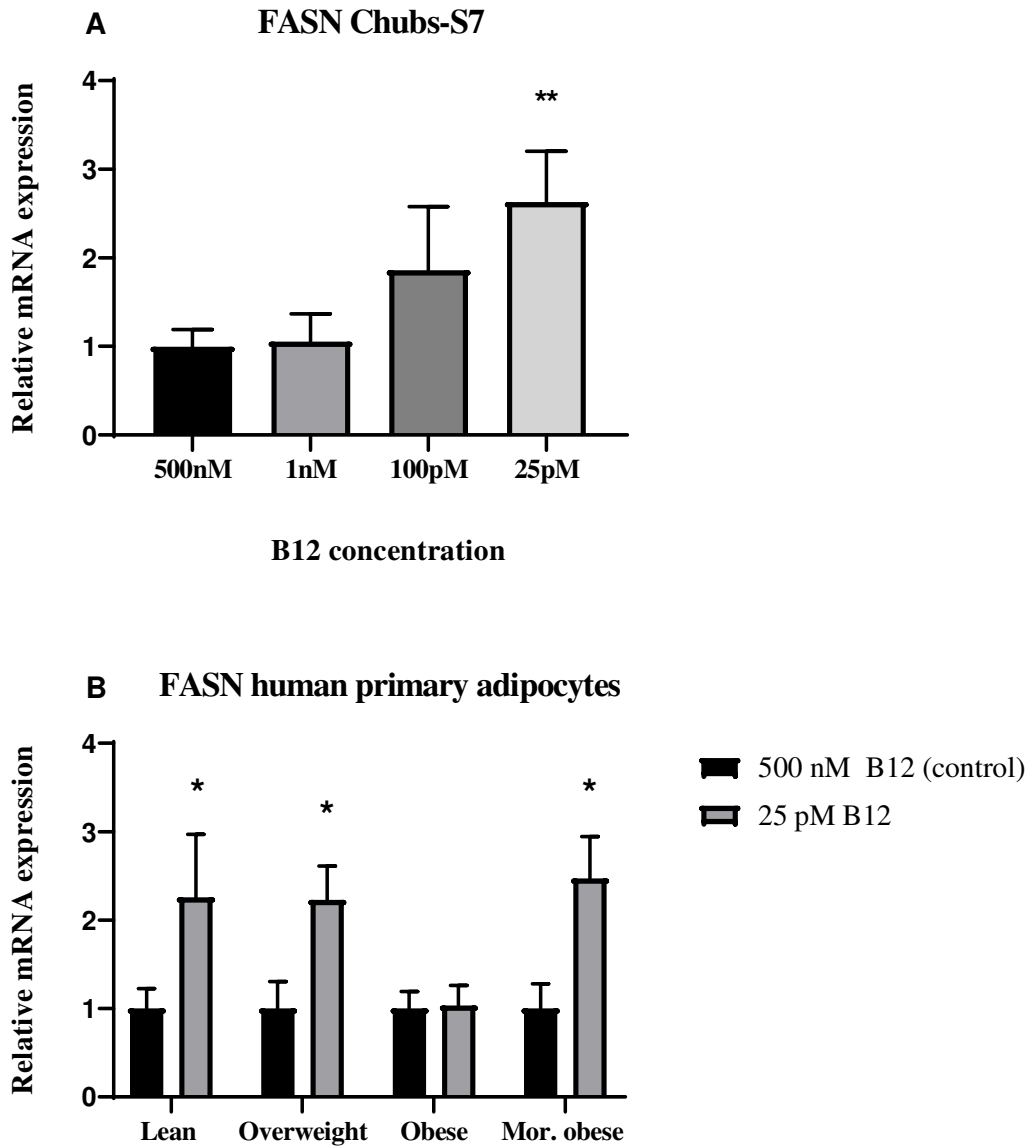


Figure 20: Gene expression of Fatty acid synthase (FASN) is increased in B12 deficient adipocytes: The graphs show mRNA expression of FASN in Chubs-S7 and primary adipocytes (three cultures per BMI group) derived from subcutaneous depots. The adipocytes were seeded and differentiated with different concentrations of B12, as indicated and mRNA expression was measured by RT-qPCR. Expression levels were normalised to L19 and bars represent the fold changes means \pm SEM and significance levels are indicated as follows; * $p < 0.05$, ** $p < 0.01$ compared to control (500nM) according to Mann-Whitney U' test.

4.2.1.3 ELOVL Family Member 6, Elongation of long chain fatty (ELOVL6)

Fatty acid elongase ELOVL6, is a membrane-bound enzyme located in the endoplasmic reticulum involved in the elongation of saturated and monounsaturated fatty acids with 12, 14 and 16 carbons using malonyl-CoA as a 2-carbon donor in the first and rate-limiting step of FA elongation (Fig 21).

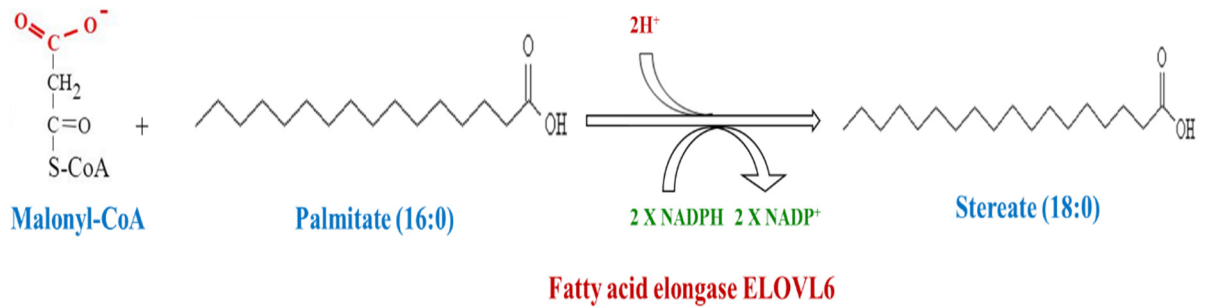


Figure 21: Catalytic action of Fatty acid elongase

As shown in figure 22A B12 deficiency has induced a significant upregulation of ELOVL6 in Chubs-S7 adipocytes compared to control. Same results were obtained in B12 deficient primaries derived from different metabolic phenotypes where a statistical significance was reached in all the categories of BMI (figure 22B).

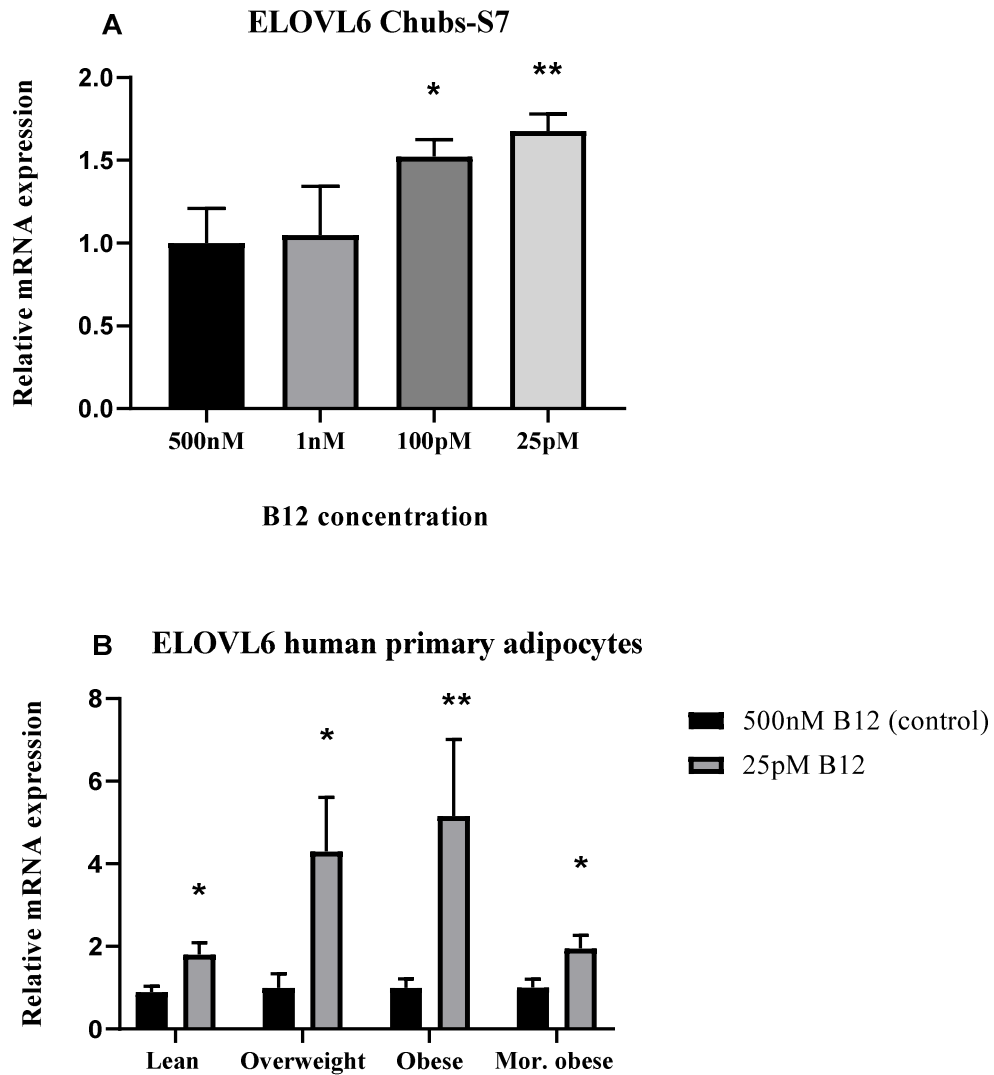


Figure 22: Gene expression of Fatty acid elongase ELOVL6 is increased in B12 deficient adipocytes: The graphs show mRNA expression of ELOVL6 in Chubs-S7 and primary adipocytes (three cultures per BMI group) derived from subcutaneous depots. The adipocytes were seeded and differentiated with different concentrations of B12 and mRNA expression was measured with RT-qPCR. Expression levels were normalised to L19, bars represent the fold changes means \pm SEM and significance levels are indicated as follows; * $p < 0.05$, ** $p < 0.01$, *** compared to control (500nM) according to Mann-Whitney U' test.

4.2.1.4 Stearoyl-CoA Desaturase (SCD)

Stearoyl-CoA desaturase is an integral membrane protein located in the endoplasmic reticulum and plays an important role in lipid biosynthesis catalysing the synthesis of unsaturated FAs. It catalyses, through a series of redox reactions, the formation of a single double bond between C8 and C9 of a long-chain fatty acyl-CoA, giving rise to palmitoleate (16:0) from palmitate and Oleate (18:0) from stearate (Fig 23).

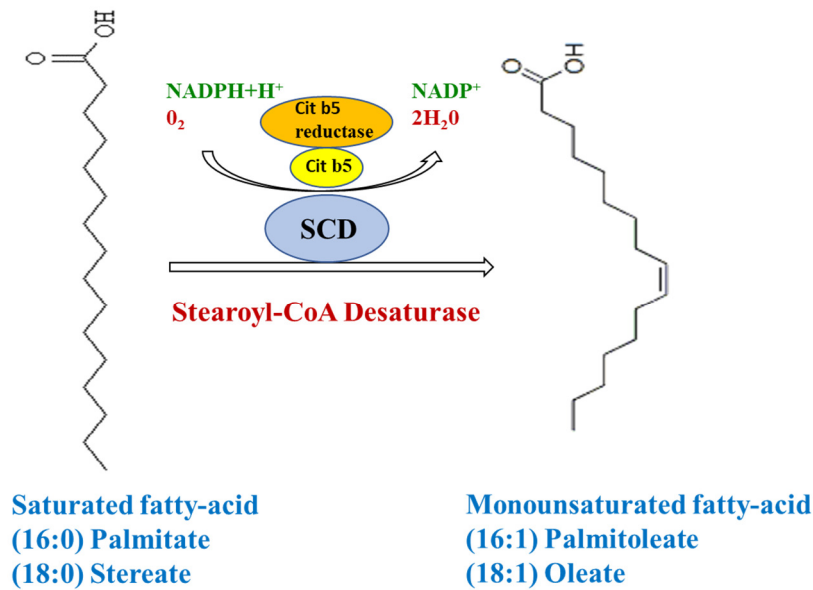


Figure 23: Catalytic action of Stearoyl-CoA Desaturase

As described in figure 24, SCD gene expression significantly upregulated in B12 deficient Chubs-S7 adipocytes. Similar results have been found in B12 deficient primary adipocytes derived from all categories of BMI. Interestingly, adipocytes derived from overweight subjects showed a strong significant increment with a fold increase of up to 26 times.

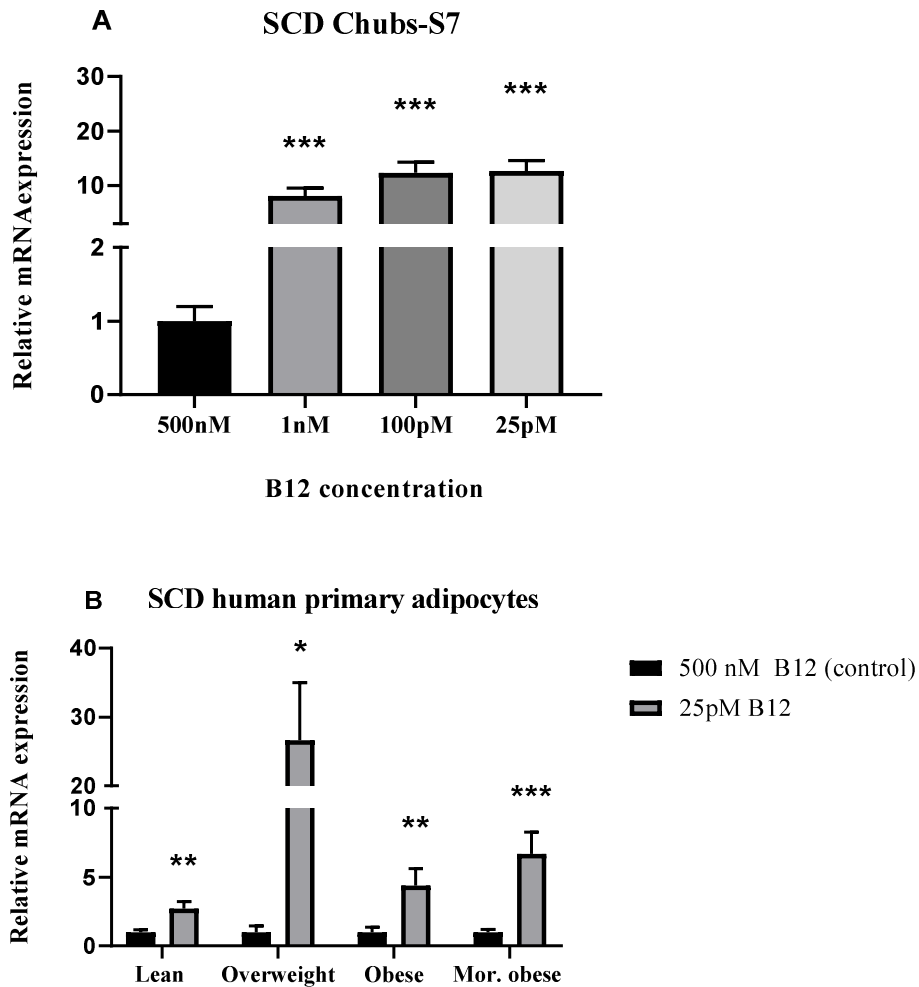


Figure 24: Gene expression of Stearoyl-CoA Desaturase (SCD) is increased in B12 deficient adipocytes: The graphs show SCD mRNA expression of in Chubs-S7 and primary adipocytes (three cultures per BMI group) derived from subcutaneous depots. The adipocytes were seeded and differentiated with different concentrations of B12, as indicated and mRNA expression was measured by RT-qPCR. Expression levels were normalised to L19, bars represent the fold changes means \pm SEM and significance levels are indicated as follows; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control (500nM) according to Mann-Whitney U's test.

4.2.1.5 Glycerol-3-Phosphate Acyltransferase (GPAT2)

Glycerol-3-phosphate acyltransferase (GPAT2) is an enzyme localized in the mitochondrial outer membrane and participates in the catalytic reaction of glycerolipid synthesis. In particular, it esterifies the acyl-group from acyl-ACP to the sn-1 position of glycerol-3-phosphate, an essential step in glycerolipid biosynthesis (Fig 25).

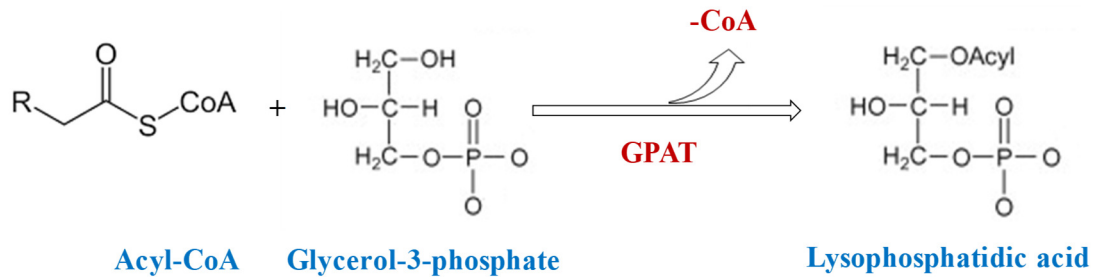


Figure 25: Catalytic action of Glycerol-3-phosphate acyltransferase

In vitro experiments showed a strong upregulation of GPAT gene expression in low B12 treated Chubs-S7 compared to the control (figure 26A.). This result was confirmed in all the metabolic phenotype derived adipocytes with a maximum increase in adipocytes derived from overweight subjects (6.6 folds higher, figure 26B).

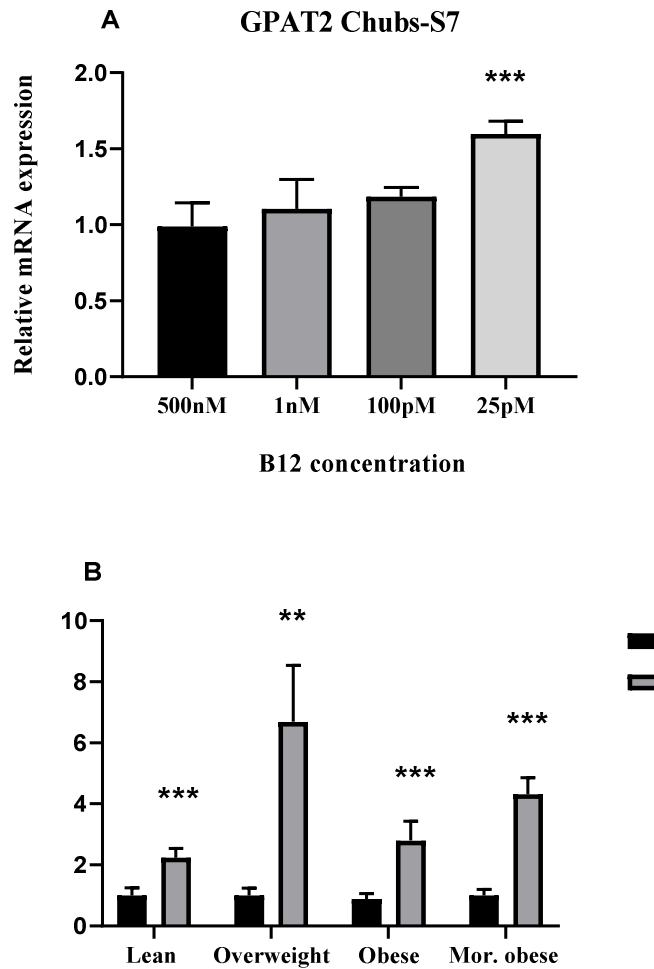


Figure 26: Gene expression Glycerol-3-Phosphate Acyltransferase (GPAT2) is upregulated in B12 deficient adipocytes: The graphs show GPAT2 mRNA expression of in Chubs-S7 and primary adipocytes (three cultures per BMI group) derived from subcutaneous depots. The adipocytes were seeded and differentiated with different concentrations of B12 and mRNA expression was measured by RT-qPCR. Expression levels were normalised to L19, bars represent the fold changes means \pm SEM and significance levels are indicated as follows; ** $p < 0.01$, *** $p < 0.001$, compared to control (500nM) according to Mann-Whitney U'test.

4.2.1.6 Phosphatidate Phosphatase LIPIN1

Phosphatidate phosphatase is a magnesium-ion-dependent enzyme required for adipocyte differentiation. It catalyses the dephosphorylation of phosphatidic acid, the penultimate step of the TAG biosynthesis and plays important role in controlling the metabolism of FAs at different levels (Fig 27).

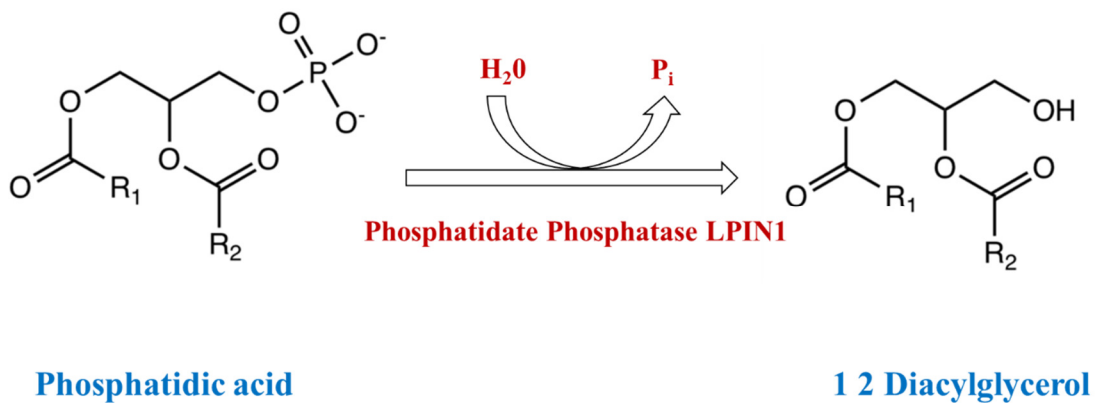


Figure 27: Catalytic action of Phosphatidate phosphatase

As described in figure 28 *LIPIN1* gene expression is progressively increased in Chubs-S7 cultured in decreasing B12 conditions (significance reached in 25pM condition, figure 28A) A strong significant increment was observed in the adipocytes derived from all the four metabolic phenotype categories and particularly, a fold increase up to 17 times has been reached in adipocytes derived from overweight subjects (figure 28B).

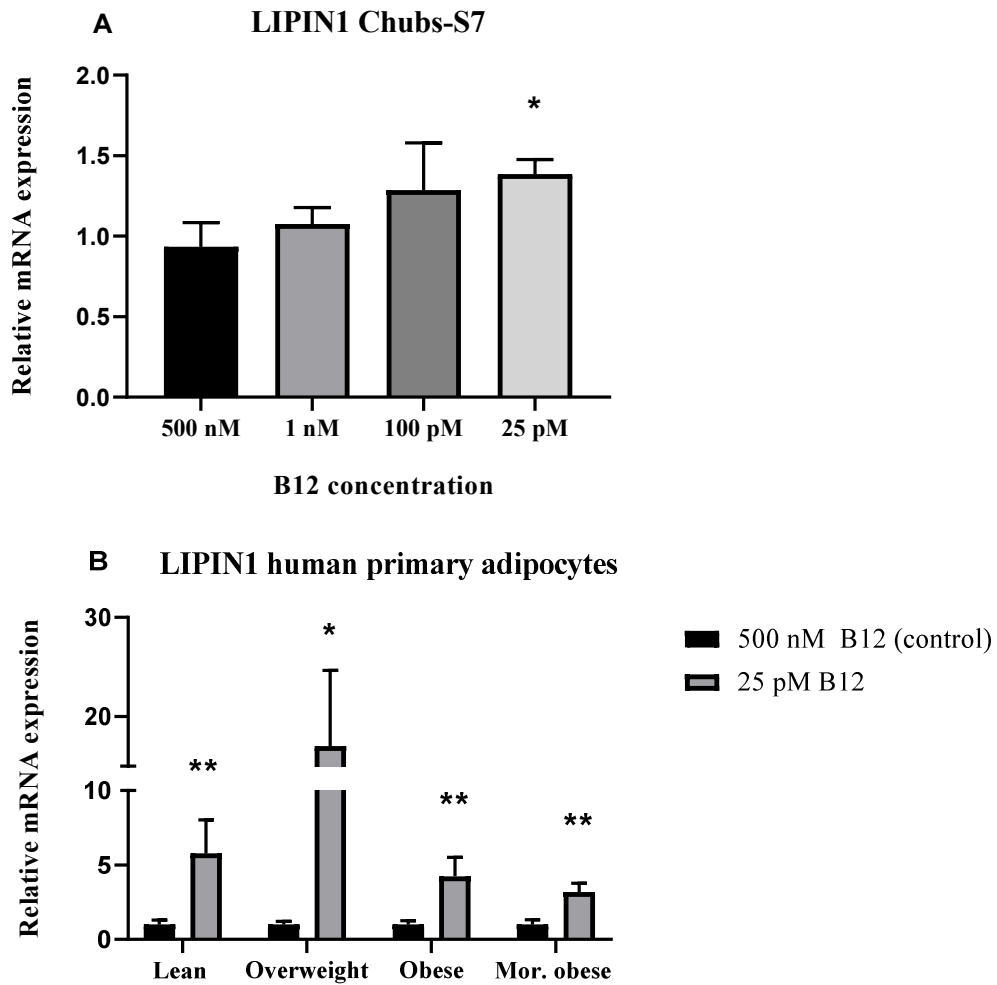


Figure 28: Phosphatidate Phosphatase LIPIN1 gene expression is upregulated in B12 deficient adipocytes: The graphs show *LIPIN1* mRNA expression of in Chubs-S7 and primary adipocytes (three cultures per BMI group) derived from subcutaneous depots. The adipocytes were seeded and differentiated with different concentrations of B12 and mRNA expression was measured by RT-qPCR. Expression levels were normalised to L19, bars represent the fold changes means \pm SEM and significance levels are indicated as follows; * $p < 0.05$, ** $p < 0.01$, compared to control (500nM) according to Mann-Whitney U' test.

4.2.1.7 Diacylglycerol O-Acyltransferase 2 DGAT2

Diacylglycerol O-Acyltransferase 2 is membrane-embedded hairpin acyltransferase localised on the ER-membrane which catalyses the final reaction in the synthesis of TAGs in which diacylglycerol is covalently bound to long chain fatty acyl-CoAs. It plays a central role in the synthesis and storage of TAGs (Fig 29).

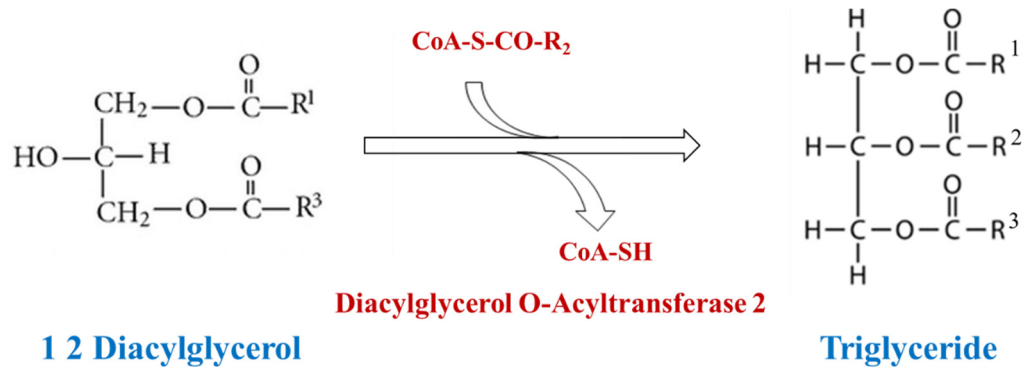


Figure 29: Catalytic action of Diacylglycerol O-Acyltransferase 2

As described in figure 30A in vitro experiments showed a significant upregulation of DGAT2 gene expression in Chubs-S7 cultured in B12 deficient media with a 7-fold increase. B12 deficient adipocytes from all the categories of BMI primary adipocytes showed a significant increase in DGAT2 gene expression and particularly the adipocytes derived from morbid obese subjects showed a 21-fold increase compared to the control (figure 30B).

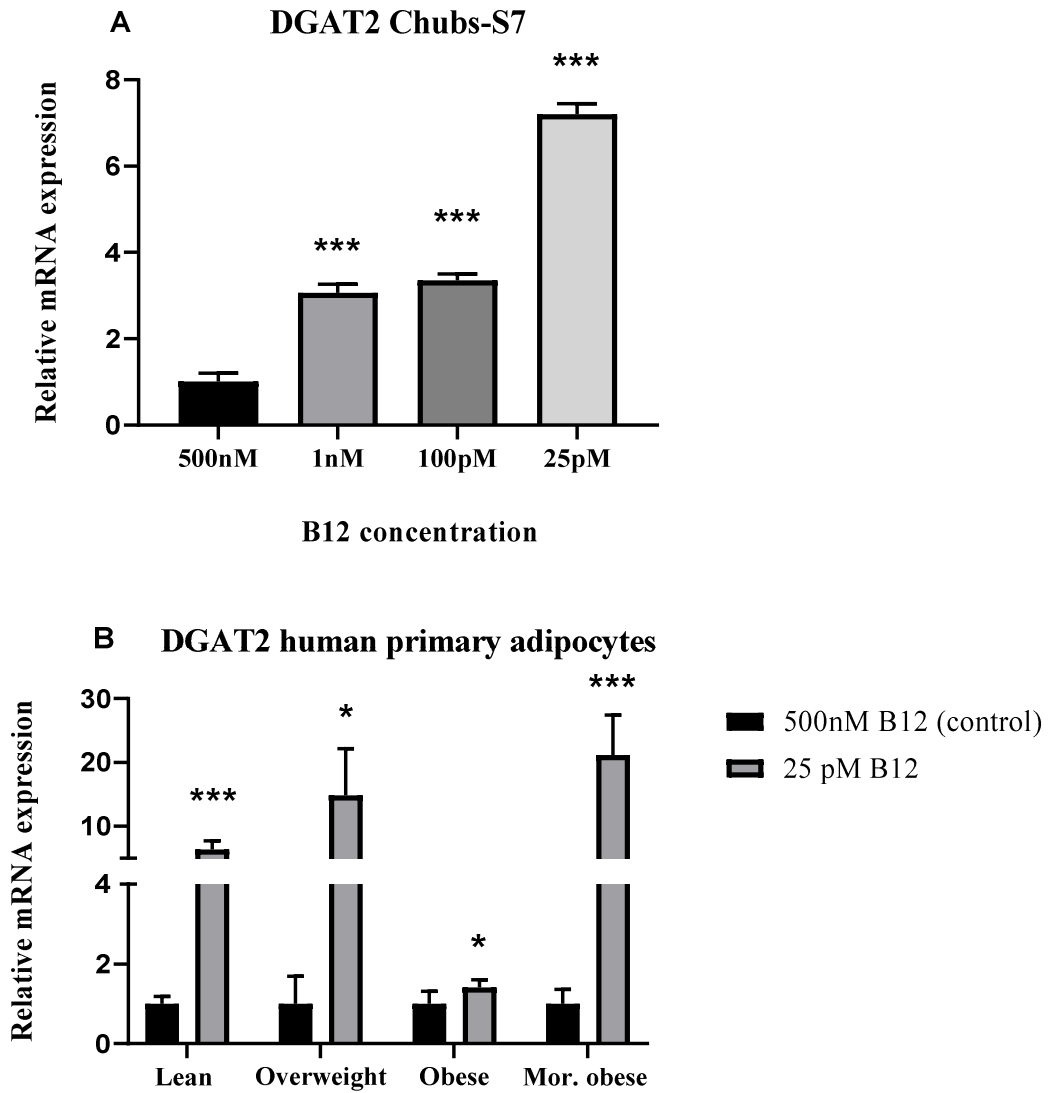


Figure 30: Diacylglycerol O-Acyltransferase 2 DGAT2 gene expression is significantly increased in B12 deficient adipocytes: The graphs show DGAT2 mRNA expression of in Chubs-S7 and primary adipocytes (three cultures per group of BMI) derived from subcutaneous depots. The adipocytes were seeded and differentiated respectively with four and two different concentrations of B12 and mRNA expression was measured with RT-qPCR. Expression levels were normalised to L19, bars represent the fold changes means \pm SEM and significance levels are indicated as follows; * $p < 0.05$, *** $P < 0.001$ compared to control (500nM) according to Mann-Whitney U's test.

4.2.2 In vitro experiments: Flux assay

In mammalian cells TAGs are stored as lipid droplets mainly in the AT, acting as energy reservoir. However, overaccumulation of FAs is toxic and leads to local inflammation (Duncan, 2008). The gene expression data pointed to elevated TAGs synthesis. We wanted to quantify this finding and to further explore whether the actual biosynthesis of triglycerides is upregulated in low B12 conditions. We determined this by assessing the uptake and incorporation of FA into TAGs. We estimated the *de novo* TAG synthesis in low B12 treated cell lines (Chubs-S7) first and then in primary adipocytes compared to control by quantification of the radio-labelled TAGs extracted from adipocytes after cellular incorporation of ^{14}C -oleate for two hours at 37 °C. Adipocyte cells exposed in B12 deficient and control (500nM) conditions were subjected to incubation of ^{14}C -oleate in the presence of L-carnitine.

As in figure 31, cellular uptake of radio-labelled fatty acid (^{14}C -oleate) significantly increased ($p < 0.001$) in Chubs-S7 cultured in decreasing B12 conditions (100pM and 25pM). These results were confirmed in both obese and lean derived adipocytes where the cellular uptake for *de novo* TAG biosynthesis was significantly higher ($p < 0.01$) in low B12 condition, indicating increased expression of genes involved in TAG biosynthesis in adipocytes (figure 32).

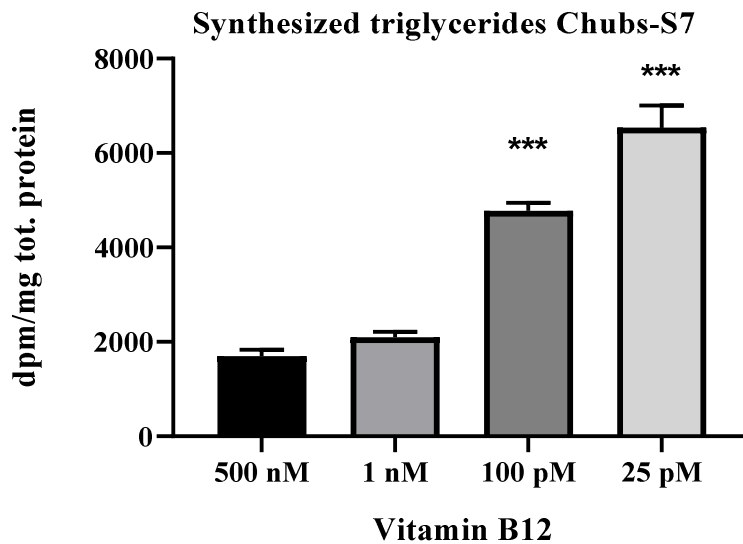
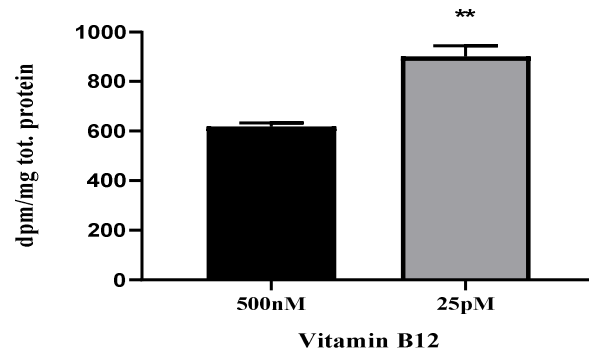


Figure 31: Increased real time triglycerides biosynthesis by primary adipocytes under B12 deficiency

Chubs-S7 adipocytes were seeded and differentiated in four different concentrations of B12 and exposed to ^{14}C -oleate. Expression levels were normalised to protein concentration. Bars represent the means \pm SEM and significance levels are indicated as follows; *** $P < 0.001$ compared to control (500nM) according to Mann-Whitney U's test.

Synthesized triglycerides in human adipocytes from lean subjects



Synthesized triglycerides in human adipocytes from obese subjects

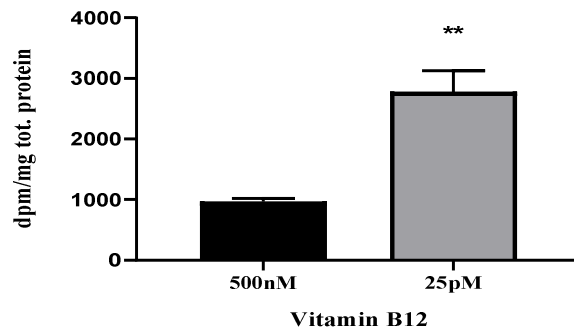


Figure 32: Amount of triglycerides synthesized in real time by primary adipocytes derived from lean and obese patients cultured under low B12 (25pM) compared with control (500nM)

Primary adipocytes (three cultures per BMI group) derived from subcutaneous depots were seeded and differentiated in two different concentrations of B12 and exposed to radiolabelled oleate. Expression levels were normalised to protein concentration. Bars represent the means \pm SEM and significance levels are indicated as follows; **P<0.01 compared to control (500nM) according to Mann-Whitney U's test.

4.2.3: Experiments on the human AT

Effect of B12 on mature human adipose tissue - omental and subcutaneous adipose tissue

Multiple genetic, environmental and behavioral factors contribute to the increasing trend of obesity. It is a common assumption that vitamin and mineral deficiencies are rare in developed countries, however the increased availability of unhealthy high processed food rich in carbohydrates but with poor nutritional value can lead to micronutrient deficiency. Micronutrients therefore play a very important role and would lead to metabolic dysfunctions. As our study in human Chubs-S7 pre-adipocyte cell line and pre-adipocytes derived from human AT treated with low and normal B12, as well as different degrees of obesity (lean-overweight-obese-morbidly obese), showed that these adipocytes in low B12 condition lipogenesis is induced, we would like to further explore the gene expression of lipogenesis in mature human AT exposed to different levels of B12. To date, no clinical studies have examined the gene expression of *de novo* lipid synthesis and TAG biosynthesis enzymes genes in the AT of non-diabetic pregnant women. Therefore, to further investigate the extent of *in vitro* data obtained in Chubs-S7 cell line and human primary adipocytes, gene expression was performed on human OmWAT and ScWAT tissue-derived from our cohort study.

Our findings revealed, as confirmed by the observations in cell line and primary adipocytes, a significant increase in mRNA expression of genes involved in the *de novo* FA synthesis pathway (ACACA and FASN), elongation and desaturation process (ELOVL6 and SCD) and TAG biosynthesis pathway (GPAT2, LIPIN1 and DGAT2) in both OmWAT and ScWAT of B12 deficient patients compared with subjects with adequate levels of the B12 (figure 33). Though, a significant upregulation of DGAT2 gene expression has been seen in OmWAT of B12 deficient patients, no significant difference was observed in the ScWAT.

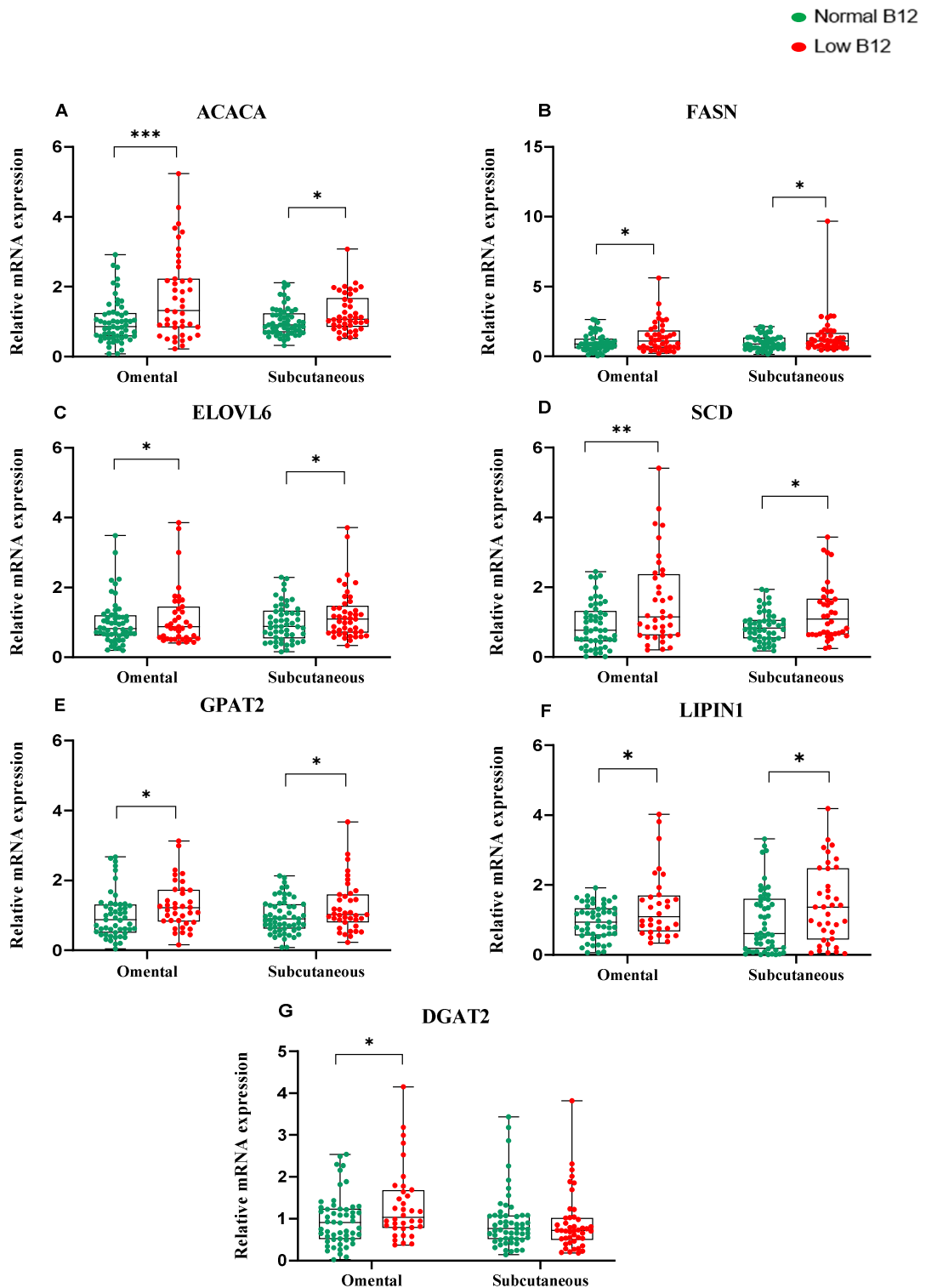


Figure 33: Effect of B12 deficiency on de novo fatty acid synthesis and triglyceride biosynthesis in omental and subcutaneous adipose tissue. Omental and subcutaneous adipose tissue obtained from 106 pregnant women were used to assess gene expression of enzymes involved in fatty acid and triglyceride biosynthesis through RT-qPCR. Patients were divided into two groups according to their vitamin B12 levels (Normal B12= 60 patients(green) and LowB12= 46 patients (red)) and gene expression assay was assessed. Data are not-normally distributed and reported as box charts. *P*-value derived from Mann-Whitney U-test is indicated as * $p < 0.05$, *** $p < 0.001$. Normal B12 (> 200pg/ml) low B12 (<200pg/ml).

4.2.4 Correlation between relative mRNA expression of de novo lipid synthesis and biochemical values.

In order to investigate the relation between the gene expression of lipogenesis, specific to mature AT and the serum levels of B12 and the biochemical parameters, we performed correlation analysis.

Relationship in ScAT

As shown in table 3 and in figure 34, in the human ScAT the correlation analysis showed a negative association between B12 and gene expression of ACACA ($r=-0.235$, $p=0.012$) and FASN ($r=-0.205$, $p=0.030$). A positive correlation has been found between ScACACA and cholesterol ($r=0.201$, $p=0.036$), LDL ($r=0.221$, $p=0.031$) triglycerides ($r=0.211$, $p=0.031$). A positive correlation has also been found between ScGPAT2 and glucose ($r=0.222$, $p=0.019$) (table 3).

Relationship in OmAT

In the human OmAT (table 4 and figure 35), a negative correlation between B12 and gene expression of ACACA ($r=-0.235$, $p=0.0123$), FASN ($r=-0.261$, $p=0.005$), SCD ($r=-0.202$, $p=0.032$), GPAT2 ($r=-0.192$, $p=0.042$), LIPIN1 ($r=-0.201$, $p=0.035$) and DGAT2 ($r=-0.262$, $p=0.005$). A negative correlation between OmACACA and HDL ($r=-0.209$, $p=0.040$) has been found. Moreover, positive correlation between OmFASN and TAGs ($r=0.220$, $p=0.025$) has been seen. OmELOVL6 positively correlated with cholesterol ($r=0.247$, $p=0.009$), LDL ($r=0.298$, $p=0.002$) and TAGs ($r=0.210$, $p=0.027$). Additionally, OmSCD positively correlated with TAGs ($r=0.220$, $p=0.022$), OmLIPIN1 negatively correlated with HDL ($r=-0.217$, $p=0.036$), and OmDGAT2 positively correlated with triglycerides ($r=0.194$, $p=0.044$).

Relative Subcutaneous mRNA expression	Serum biochemical values					
	B12 (pg/ml)	Glucose (mmol/l)	Cholesterol (mmol/l)	HDL (mmol/l)	LDL (mmol/l)	Triglycerides (mmol/l)
ACACA	-0.234*	0.115	0.201*	0.002	0.221*	0.211*
FASN	-0.205*	-0.040	0.092	0.097	0.107	0.083
ELOVL6	0.028	0.060	0.011	0.136	0.005	-0.057
SCD	0.009	0.048	-0.013	0.066	0.021	-0.092
GPAT2	-0.100	0.222*	-0.053	0.003	-0.073	-0.042
LIPIN1	-0.149	-0.025	-0.086	-0.064	-0.051	-0.163
DGAT2	0.132	0.102	0.066	-0.000	0.075	-0.097

Table 3: Subcutaneous lipid synthesis gene expression correlate with serum biochemical metabolic related values

Correlation between subcutaneous *de novo* FA synthesis and TAGs biosynthesis pathway gene expression and serum metabolic related biochemical values. Data are represented as Spearman's R values. Abbreviation: ACACA Acetyl-CoA Carboxylase Alpha, FASN Fatty Acid Synthase, ELOVL6 ELOVL Fatty Acid Elongase 6, SCD Stearoyl-CoA Desaturase, GPAT2 Glycerol-3-Phosphate Acyltransferase 2, Mitochondrial, LIPIN1 Phosphatidate Phosphatase LPIN1 DGAT2 Diacylglycerol O-Acyltransferase 2, B12 vitamin B12, HDL high-Density Lipoprotein, LDL Low-Density Lipoprotein.

Relative Omental mRNA expression	Serum biochemical values					
	B12 (pg/mL)	Glucose (mmol/l)	Chol. (mmol/l)	HDL (mmol/l)	LDL (mmol/l)	Triglycerides (mmol/l)
ACACA	-0.235*	-0.084	0.047	-0.209*	0.085	0.024
FASN	-0.261**	-0.040	-0.032	-0.090	-0.046	0.220
ELOVL6	-0.054	-0.118	0.247**	-0.010	0.298**	0.210*
SCD	-0.203*	-0.113	0.023	-0.102	0.105	0.220*
GPAT2	-0.192*	-0.028	-0.051	-0.014	-0.109	-0.001
LIPIN1	-0.201*	-0.038	-0.103	-0.217*	-0.071	0.009
DGAT2	-0.262**	0.004	0.018	0.044	0.035	0.194*

Table 4: Omental lipid synthesis gene expression correlates with serum biochemical metabolic related values

Correlation between omental *de novo* FA synthesis and TAG biosynthesis pathway gene expression and serum metabolic related biochemical values. Data are represented as Spearman's R values. Abbreviation: ACACA Acetyl-CoA Carboxylase Alpha, FASN Fatty Acid Synthase, ELOVL6 ELOVL Fatty Acid Elongase 6, SCD Stearoyl-CoA Desaturase, GPAT2 Glycerol-3-Phosphate Acyltransferase 2, Mitochondrial, LIPIN1 Phosphatidate Phosphatase LPIN1 DGAT2 Diacylglycerol O-Acyltransferase 2, B12 vitamin B12, HDL high-Density Lipoprotein, LDL Low-Density Lipoprotein.

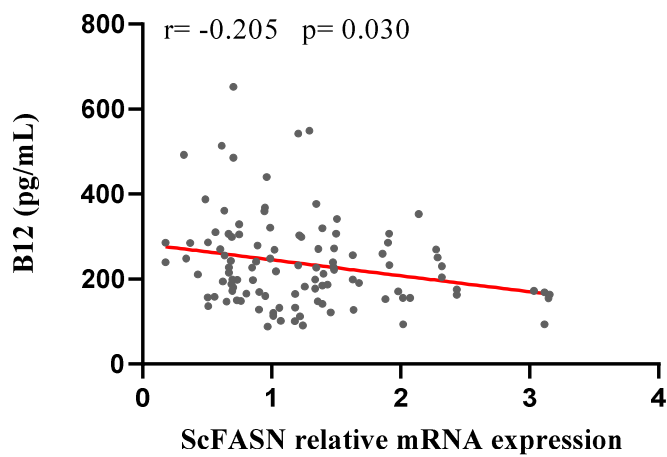
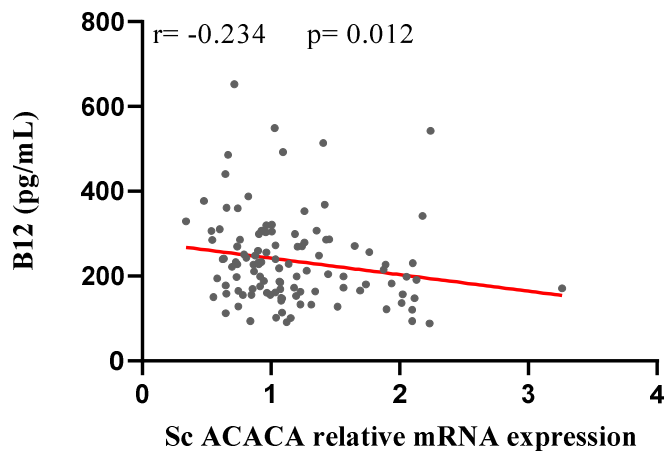


Figure 34: Subcutaneous lipid synthesis relative gene expression of ACACA and FASN correlate with serum B12

Correlation between ScACACA, and ScFASN and serum B12. Dots depict individual study subjects and data are presented as Spearman's R values. ACACA Acetyl-CoA Carboxylase Alpha, FASN Fatty Acid Synthase.

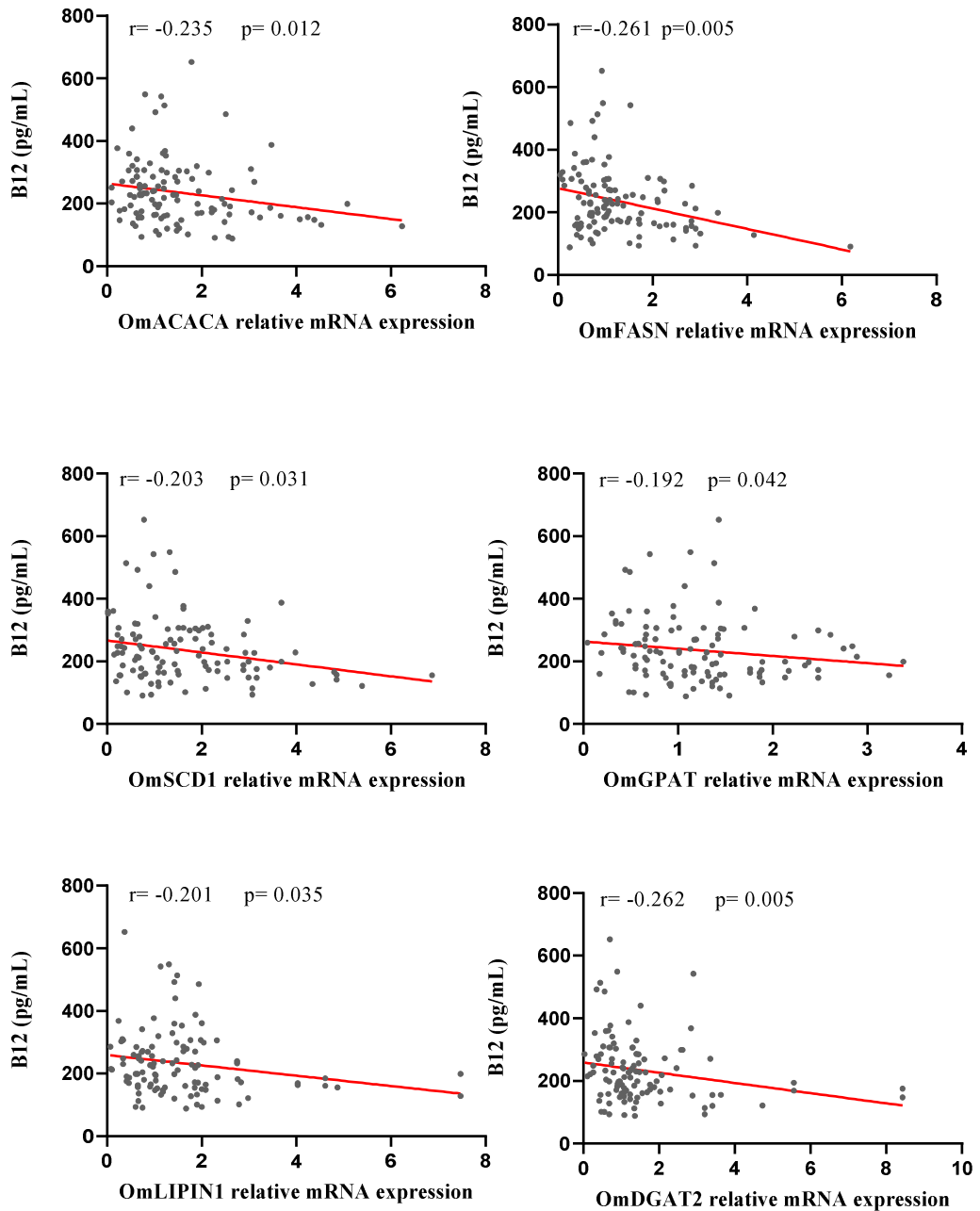


Figure 35: Omental lipid synthesis relative mRNA expression correlate with serum B12

Correlation between omental ACACA, FASN, SCD1, GPAT, LIPIN1, DGAT2, and serum vitamin B12. Dots depict individual study subjects and data are presented as Spearman's R values: ACACA Acetyl-CoA Carboxylase Alpha, FASN Fatty Acid Synthase, ELOVL6 ELOVL Fatty Acid Elongase 6, SCD Stearoyl-CoA Desaturase, GPAT2 Glycerol-3-Phosphate Acyltransferase 2, Mitochondrial, LIPIN1 Phosphatidate Phosphatase LPIN1 DGAT2 Diacylglycerol O-Acyltransferase 2.

4.3 Discussion

In this study, our key findings are, 1) B12 deficiency in human adipocyte cell lines (Chubs-S7) showed higher mRNA expressions of genes involved in FA synthesis, fatty acid elongation and TAG synthesis; 2) Higher synthesis of actual amount of FAs; 3) Confirmation of these findings in adipocytes from humans with B12 deficiency and in adipocytes extracted from AT of humans with varying degrees of obesity; 4) Quantification of the lipogenesis in both ScWAT and OmWAT and 5) The association of these with lipid profiles in the circulation.

Micronutrients play an important role in metabolism and a deficiency can bring about cellular dysfunction. Among the micronutrients, vitamin B12, a fundamental component together with folate in the one-carbon metabolism, is essential for several metabolic processes including energy metabolism. In the last decade clinical evidences of a correlation between B12 deficiency and adverse metabolic health profiles triggered many scientists to study the role of this micronutrient in the development of obesity. Recently a population study involving more than 9000 Danish people showed a negative correlation between B12, BMI waist circumference and body fat percentage (Allin *et al.*, 2017). A clinical study conducted in Israel revealed a low B12 concentration in obese children compared with normal weight group (Pinhas-Hamiel *et al.*, 2006). Similar results were observed in adult patients with morbid obesity (Madan *et al.*, 2006). Interesting studies conducted on pregnant women characterized by B12 deficiency confirmed the correlation between this condition and increase risk of developing IR, GDM, adverse HDL levels and obesity (Adaikalakoteswari *et al.*, 2015c; Knight *et al.*, 2015). These data suggest therefore that B12 influences lipid metabolism and may have an important role in obesity development.

Acetyl-CoA Carboxylase alpha (ACACA) has been the first target of investigation, it's involved in the FA synthesis pathway together with FASN and is considered a promising target for the treatment of obesity. In this study, we observed that B12 deficiency, in pre-adipocytes cultures (cell lines and primaries derived from different metabolic phenotypes), has induced a significant upregulation of ACACA. These observations were confirmed in the two compartments of mature AT derived from B12 deficient patients, where the OmAT resulted the most affected compartment. These findings are in agreement with in vivo studies conducted in vitro and on ACACA knockout mice

showing a significant reduction in malonyl-CoA levels in skeletal muscle, AT and heart tissue, along with corresponding increase in FAO (Bourbeau & Bartberger, 2015). These findings are in accordance with in vitro studies by Adaikalakoteswari on Chubs-S7 adipocytes showing an increase in cholesterol biosynthesis in B12 deficient cells due to an increase expression of SREBPF-1 and subsequent hypomethylation of this transcription factor which controls the genes encoding for cholesterol and TAG enzymes (Adaikalakoteswari *et al.*, 2017).

We then focused our attention on the study of Fatty acid synthase (FASN) gene, a multienzyme involved in the catalysis of the synthesis of palmitate from acetyl-CoA and malonyl-CoA. It plays a role in the pathophysiology of human obesity and its related metabolic alterations. In this study, we observed that B12 deficiency, in pre-adipocytes cultures (cell lines and primaries derived from different metabolic phenotypes), has induced a significant upregulation of FASN. These observations were also confirmed in the two compartments of mature AT derived from B12 deficient patients. The current study is in line with experiments that have shown association between FASN gene expression in AT and increased omental fat accumulation and impaired insulin sensitivity (Mayas *et al.*, 2010). Mobbs *et al.* confirmed in FASNKO mice, that the regulation of some nutrition-sensitive hypothalamic pathways, control the thermogenic programming both in WAT and BAT (Guilherme *et al.*, 2017; Mobbs & Makimura, 2002).

We further observed the effect of B12 deficiency on the elongation pathway by analysing the key gene involved in this pathway, the Fatty acid elongase ELOVL6 which plays an important role in FA synthesis and insulin sensitivity. It has been shown, in this study, a significant increase of the gene expression in low B12 treated adipocytes (both cell line and primaries) and confirmed in human ScWAT and OmWAT. These findings are similar with experiments conducted on cultured human hepatocytes and murine models of hyperhomocysteinemia (which is caused by B12 deficiency) with an increase in ER stress and local accumulation of lipids (Werstuck *et al.*, 2001). This study also observed the desaturation process of the FA by analysing Stearoyl-CoA desaturase (SCD), showing an upregulation of the gene expression in both B12 deficient adipocytes and on both human AT compartments derived from B12 deficient patients. These findings are in accordance with animal and human studies reporting that SCD seems to play a role in the development of metabolic diseases increasing AT inflammation by inducing methylation changes in inflammatory genes (Malodobra-Mazur *et al.*, 2014; Thon *et al.*, 2017) and inducing leptin resistance in neuronal cells associated with obesity (Thon *et al.*, 2017)

We further analysed the effect of B12 deficiency on TAG biosynthesis analysing the gene expression of the enzymes involved in the process (GPAT2, LIPIN1, DGAT2), on adipocytes and AT derived from the population study confirming a strong upregulation under B12 deficiency status. These results are in line with several *in vitro* and *in vivo* studies confirming the pivotal role of those gene in the TAG and phospholipid synthesis and their important role in development of obesity (Kuhajda *et al.*, 2011) (Yu *et al.*, 2018), IR, lipid biosynthesis and systemic inflammatory responses (Kajimoto *et al.*, 2016; Meana *et al.*, 2014), adipocyte hypertrophy and lipid accumulation (Suzuki *et al.*, 2005; Zhao *et al.*, 2017). A population study conducted in north India showed an association between low levels of B12 and HDL, although no gene expression has been taken in consideration (Saraswathy *et al.*, 2018) and similar finding also obtained in a study conducted on British and Indian subjects with T2DM (Adaikalakoteswari *et al.*, 2014b). B12 deficiency leads to epigenetic changes in genes associated in lipid metabolism, as suggested by recent studies (Adaikalakoteswari *et al.*, 2017; Fukuoka & Kubota, 2018). In this study, it has been observed that B12 deficiency in both primary adipocytes and mature AT is associated with an increase gene expression of enzymes involved in FA synthesis. These findings are in accordance with *in vitro* studies by Adaikalakoteswari on Chubs-S7 adipocytes showing an increase in cholesterol biosynthesis in B12 deficient cells due to an increase expression of SREBP-1 and subsequent hypomethylation of this transcription factor which controls the genes encoding cholesterol and TAG enzymes. Similar results were seen on experiments conducted on severely B12 deficient female mice showing a significantly higher levels of plasma cholesterol and TAGs after 4 and 12 weeks compared to control mice (Ghosh *et al.*, 2016). In support to these experiments a study conducted by a Norwegian group on zebrafish showed that parental micronutrient deficiency (folate and vitamin B12) leads to modification in DNA methylation and lipid accumulation in adult F1 male offspring (Skjærven *et al.*, 2018).

Moreover in order to support the findings that triglycerides biosynthesis is upregulated in low B12 conditions, we estimated *de novo* TAG synthesis in low B12 treated cell lines (Chubs-S7) and primary adipocytes compared to control by quantification of the amount of radio-labelled TAGs extracted. The quantification showed a significant upregulation in B12 deficient adipocytes, in line with animal studies conducted on B12 deprived rats in which an increase in odd-numbered FAs has been found in both liver and nervous tissue (Fehling *et al.*, 1978) but in contrast with a more recent study in which no increase in TAG has been seen in B12 deficient mice (Khaire *et al.*, 2016).

We have further explored the clinical and biochemical characteristics of our cohort study based on B12 status and correlated the gene expression of *de novo* lipid synthesis enzymes with serum biochemical values, in order to understand a possible relationship between metabolic disorders and B12 deficiency. In the ScWAT, the correlation approach showed that only FA synthesis genes, FASN and ACACA, were correlated with B12. Differently, in the OmWAT compartment the gene expression of all the enzymes involved in FA synthesis, elongation and TAG biosynthesis resulted to be upregulated in B12 deficiency patients, showing therefore a major exasperation in the OmWAT compartment. Significant associations between ScWAT and OmWAT mRNA expression of enzymes involved in lipid synthesis and total cholesterol, HDL, LDL have been shown, in line with a recent study conducted on British non GDM women in which B12 resulted to be positively correlated with HDL and negatively correlated with TAG but no correlation with gene expression was investigated (Knight *et al.*, 2015).

The results of the present study provide novel evidence that vitamin B₁₂ plays a vital role in the biosynthesis of *de novo* fatty acids and triglyceride biosynthesis. Specifically, we have demonstrated that low vitamin B₁₂ (a) induce an increased expression of genes involved in *de novo* fatty acid and triglyceride biosynthesis in Chubs-S7; (b) induce an increased expression of genes involved in *de novo* fatty acid and triglyceride biosynthesis in human primary adipocytes derived from different categories of BMI with a prominent increment in adipocytes derived from the overweight group (maybe due to an increased susceptibility of this category of BMI to B12 deficiency); (c) increased expression of genes responsible for *de novo* and triglyceride biosynthesis in both omental and subcutaneous human adipose tissue ; (d) increased biosynthesis of triglycerides in Chus-S7 and in human adipocytes with a prominent increment in adipose cells derived from obese subjects.

Adipose tissue is the biggest reservoir of triglycerides which is used as form of energy from the body. For instance, during pregnancy the enlistment of stored fat depots provide a source of FAs, essential for the foetal growth. However, high levels of triglycerides in the blood circulation, and maternal hypercholesterolemia are associated with an increased risk for maternal-foetal complications such as GDM, preeclampsia, increased risk in developing metabolic related disease in the offspring, preterm birth (Wilson *et al.*, 1998). Moreover, studies also found associations between poor levels of B12 and dyslipidaemia

(Sukumar et al., 2016), hence, the diagnosis and treatment of dyslipidaemia before and during pregnancy results the best chance to reduce associated metabolic diseases.

The exact biochemical explanation by which of cellular B12 deficiency leads to an increment in triglycerides biosynthesis is still not clearly understood. Between the possible explanations would be 1) the increased levels of MMA due to a disruption of the mitochondrial methyl malonylCoA mutase reaction leading to an increase of levels of MMA and consequently enhancing increased lipogenesis, as suggested by a study conducted by Ruderman and colleagues (Ruderman *et al.*, 2003); 2) increased levels of homocysteine with an increase in ER stress and local accumulation of lipids, as previously shown on in vitro studies conducted on human hepatocytes and on murine models (Werstuck *et al.*, 2001).

Our data highlights that low B12 induces a dysregulation of fatty acid metabolism by upregulating *de novo* FA synthesis and TAGs biosynthesis, which might lead to adipocyte dysfunction suggesting therefore a possible role of B12 deficiency in metabolic disorders and opening new insights into the pathogenesis of maternal obesity and the relevance of micronutrient supplementation for pregnant mothers.

Chapter 5: Vitamin B12 and the fatty acid β -oxidation

5.1 Introduction

Fatty acid β -oxidation (FAO) is an aerobic multi-step process consisting of breaking down FAs into acetyl-CoA units. It occurs in the mitochondrial matrix and plays an important role in energy metabolism. When hormones such as glucagon and noradrenaline report an energy metabolism deficit, TAGs kept in lipid droplets in the AT, are released and metabolized in order to produce energy. During obesity, the disruption of energy balance which leads to AT expansion and excessive fat accumulation increases TAG synthesis, translating in a negative impact on health. Studies on abdominal muscle cells derived from obese patients showed a decreased FAO and an increased SCD1 implying a reduced FA metabolism in obesity (Hulver *et al.*, 2005; Thyfault *et al.*, 2004). Together, these findings imply that a reduced capacity for fat oxidation is with obesity.

Studies on micronutrient deficiency such as B12 confirmed the essential role that it plays in mitochondrial energy production. It's involved in the 1-C metabolism in the form of AdoCbl which is required for the synthesis of succinyl-CoA and in Krebs cycle. It has been also shown that a B12 deficiency leads to an increase CoA and FA synthesis (Tahiliani & Beinlich, 1991). These findings are reinforced by animal and human studies, lead to the conclusion that B12 play a role in lipid regulation and may play a role in obesity and metabolic related diseases. Regardless the role of B12 on oxidation of FA, some pre-clinical studies have shown evidence of association between B12 deficiency and FAO impairment. In an animal study conducted on rats liver tissues, a disruption in the oxidation of FAs resulting from deficiency in methyl donors (B12 and folate) was demonstrated (Pooya *et al.*, 2012)..

However, there are no studies on the role of B12 and FAO in human adipocytes, therefore we investigated the role of B12 in *in vitro* human cell line and human adipose explants. The purpose of the present study is to (1) investigate the role of B12 deficiency on FAO *in vitro* human adipocyte cell, (2) endorse these findings in cultures of human primary pre-adipocytes derived from adipose tissue of individuals with different degrees of BMI, (3) endorse these findings in mature AT from different depots of human subjects such as OmWAT and ScWAT with different levels of B12 and (5) evaluating cellular bioenergetics by determining the effect of B12 deficiency on mitochondrial function in adipocyte cells.

5.2 Results

5.2.1 *In vitro* experiments:

Genes involved in expression of fatty acid transporters and genes involved in fatty acid β -oxidation in Chubs-S7 and primary adipocytes

Methods

To investigate the gene expression of both mitochondrial FA transporters and genes involved in β -oxidation under B12 deficiency conditions, Chubs-S7 and primary pre-adipocytes were seeded at low density (30.000 cells/well in 6 well plates) and treated respectively with four (25 pM, 100 pM, 1 nM, 500 nM) and two different concentrations of B12 (25 pM and 500 nM), from the seeding to the full differentiation. In order to investigate the role of B12 deficiency on different metabolic phenotypes, human pre-adipocytes derived from lean, overweight, obese and morbidly obese subjects were selected and used for our experiments. Primary pre-adipocytes were treated with two different condition of B12 (25 pM and 500 nM) as previously explained. Gene expression detection was performed by RT-qPCR and data were analyzed with Mann-Whitney U-test.

5.2.1.1 Malonyl-CoA decarboxylase (MCD)

Malonyl-CoA decarboxylase catalyses the conversion of malonyl-CoA to acetyl-CoA and CO₂. It's localized in the cytoplasm and it plays a role in the regulation of mitochondrial FAO, by regulating the concentration of malonyl-CoA, an inhibitor of CPT1 (Fig 36).



Figure 36: Catalytic action of Malonyl-CoA decarboxylase

As shown in figure 37A, B12 deficiency has induced a significant downregulation of MCD in Chubs-S7 adipocytes. A similar significant decrease has been observed in B12 deficient adipocytes derived from different metabolic phenotypes (figure 37B).

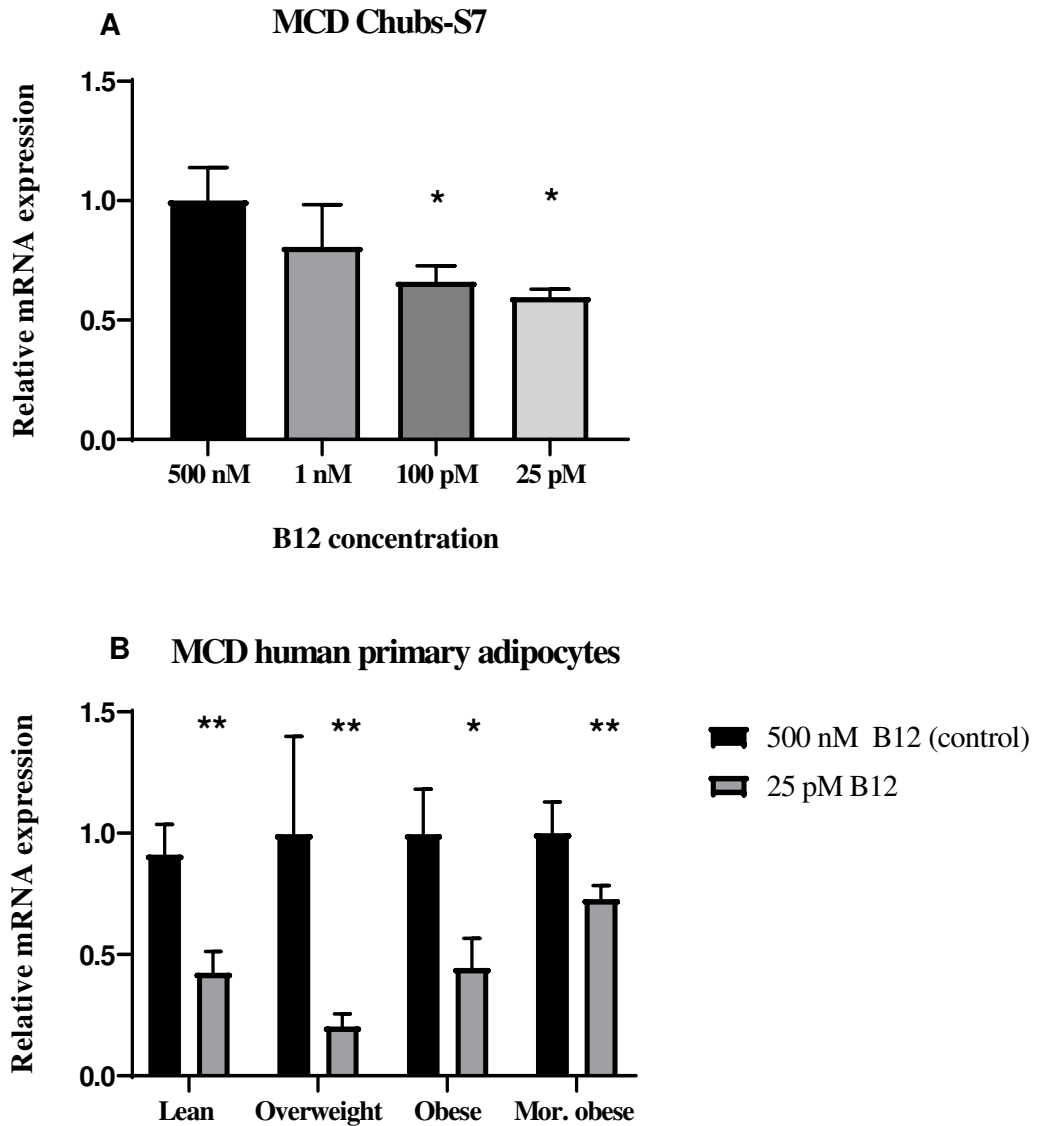


Figure 37: Gene expression of Malonyl-CoA decarboxylase (MCD) is altered in B12 deficient adipocytes: The graphs show mRNA expression of MCD in Chubs-S7 and primary adipocytes (three cultures per group of BMI) derived from subcutaneous depots. The adipocytes were seeded and differentiated respectively with four and two different concentrations of B12 and mRNA expression was measured with RT-qPCR. The mRNA levels were normalized to L19. Data are expressed as mean \pm SE and significance levels are indicated as follows; * $p < 0.05$, ** $p < 0.01$ compared to control (500nM) according to Mann-Whitney U's test.

5.2.1.2 Carnitine palmitoyltransferase-1 (CPT1 β)

Carnitine palmitoyltransferase-1 (CPT1 β) is a carnitine-dependent enzyme located on the outer mitochondrial membrane involved in the transport of long-chain FA into mitochondria for β -oxidation, hence a key factor in the mitochondrial FAO (Warfel *et al.*, 2016) (Fig 38).

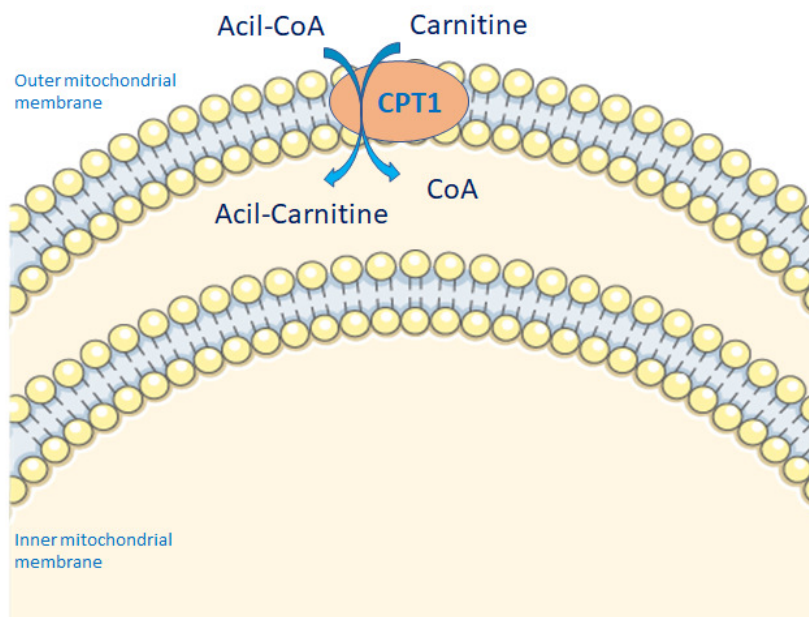


Figure 38: Acyl-CoA transport from cytosol to the inner mitochondrial membrane

The in vitro experiments showed a strong downregulation of CPT1- β gene in low B12 treated Chubs-S7 (figure 39A) compared to the control. This result was confirmed in all the metabolic phenotype derived adipocytes where a significant decrease up to 70% in the gene expression was observed compared to lean derived adipocytes (figure 39B).

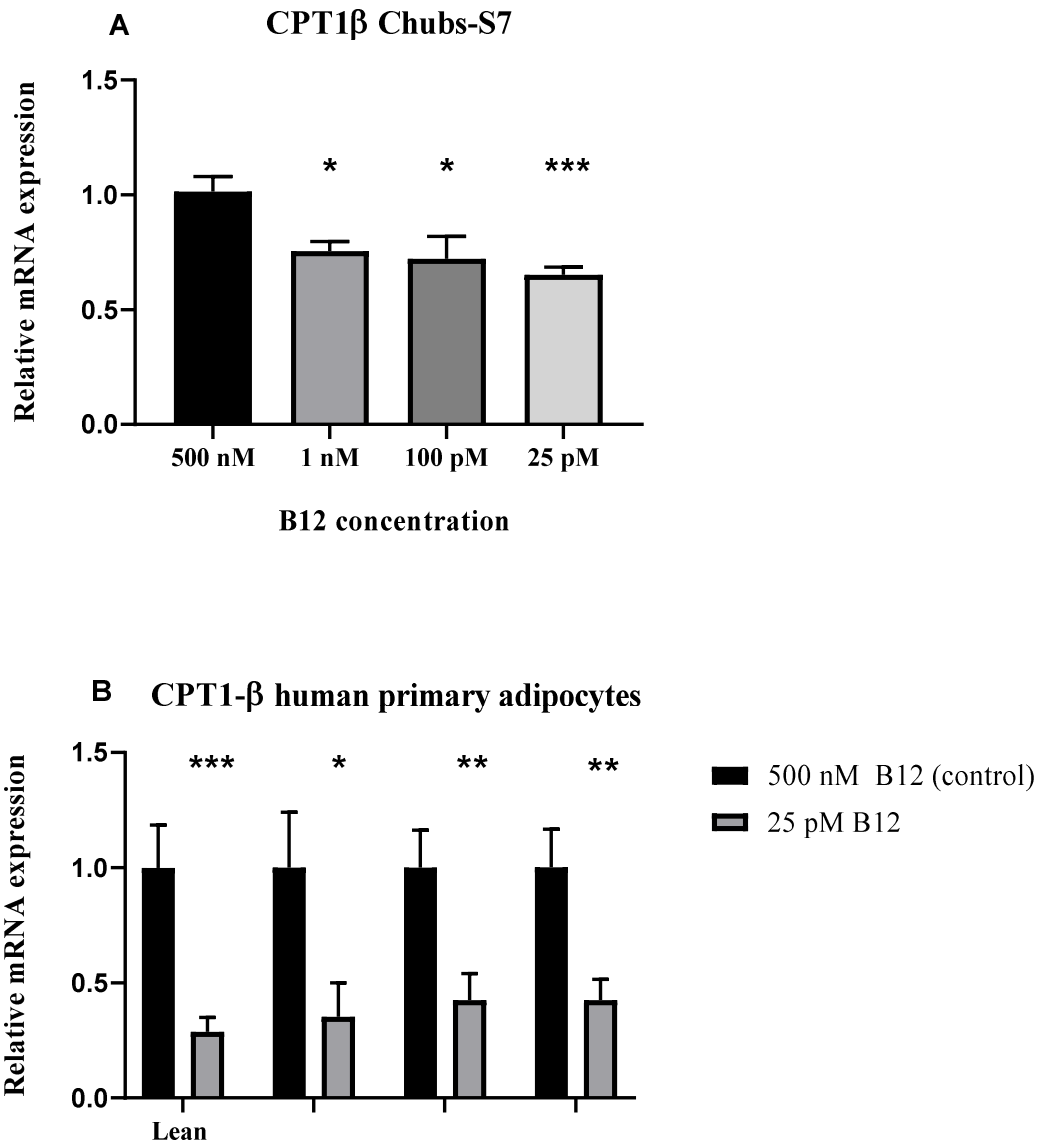


Figure 39: Gene expression of Carnitine palmitoyltransferase-1 (CPT1- β) is downregulated in B12 deficient adipocytes: The graphs show mRNA expression of CPT1- β in Chubs-S7 and primary adipocytes (three cultures per group of BMI) derived from subcutaneous depots. The adipocytes were seeded and differentiated respectively with four and two different concentrations of B12 and mRNA expression was measured with RT-qPCR. The mRNA levels were normalized to L19. Data are expressed as mean \pm SE and significance levels are indicated as follows; * p <0.05, ** p <0.01, *** p <0.001 compared to control (500nM) according to Mann-Whitney U's test.

5.2.1.3 Carnitine palmitoyltransferase-2 (CPT2)

Carnitine palmitoyltransferase 2 (CPT2) is a nuclear protein which is transported to the mitochondrial inner membrane together with carnitine palmitoyltransferase 1 is involved in the transport and oxidation of long-chain FA in the mitochondria (Fig 40).

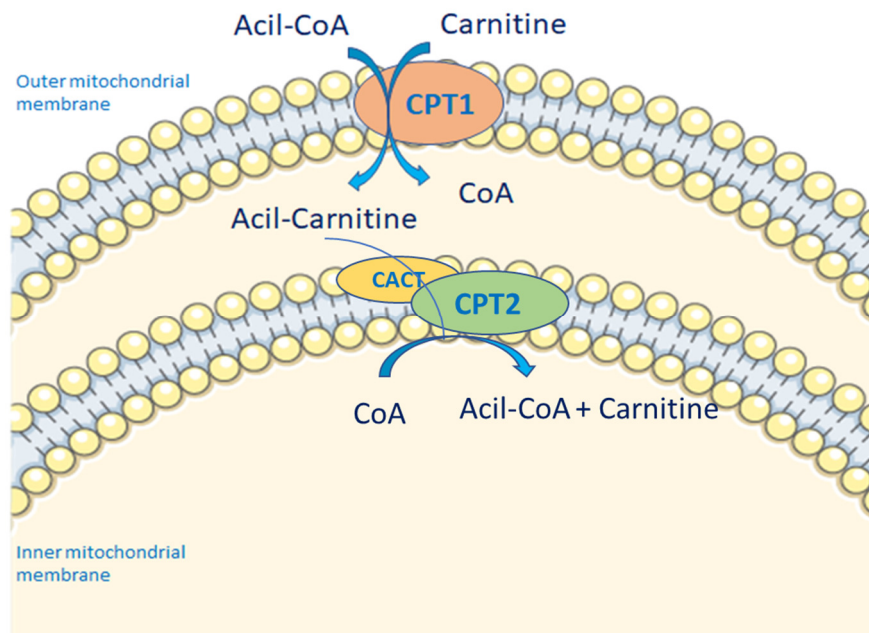


Figure 40: Schematic representation of Acyl-CoA transport from cytosol to the mitochondrial matrix

As described in figure 41, CPT2 relative mRNA expression was progressively reduced in Chubs-S7 cultured in low B12 conditions with significance reached in 100pM and 25pM conditions (figure 41A). These results were also confirmed in B12 deficient primary adipocytes where a 50% reduction was shown in lean, obese and morbid obese derived cells compared to control.

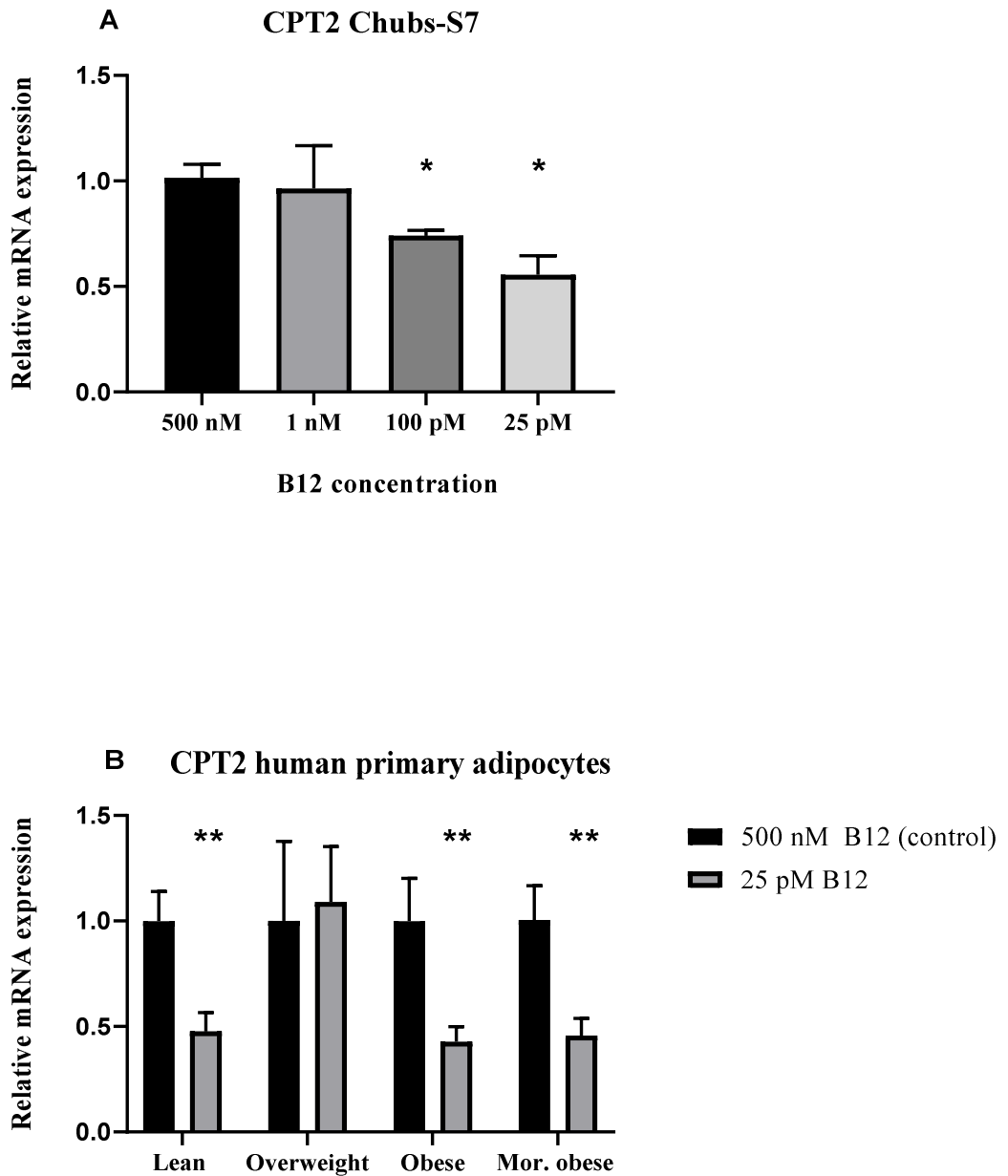


Figure 41: Gene expression of Carnitine palmitoyltransferase-2 (CPT2) is reduced in B12 deficient adipocytes: The graphs show mRNA expression of CPT2 in Chubs-S7 and primary adipocytes (three cultures per group of BMI) derived from subcutaneous depots. The adipocytes were seeded and differentiated respectively with four and two different concentrations of B12 and mRNA expression was measured with RT-qPCR. The mRNA levels were normalized to L19. Data are expressed as mean \pm SE and significance levels are indicated as follows; * $p < 0.05$, ** $p < 0.01$, compared to control (500nM) according to Mann-Whitney U's test.

5.2.1.4 Acyl-CoA Dehydrogenase Long Chain (ACADL)

Acyl-CoA Dehydrogenase Long Chain (ACADL) is an enzyme that belongs to the acyl-CoA dehydrogenase family, mitochondrial enzymes that function to catalyse the initial step of the fatty acid β -oxidation involved in the introduction of trans double-bond between C2 and C3 (α,β -dehydrogenation) of the acyl-CoA thioester substrate (Indo *et al.*, 1991). It has a homotetrameric structure with each monomer containing a FAD molecule required for the oxidation reaction (Fig 42).

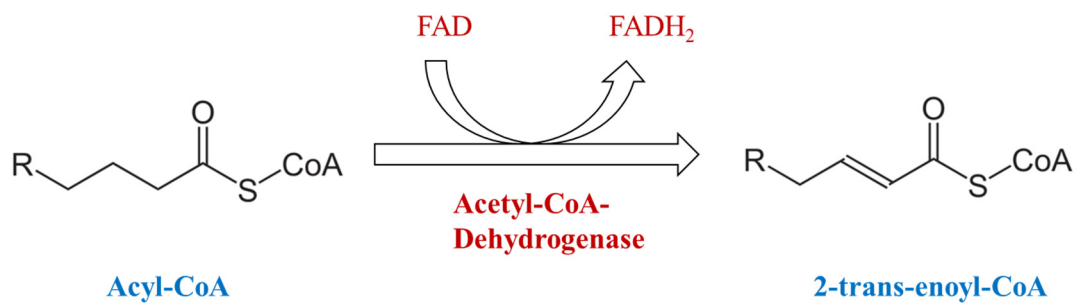


Figure 42: Catalytic action of Acyl-CoA Dehydrogenase

As seen in figure 43, Chubs-S7 cultured in low B12 conditions showed a progressive decrease in ACADL gene expression with a statistic significance reached in 100pM and 25pM conditions (figure 43A). A similar result was obtained in B12 deficient primary cells, reaching more than 80% reduction in adipocytes derived from obese individuals.

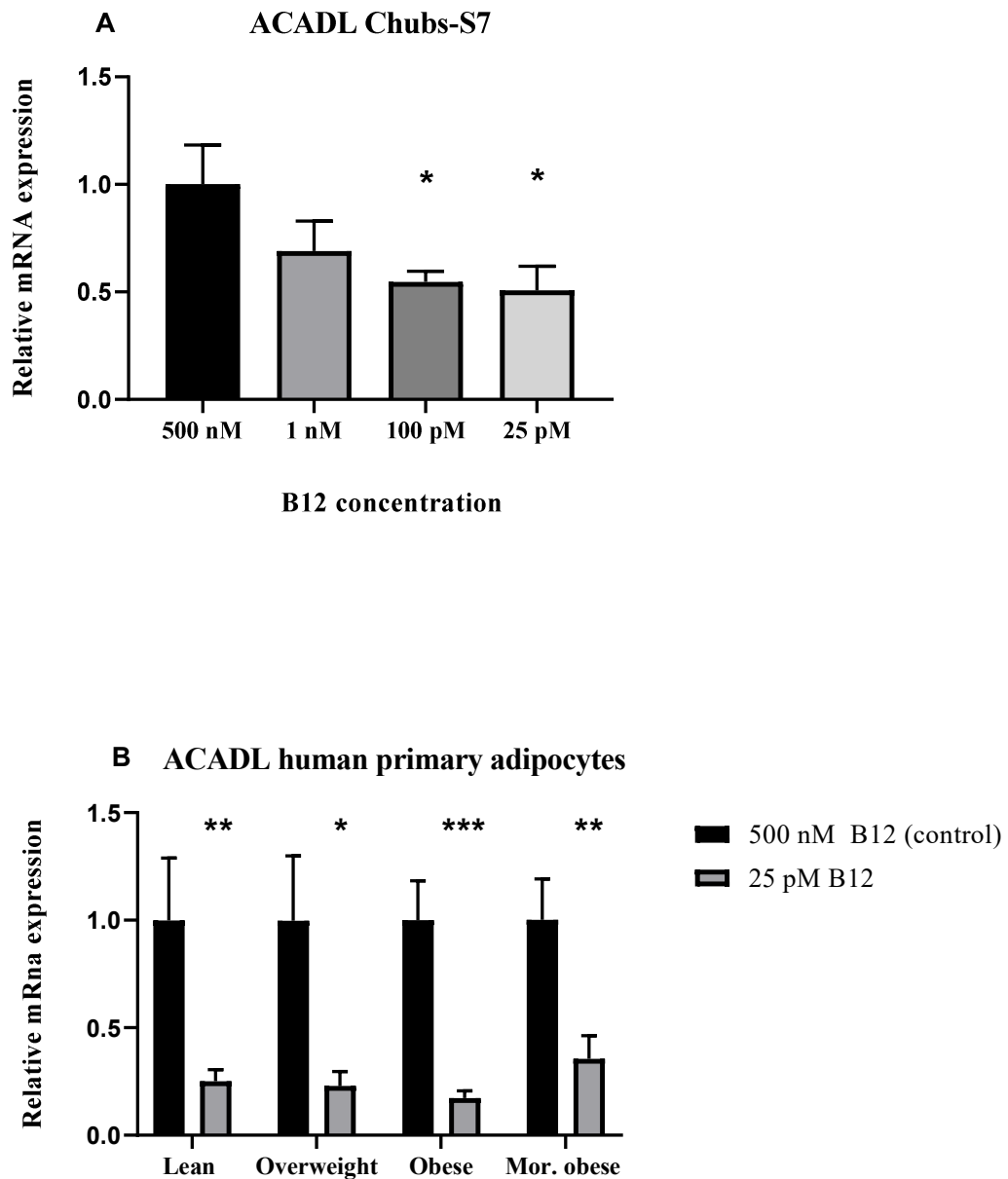


Figure 43: Gene expression of Acyl-CoA Dehydrogenase Long Chain (ACADL) is altered in B12 deficient adipocytes: The graphs show mRNA expression of ACADL in Chubs-S7 and primary adipocytes (three cultures per group of BMI) derived from subcutaneous depots. The adipocytes were seeded and differentiated respectively with four and two different concentrations of B12 and mRNA expression was measured with RT-qPCR. The mRNA levels were normalized to L19. Data are expressed as mean \pm SE and significance levels are indicated as follows; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control (500nM) according to Mann-Whitney U's test.

5.2.1.5 Enoyl-CoA Hydratase, Short Chain 1 (ECHS1)

The protein encoded by this gene catalyses the second step of the mitochondrial FAO pathway. This enzyme is localized in the mitochondrial matrix and catalyses the hydration of 2-trans-enoyl-coenzyme A to L-3-hydroxyacyl-CoAs by the addition of a water molecule to the double bond of a *trans*-2-enoyl-CoA thioester (Fig 44).

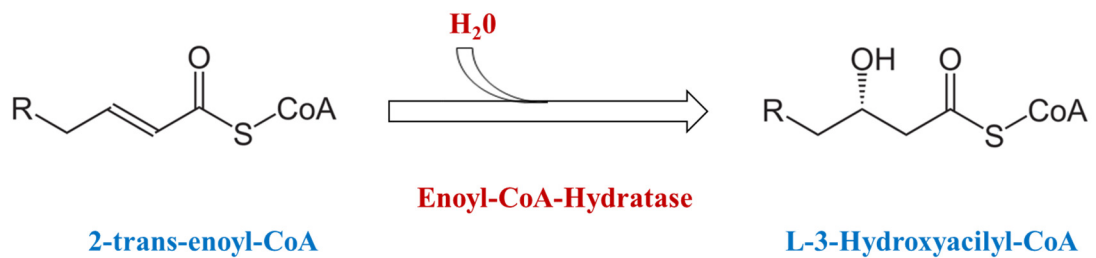


Figure 44: Catalytic action of Enoyl-CoA Hydratase

As described in figure 45A ECHS1 relative mRNA expression results to be progressively reduced in Chubs-S7 cultured in low B12 conditions with significance reached in 100pM and 25pM conditions. These results were confirmed in B12 deficient primary adipocytes where a 50% reduction has been shown in lean, obese and morbid obese derived cells (figure 45B).

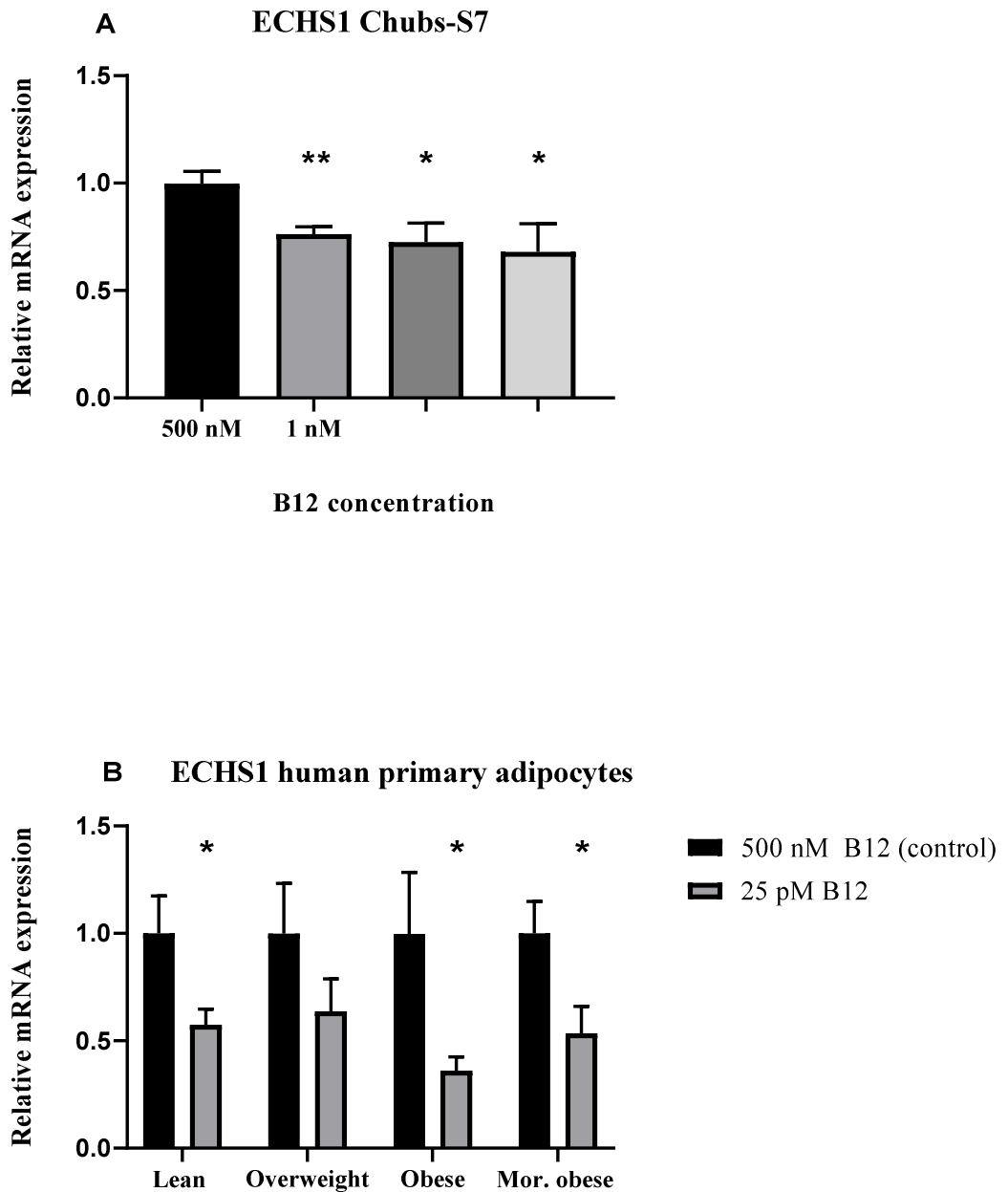


Figure 45: Gene expression of Enoyl-CoA Hydratase, Short Chain 1 is decreased in B12 deficient adipocytes. The graphs show mRNA expression of ECHS1 in Chubs-S7 and primary adipocytes (three cultures per group of BMI) derived from subcutaneous depots. The adipocytes were seeded and differentiated respectively with four and two different concentrations of B12 and mRNA expression was measured with RT-qPCR. The mRNA levels were normalized to L19. Data are expressed as mean \pm SE and significance levels are indicated as follows; * $p < 0.05$, ** $p < 0.01$, compared to control (500nM) according to Mann-Whitney U's test.

5.2.1.6 Acetyl-CoA Acyltransferase 2 (ACAA2)

Acetyl-CoA Acyltransferase 2 also known as 3-Ketoacyl-CoA Thiolase catalyses the last step of the mitochondrial FAO spiral. It catalyses, using one molecule of coenzyme CoA, the thiolytic cleavage of medium- to long-chain unbranched 3-oxoacyl-CoAs into acetyl-CoA resulting in a fatty acyl-CoA shortened by two carbon atoms (Fig 46).

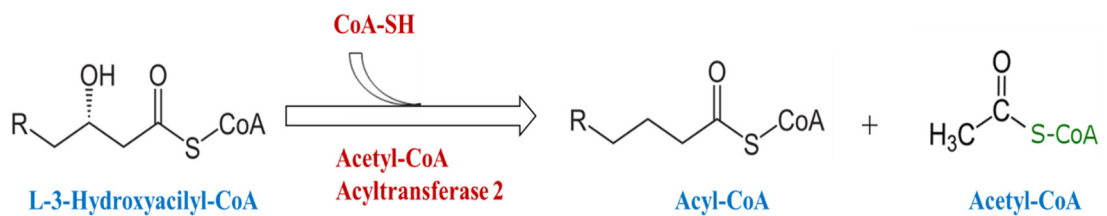


Figure 46: Catalytic action of Acetyl-CoA Acyltransferase 2

The *in vitro* experiments showed a significant downregulation of the relative mRNA expression ACAA2 in B12 deficient Chubs-S7 compared to the control (figure 47A). This result was confirmed in all the metabolic phenotype derived adipocytes where the gene expression was shown in a 4-fold decrease in adipocytes derived from obese subjects compared to adipocytes derived from lean subjects (figure 47B).

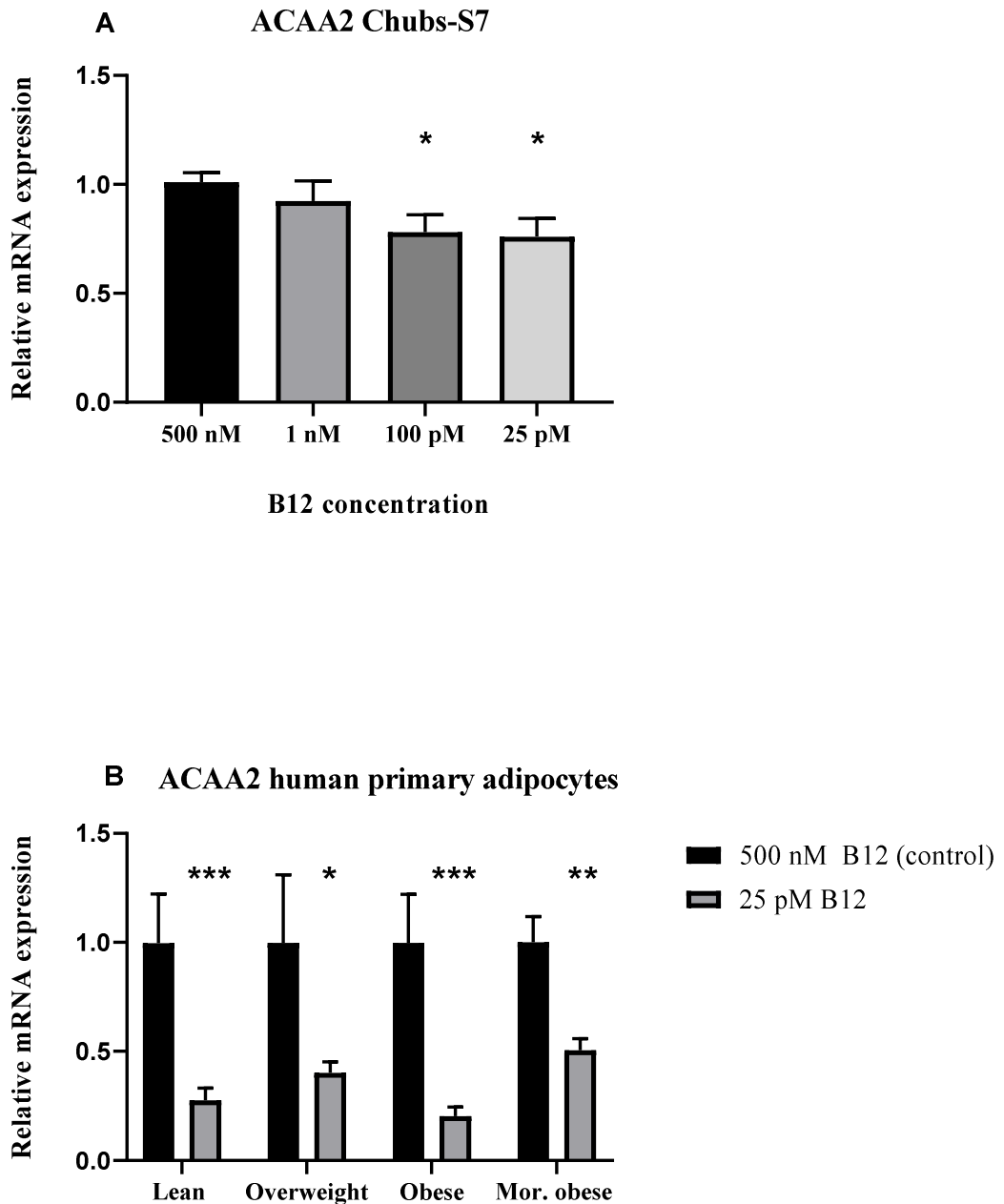


Figure 47: Gene expression of Enoyl-CoA Hydratase, Short Chain 1 is decreased in B12 deficient adipocytes. The graphs show mRNA expression of ECHS1 in Chubs-S7 and primary adipocytes (three cultures per group of BMI) derived from subcutaneous depots. The adipocytes were seeded and differentiated respectively with four and two different concentrations of B12 and mRNA expression was measured with RT-qPCR. The mRNA levels were normalized to L19. Data are expressed as mean \pm SE and significance levels are indicated as follows; * $p < 0.05$, ** $p < 0.01$, *** $P < 0.001$ compared to control (500nM) according to Mann-Whitney U's test.

5.2.2 Effect of B12 deficiency on mitochondrial bioenergetic profiling of human adipocytes

Cells require energy in the form of ATP to support the biological processes of life, like growth, division, differentiation and many physiological activities. Mitochondria plays a central role in a variety of essential cell functions including FAO and oxidative phosphorylation and hence measurement of mitochondrial function would provide information of the cellular energy metabolism.

Chubs-S7 and primary pre-adipocytes were seeded at low density (10, 000 cells/well in 24 well Seahorse XF plates) and treated respectively with four (25 pM, 100 pM, 1 nM, 500 nM) and two different concentrations of B12 (25 pM and 500 nM), from the seeding to the full differentiation.

Analysing oxygen consumption rate (OCR) results, following application of stress test chemicals, provided information on aspects of mitochondrial function, as described in Figure 48. In Chubs-S7 adipocytes, an overall decrease of (OCR) was found in B12 deficient cells. Adipocytes cultured in 25 pM B12 media showed a 160% decrease in OCR, whilst cells grown in 100 pM media showed a 70% decrease compared to control (500 nM) as shown in figure 48 A. Similar results were obtained in primary adipocytes where B12 deficiency (25 pM) resulted in a 320% decrease of OCR compared with the control (figure 49 A).

We further analyzed the spare respiratory capacity, a measure of the ability of the cell to respond to an increased energy demand under stressful conditions. Spare respiratory capacity was progressively reduced in Chubs-S7 cultured in low B12 conditions and was significantly lower in 100pM and 25pM conditions (figure 48B). Similar results were obtained also in primary adipocytes where cell treated in B12 deficient media showed 140% decrease in spare respiratory capacity compared with the control as seen in figure 49B.

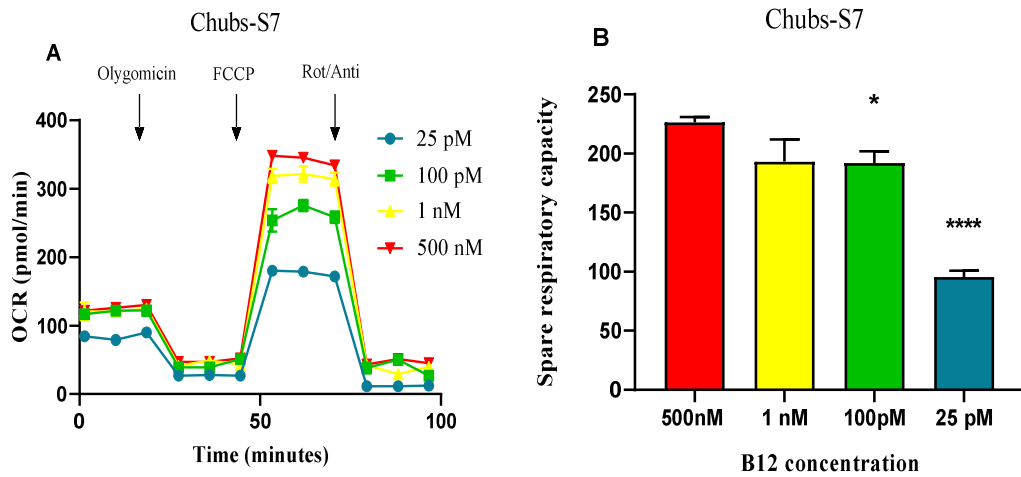


Figure 48: Low B12 compromises mitochondrial functional integrity of Chubs-S7 in rich-substrate media: The spare respiratory capacity (A) and oxygen consumption rate (OCR) (B) of Chubs-S7 in various conditions of B12 such as [500nM (control), 1nM (1000pM), 100pM and 25pM] and a rich-substrate KHB media containing glucose (2.5mM), pyruvate (1mM), amino acid (L-Glutamine) (2mM) and BSA (0.1%) at pH 7.4 and 37°C temperature,. The data is representative of mean \pm SEM (n=6), and * indicates significance compared to control; * p< 0.05, *** p< 0.001 according to Mann-Whitney U's test.

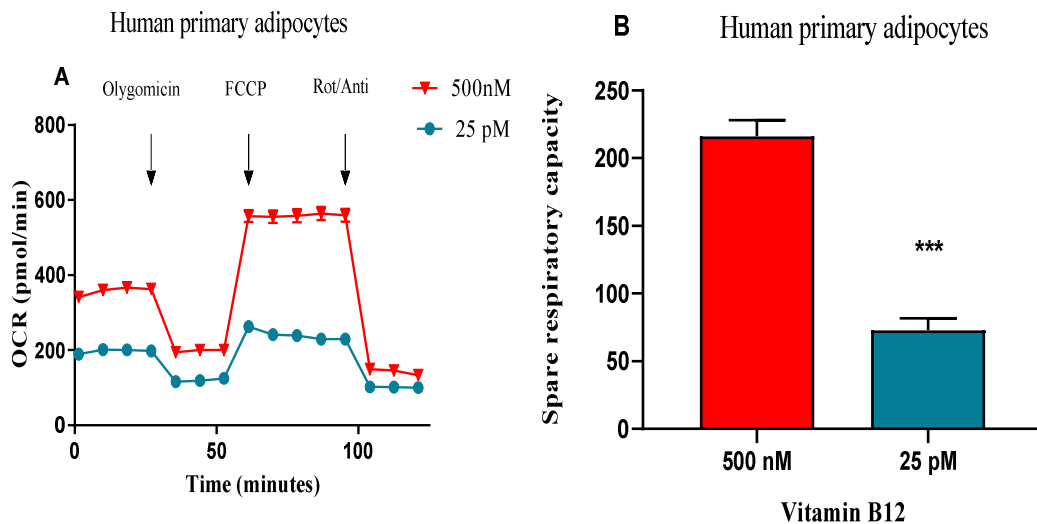


Figure 49: Low B12 compromises mitochondrial functional integrity of primary adipocytes in rich-substrate media: The spare respiratory capacity (A) and oxygen consumption rate (OCR) (B) of primary adipocytes in various conditions of B12 such as [500nM (control) and 25pM] and a rich-substrate KHB media containing glucose (2.5mM), pyruvate (1mM), amino acid (L-Glutamine) (2mM) and BSA (0.1%) at pH 7.4 and 37°C temperature. The data is representative of mean \pm SEM (n=6), and * indicates significance compared to control; *** p< 0.001 according to Mann-Whitney U's test.

5.2.3 Effect of low B12 on fatty acid-driven oxygen consumption in human adipocytes

A negative modulation of fatty acid beta oxidation can be related to adverse effects such as liver injury, T2DM and obesity. Hence, in vitro assays that are able to identify elements that affect FAO results to be valuable. A disruption in FAO enzymes gene expression by B12 deficiency has been previously shown in this chapter. Therefore, in order to measure fatty acid-driven oxygen consumption, Seahorse XF FAO assay was used in Chubs-S7 and primary adipocytes as described in section 2.2.5. After the basal respiration (OCR) was measured in adipocytes, the cells were then exposed to 200 μ M Palmitate or 33.3 μ M BSA (basal control) in the substrate medium to assess how adipocytes efficiently uptake palmitate for ATP production. As shown in figure 50, fatty acid stress test using palmitate revealed that Chubs-S7 and primary adipocytes cultured in B12 deficient media have a lower spare respiratory capacity increase in response to palmitate than adipocytes cultured in 500 nM B12 media (control group) where a significant increase in the oxygen consumption has been shown compared to basal control (BSA). These data suggest a disruption of FAO in B12 deficient adipocytes.

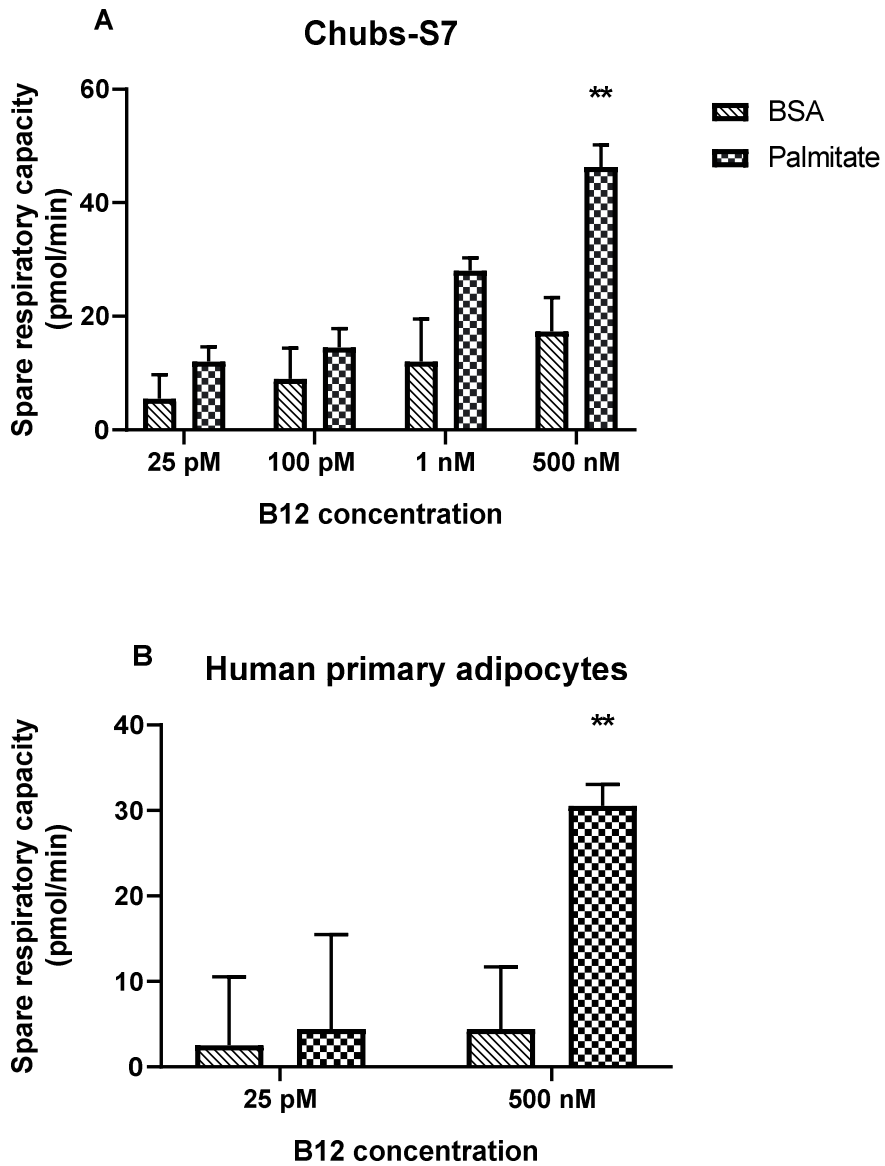


Figure 50: B12 deficiency downregulates fatty acid oxidation of Chubs-S7 and primary adipocytes. Measurement and quantification of mitochondrial spare respiratory capacity in Chubs-S7 and primary adipocytes with fatty acid stress test using palmitate versus BSA control. The data is representative of mean \pm SEM (n=6), and * indicates significance compared to control; ** p< 0.01 according to Mann-Whitney U's test.

5.2.4 Experiments in human AT:

Gene expression of fatty acid transporters and genes involved in fatty acid β -oxidation in human OmWAT and ScWAT

To further investigate the extent of the *in vitro* data obtained in Chubs-S7 cell line and human primary adipocytes, expression of genes involved in FAO was performed on OmWAT and ScWAT derived from our cohort study.

After blood analysis test, patients' AT were divided into two groups according to their B12 values (<200pg/mL and >200pg/mL) and gene expression assay was undertaken.

Our findings revealed, as confirmed by the observations in Chubs-S7 and primary adipocytes, that the relative mRNA expression of FAO enzymes MCD, CPT1 β , CPT2, ACADL, ECHS1 and ACAA2 significantly decreased in both OmWAT and ScWAT of B12 deficient patients compared with subjects with adequate levels of B12 (figure 51).

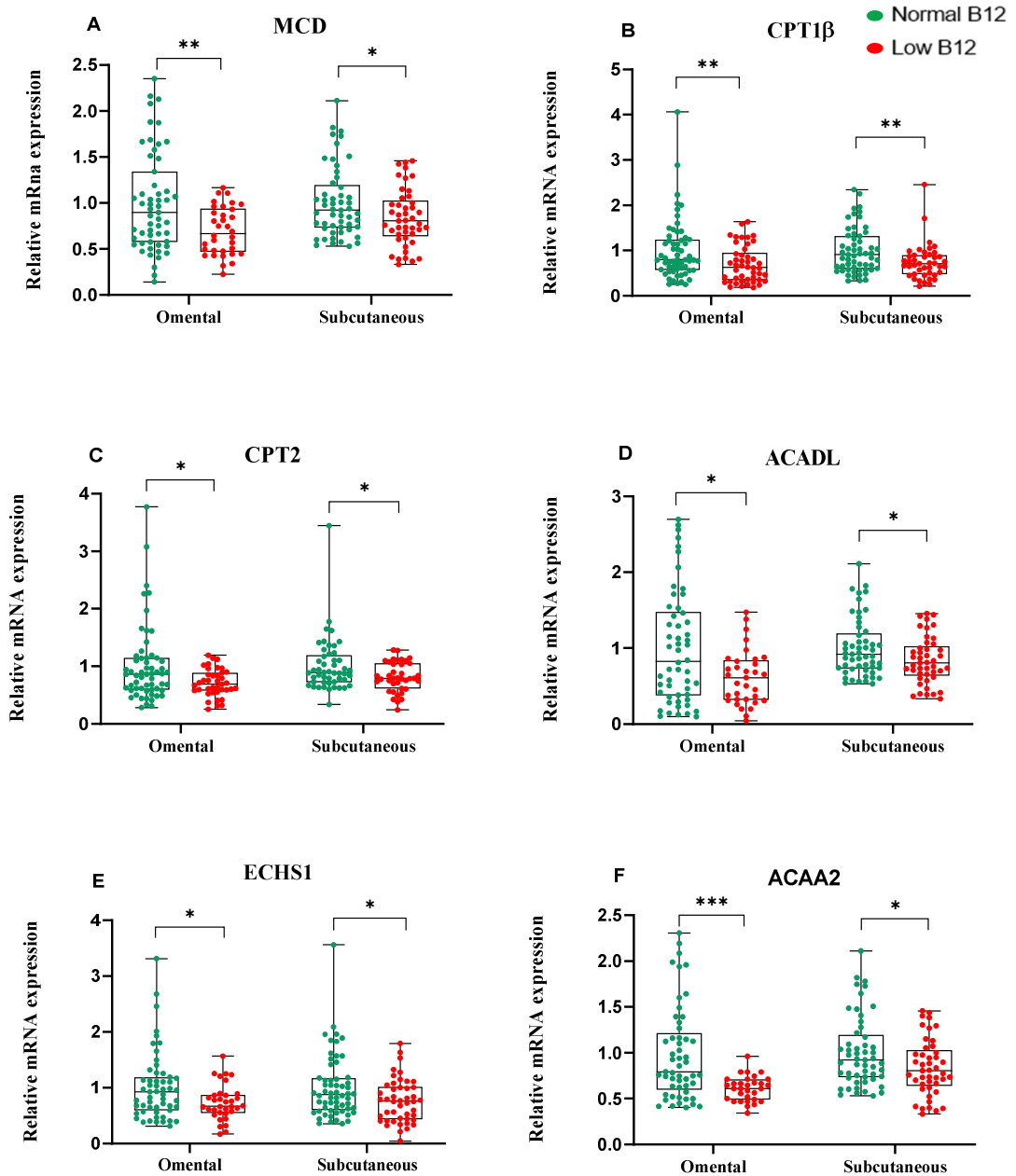


Figure 51: Effect of B12 deficiency on genes involved in fatty acid oxidation in human omental and subcutaneous adipose tissue. Human OmWAT and ScWAT obtained from 106 pregnant women were used to assess gene expression of FAO enzymes through RT-qPCR. Patients were divided into two groups according to their vitamin B12 levels [Normal B12 (green)= 60 patients and LowB12 (red) = 46 patients] and gene expression assay was assessed. Data are not-normally distributed and reported as box charts. *P*-value derived from Mann-Whitney U-test is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Normal B12 ($> 200\text{pg/ml}$) low B12 ($< 200\text{pg/ml}$).

5.2.5 Correlation between human OmWAT and ScWAT fatty acid β -oxidation enzymes and biochemical parameters.

The correlation analysis revealed the relationship between biochemical values and FAO enzymes gene expression both in OmWAT and ScWAT.

As shown in table 5 and figure 53, in the human ScWAT, a positive correlation between B12 and gene expression of MCD ($r=0.203$, $p=0.033$), CPT1 β ($r=0.212$, $p=0.026$) CPT2 ($r=0.325$, $p=0.0004$), ACADL ($r=0.213$, $p=0.027$), ECHS1 ($r=0.222$, $p=0.023$) and ACAA2 ($r=0.200$, $p=0.036$) has been found. A positive correlation was found between HDL and ScCPT2 ($r= 0.231$, $p= 0.015$) and ScECHS1 ($r=0.192$, $p=0.044$).

Furthermore, In the human OmWAT (table 6 and figure 52), the correlation analysis showed a positive association between B12 and gene expression of MCD ($r=0.199$, $p=0.039$), CPT1 β ($r=0.209$, $p=0.026$), CPT2 ($r=0.195$, $p=0.046$) and ACAA2 ($r=0.289$, $p=0.001$).

Relative Subcutaneous mRNA expression	Serum biochemical values			
	B12 (pg/mL)	Glucose (mmol/L)	Triglycerides (mmol/L)	HDL (mmol/L)
MCD	0.203*	0.041	0.022	0.129
CPT1β	0.212*	0.010	0.086	-0.114
CPT2	0.325***	-0.062	-0.067	0.231*
ACADL	0.213*	0.014	0.048	0.114
ECHS1	0.222*	0.052	-0.009	0.192*
ACAA2	0.200*	0.014	0.048	0.114

Table 5: Subcutaneous fatty acid β -oxidation gene expression correlates with serum biochemical metabolic related values: Correlation between subcutaneous FAO gene expression and serum metabolic related biochemical values. Data are represented as Spearman's R values. Abbreviation: MCD Malonyl-CoA decarboxylase, CPT1 β Carnitine palmitoyltransferase-1, CPT2 Carnitine palmitoyltransferase-2, ACADL Acyl-CoA Dehydrogenase Long Chain, ECHS1 Enoyl-CoA Hydratase, Short Chain 1, ACAA2 Acetyl-CoA Acyltransferase 2, B12 vitamin B12. ECHS1 Enoyl-CoA Hydratase, Short Chain 1, ACAA2 Acetyl-CoA Acyltransferase 2, B12 vitamin B12, HDL high-Density lipoprotein.

Relative Omental mRNA expression	Serum biochemical values			
	B12 (pg/mL)	Glucose (mmol/L)	Triglycerides (mmol/L)	HDL (mmol/L)
MCD	0.199*	-0.050	0.113	-0.072
CPT1β	0.209*	0.155	0.047	0.019
CPT2	0.195*	-0.202*	-0.217*	0.106
ACADL	0.004	0.074	0.079	0.088
ECHS1	0.095	0.007	-0.066	0.113
ACAA2	0.289**	0.023	-0.215*	-0.025

Table 6: Omental fatty acid β -oxidation gene expression correlates with serum biochemical metabolic related values: Correlation between omental FAO gene expression and serum metabolic related biochemical values. Data are represented as Spearman's R values. Abbreviation: MCD Malonyl-CoA decarboxylase, CPT1 β Carnitine palmitoyltransferase-1, CPT2 Carnitine palmitoyltransferase-2, ACADL Acyl-CoA Dehydrogenase Long Chain, ECHS1 Enoyl-CoA Hydratase, Short Chain 1, ACAA2 Acetyl-CoA Acyltransferase 2, B12 vitamin B12, HDL high-Density lipoprotein

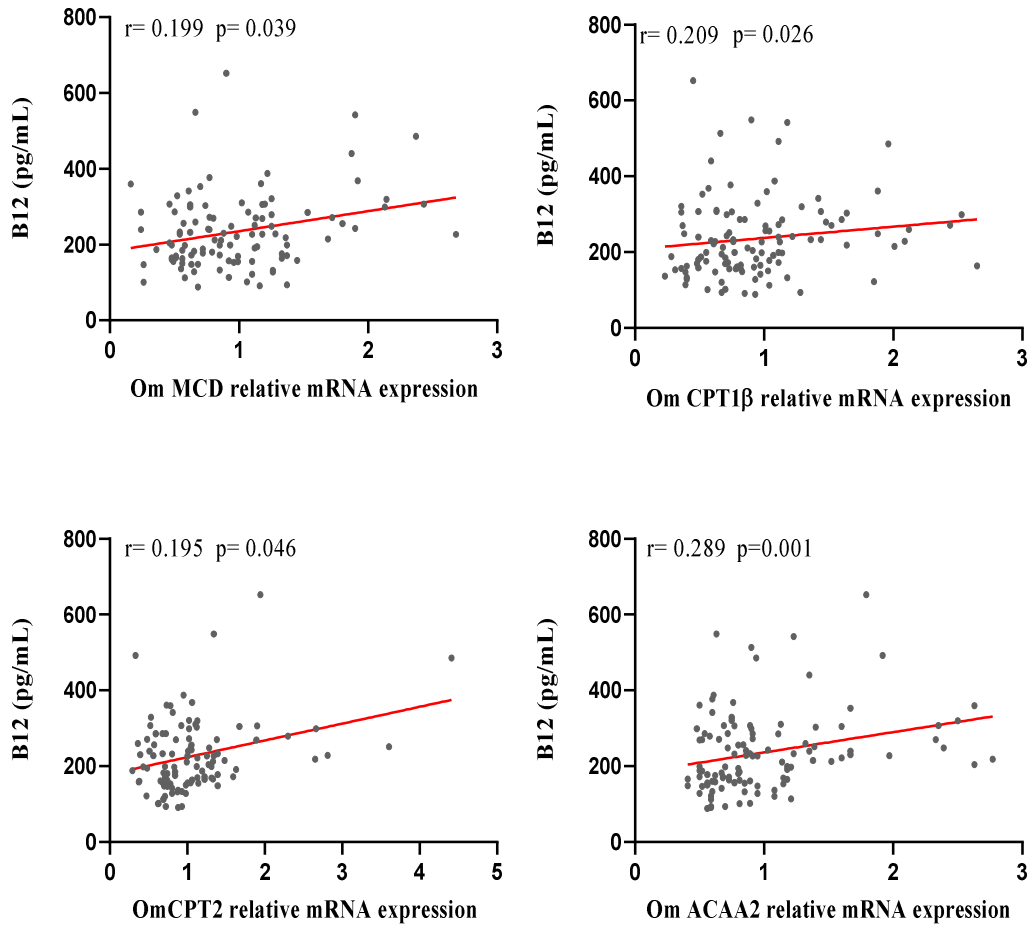


Figure 52: Omental fatty acid oxidation gene expression correlate with serum B12 levels

Correlation between omental MCD, CPT1 β , CPT2, ACAA2 and serum B12. Dots depict individual study subjects and data are presented as Spearman's R values. MCD Malonyl-CoA decarboxylase, CPT1 β Carnitine palmitoyltransferase-1, CPT2 Carnitine palmitoyltransferase-2, ACAA2 Acetyl-CoA Acyltransferase 2.

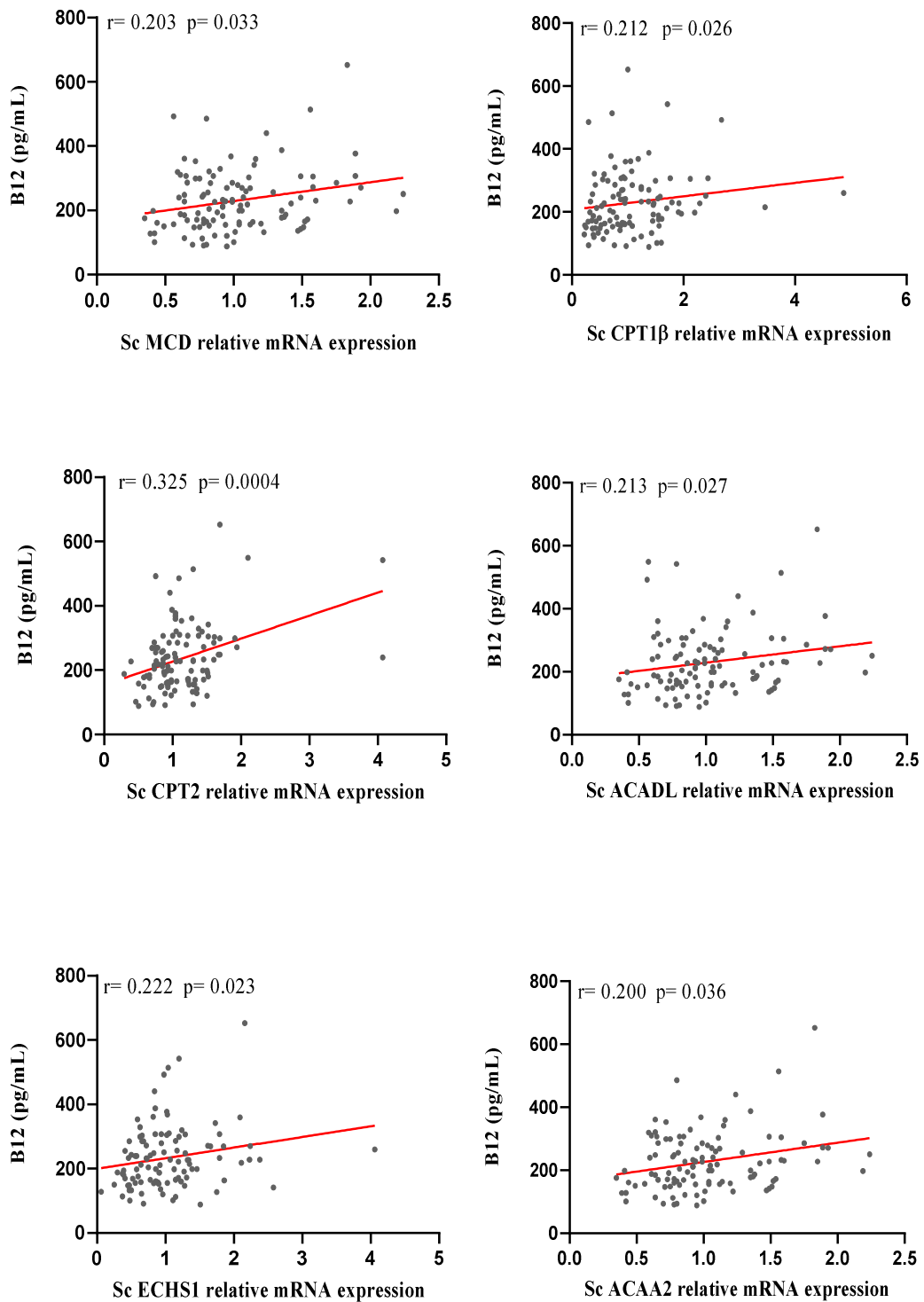


Figure 53: Subcutaneous fatty acid oxidation gene expression correlate with serum B12 levels

Correlation between subcutaneous MCD, CPT1 β , CPT2, ACADL, ECHS1, ACAA2 and serum vitamin B12. Dots depict individual study subjects and data are presented as Spearman's R values. MCD Malonyl-CoA decarboxylase, CPT1 β Carnitine palmitoyltransferase-1, CPT2 Carnitine palmitoyltransferase-2, ACADL Acyl-CoA Dehydrogenase Long Chain, ECHS1 Enoyl-CoA Hydratase, Short Chain 1 ACAA2 Acetyl-CoA Acyltransferase.

5.3 Discussion

The purpose of this study was to examine the effects of vitamin B12 deficiency on FAO. Here we have demonstrated that human pre-adipocytes cell line and the pre-adipocytes derived from different metabolic phenotypes when exposed to low levels of B12 showed lower levels of expression of genes involved in FAO. Similarly, human mature AT from different depots, such as OmWAT and ScWAT, showed a decrease in FAO in patients with B12 deficiency. Moreover, we have demonstrated, in both cell line and primary adipocytes, that low B12 resulted in reduced mitochondrial function. In addition, correlation study findings indicate an association between FAO gene expression and B12 in both depots of AT.

Decrements in FAO capacity have been reported in obese humans and rodents demonstrating lipid accumulation and lipotoxicity (Adams *et al.*, 2009; Tanaka *et al.*, 2013). FAO plays an important role in energy metabolism and among obesity-derived AT dysfunctions, has been hypothesized to contribute to metabolic related disorders. For this reason, we investigated the role of B12 deficiency on the gene expression of each key metabolic enzyme involved in this mechanism.

Malonyl-CoA decarboxylase (MCD) has been the first target of investigation because it reduces the concentration of malonyl-CoA, a potent endogenous inhibitor of carnitine palmitoyl transferase 1, the rate-limiting enzyme of FAO (Ussher & Lopaschuk, 2008). In this study, we observed that B12 deficiency, in pre-adipocytes cultures (cell lines and primaries), has induced a significant downregulation of MCD. These observations were confirmed in the two compartments of mature AT derived from B12 deficient patients, where the OmWAT resulted to be more affected by B12 deficiency. These findings are in line with animal model studies in which an acute inhibition of FAO has been related with a decrease in FAO in the heart (Ussher *et al.*, 2016). This study is also in line with clinical studies conducted on IR muscle in which elevated malonyl-CoA levels and decreased FAO has been observed (Bandyopadhyay *et al.*, 2006) and on ScWAT of patients with T2DM where an increase of malonyl-CoA decarboxylase has been observed after treatment with Thiazolidenediones (Bogacka *et al.*, 2005). In this view, a deficiency of B12 would be involved in a decrease of malonyl-CoA by downregulating MCD, and upregulating ACACA, as shown in the previous chapter.

We then focused our interest on the study of carnitine palmitoyltransferase 1 (CPT1- β), the rate limiting enzyme that controls the entry of long-chain fatty acyl CoA into mitochondria. a strong downregulation of the gene expression has been observed in both Chubs-S7 cell lines and primary adipocytes treated in low B12 conditions. . These observations were established in OmWAT and ScWAT derived from B12 deficient patients. This study is in line with animal studies on CPT1 knockout mice which showed a diminished fat oxidation capacity and an increased lipid levels (Wicks *et al.*, 2015). A study conducted on liver tissue of wistar rat offspring where a deficiency of B12 led to increased cholesterol levels and reduced liver mRNA levels of CPT-1 (Khaire *et al.*, 2015). In the current study, the downregulation of CPT1- β due to reduced B12 levels would reduce the passage of Acyl-CoA for β -oxidation leading therefore to lipid accumulation.

In this study, a significant downregulation of carnitine palmitoyltransferase 2 (CPT2) gene expression, gene involved in the transport of long-chain FAs in the mitochondria has been observed in low B12 treated adipocytes and in both human AT compartments of B12 deficient patients. This study is in line with recent *in vitro* studies on hepatocytes in which a downregulation of CPT2 has been associated with acylcarnitine accumulation causing FA accumulation and obesity driven steatohepatitis (Fujiwara *et al.*, 2018). An accumulation of acylcarnitine has been also observed in B12 deficient rats in which an increase has been observed in serum and liver, confirming that changes in carnitine metabolism were consistent with the changes in CoA metabolism known to occur with B12 deficiency (Brass & Stabler, 1988).

Then we analysed the expression of Acyl-CoA Dehydrogenase Long Chain (ACADL), the mitochondrial enzyme that function to catalyse the initial step of the FAO (Indo *et al.*, 1991). In low B12 conditions, a downregulation of the gene expression in both Chubs-S7 cell lines and primary adipocytes cultured in B12 deficient conditions has been shown. These observations were confirmed in the two compartments of mature AT derived from B12 deficient patients. ACAD family enzymes has been negatively associated with T2DM and metabolic related diseases as confirmed by a study on ACAD10 deficient mice where impaired glucose tolerance, peripheral IR, and abnormal weight gain has been shown (Bloom *et al.*, 2018).

The effect of B12 deficiency on the second step of the mitochondrial FAO Enoyl-CoA Hydratase, Short Chain 1 (ECHS1), has been analysed on human adipocytes cell line, primaries and in OmWAT and ScWAT where a significant downregulation has been seen. A decrease in ECHS1 gene expression by B12 deficiency would lead to a reduction of FA metabolism, translating in lipid accumulation and then in obesity. In support to this is an *in vitro* study conducted on the AT of induced obese mice where a dysregulation of ECHS1 has been seen (Moreno-Viedma *et al.*, 2016).

We lastly investigated the role of vitamin B12 deficiency on Acetyl-CoA Acyltransferase 2 (ACAA2), the enzyme that catalyses the last step of mitochondrial FAO, discovering a downregulation of the gene expression both in human adipocytes and in OmWAT and ScWAT. This study is in line with clinical studies where a dysregulation of this enzyme has been associated with increased risk for coronary artery diseases and abnormal blood lipid levels (Kathiresan *et al.*, 2008; Willer *et al.*, 2008). Similar findings were observed in rat models where B12 deficiency resulted in decreased oxidation rate of palmitoyl-CoA and palmitoyl-L-carnitine in liver (Pooya *et al.*, 2012)..

B12 is required for the conversion of MMA to succinyl-CoA through the action of methylmalonyl-CoA mutase. During a B12 deficiency state the enzyme function of methylmalonyl-CoA mutase is inhibited, causing therefore an accumulation of MMA which is, along with other health diseases, hypothesized to affect mitochondrial respiration decreasing mitochondrial complexes II and I activity (Brito *et al.*, 2017; Kolker *et al.*, 2003; Preynat *et al.*, 2010; Wajner & Coelho, 1997). These data suggest the key role that B12 might play in the cellular respiration. According to these findings, the effect of B12 deficiency on mitochondrial health of human cell line and primary adipocytes has been explored through Seahorse XF technology. As predicted low B12 decreased the oxygen consumption rate and the spare respiratory capacity, translating in a reduced ability of the cell to respond to an increased energy demand under stressful conditions. This finding is in line with studies on rat liver where an inhibition of succinate dehydrogenase (respiratory complex II) has been shown after mitochondrial MMA accumulation secondary to vitamin B12 deficiency (Toyoshima *et al.*, 1995) and an *in vivo* and *in vitro* study conducted on B12 deficient mice in which a decreased respiration rate has been shown in liver mitochondria (Gessler *et al.*, 1992). Furthermore, this study highlighted through FA stress test, that B12 deficient adipocytes have a lower spare respiratory capacity increase in response to palmitate than adipocytes cultured in 500 nM B12 media (control group) confirming therefore that adipocytes under B12 deficiency are

inefficient in oxidising the fatty acid palmitate thereby implying FAO disruption in low B12 status.

In order to understand a possible relationship between B12 deficiency and metabolic disorder, we have further explored the clinical and biochemical characteristics of our cohort study based on B12 status and correlated with β -oxidation gene expression. Interestingly, in ScWAT all the genes involved in β -oxidation and taken in consideration in this study, result to be positively correlated with B12. A similar result has been observed also in OmWAT where MCD, CPT1 β , CPT2 and ECHS1 genes are associated positively with B12. This result validates the hypothesis of the important impact of B12 on management of lipid metabolism.

The correlation approach showed a positive association between ScWAT mRNA expression of CPT2 and ECHS1 with serum HDL levels, whereas in the OmWAT, a negative correlation has been observed between CPT2 and Glucose. Omental CPT2 gene expression, as well as ACAA2 negatively correlated with TAGs. These findings are in accordance with the theory that obesity decreases FAO and increases dyslipidaemia (Berggren *et al.*, 2008; Houmard, 2008; Serra *et al.*, 2013).

The exact biochemical explanation by which of cellular B12 deficiency leads to impairment in FAO and mitochondrial respiration is still well understood. According to the result of the present study the possible explanations that would be suggested are the following:

- 1) Decreased gene expression of malonyl-CoA decarboxylase that leads to increased malonyl-CoA levels that brings to downregulation of carnitine palmitoyl transferase (CPT-1) (Ruderman *et al.*, 2003);
- 2) Impairment in the biosynthesis of carnitine, molecule that plays an important role in facilitating the entry of long-chain FAs into the mitochondria via the carnitine shuttle (Brito *et al.*, 2017)..
- 3) Increase in MMA levels due to reduced mitochondrial chain respiration, translating therefore in a decreased respiratory capacity and mitochondrial health (Kolker *et al.*, 2003). Our data highlights therefore that low B12 induces a dysregulation of FA metabolism by downregulating FAO genes and inducing mitochondrial damage, which might lead to adipocyte dysfunction and suggest a possible role of B12 deficiency in metabolic disorders.

Chapter 6: Vitamin B12 and inflammation

6.1 Introduction

The adipose tissue is highly heterogeneous and besides its role as energy reservoir and endocrine organ, it's recognized as an important immune organ. AT is able to secrete both anti- and pro-inflammatory adipokines and comprises a large diversity of immune cells which allows the crosstalk between metabolism and immunity (Stolarczyk, 2017). The primary role of AT is to control energy balance and secrete a large number of hormones, proteins and lipids to coordinate and regulate other tissues. During over nutrition status, adipocytes enlarge in size for extra lipid storage causing hypertrophy and also ectopically accumulates in perivascular regions and liver (Reilly & Saltiel, 2017). The increased volume of adipocytes leads to an abnormal adipokine production, mainly cytokines, which results in activation of some pro-inflammatory signaling pathways. This develops into a low-grade chronic inflammation, typical of many of the comorbidities of obesity, including T2DM, non-alcoholic fatty liver disease, cancer, CVD, and neurodegenerative diseases.

According to the Third National Health and Nutrition Examination Survey (NHANES III) in the United States, the prevalence of low serum vitamin B12 status was in fact 3.2% among adults and rising up to 4.4% for those aged >50 years in 2010 (Evatt *et al.*, 2010). B12 deficiency in pregnant women is increasingly common and has been shown to be associated with higher BMI, GDM, and T2DM (Adaikalakoteswari *et al.*, 2014a; Sukumar *et al.*, 2016a; Yajnik *et al.*, 2008). Moreover, recent studies have indicated that B12 is an antioxidant and lower status of B-12 might be a potential trigger contributing to increased oxidative stress, particularly in patients with T2DM, a diseased condition related to oxidative stress and chronic inflammatory disease (Birch *et al.*, 2009). This antioxidant or anti-inflammation activity of B12 might, therefore, modulate oxidative and inflammatory stress responses. However, there are no studies on the role of B12 on pro-inflammatory genes in AT.

A recent clinical study showed association between B12 deficiency and high oxidative stress and inflammatory markers in diabetic and obese patients, suggesting a crucial role of this micronutrient in the development of metabolic related diseases (Al-Daghri *et al.*, 2016). B12, is an essential micronutrient required for several metabolic reactions (Saravanan & Yajnik, 2010), it acts as an essential cofactor for the mitochondrial conversion of methylmalonyl-CoA to succinyl-CoA, and its deficiency blocks this reaction and leading to FAO inhibition, resulting in AT enlargement through lipogenesis

(Adaikalakoteswari *et al.*, 2014a). Our group has also shown that B12 deficiency dysregulates cholesterol biosynthesis which may lead to adipocyte dysfunction (Adaikalakoteswari *et al.*, 2014) and cellular inflammation (Kumar *et al.*, 2013; Sukumar *et al.*, 2016).

Several animal and human studies have demonstrated that dyslipidemia can trigger pro-inflammatory status and increase production of pro-inflammatory cytokines developing into chronic diseases such as atherosclerosis (Catapano *et al.*, 2017; Fatkhullina *et al.*, 2016; Jung & Choi, 2014). Many cytokines play in fact a crucial role in inflammation of AT. IL-1 β is responsible of hyperplasia in ScWAT and protagonist of the regulation of lipogenesis in the macrophage cell membrane playing a central role between lipid metabolism and innate immunity (Speaker & Fleshner, 2012). TGF- β is implicated in different biological processes like cell migration and adhesion, tissue remodeling and controls multiple aspects of adipose biology. It's highly expressed in AT of obese patients and contribute to AT fibrosis (Lee, 2018). IL-6 cytokine is expressed by cells of the stromal vascular fraction like pre-adipocytes, endothelial cells and monocyte-macrophages and then secreted in the circulation. It is thought that 15 to 30% of circulating IL-6 levels is derived from AT without an acute inflammation. Several studies conducted both in mice and humans have shown high expression of this molecule in AT of obese and insulin-resistant subjects and *in vivo* and *in vitro* hyperglycemic models (Bastard *et al.*, 2006; Trayhurn & Wood, 2004). IL-8 is a pro-inflammatory cytokine produced mainly by macrophages and monocytes. It induces adhesion of monocytes to endothelium causing atherosclerosis and plays an important role in the modulation of inflammatory response. It's highly expressed in the AT of obese and insulin-resistant patients and correlates positively with visceral fat mass in obese subjects (Bruun *et al.*, 2004; Cimini *et al.*, 2017). IL-18 is a pro-inflammatory cytokine expressed in both ScWAT and OmWAT tissue and highly correlated with obesity and IR. Studies reported that IL-18 predict cardiovascular events and mortality in a population with metabolic syndrome (Ahmad *et al.*, 2017; Wood *et al.*, 2005). MCP1 is involved in inflammatory cells recruitment in AT of obese subjects. Meijer *et al* reported that adipocyte-derived MCP-1 can stimulate inflammation in human AT independently of macrophages. Large cohort studies conducted in Caucasians and Chinese population indicated that an increase of CCL2/MCP-1 in T2DM subjects (Meijer *et al.*, 2011; Raina *et al.*, 2015). TNF- α is mainly secreted by subcutaneous stromal vascular fraction cells. It has an autocrine and paracrine influence in a range of processes and plays a pivotal role in the stimulation and

production of other adipokines like IL-6. Several studies conducted on mice confirmed the increase of TNF- α expression in obese models and is linked to IR through the inhibition of the insulin receptor (Hotamisligil, 2003). Studies in humans show expression of TNF- α is increased in obesity and IR (Jung & Choi, 2014).

Since the association between B12 deficiency and BMI and lipid metabolism is shown in previous chapters, we hypothesize that B12 deficiency might play a role in inflammation. Hence, we decided to study the role of B12 on the major cytokines that play an important role in obesity and metabolic diseases. The aim of this study is to investigate and understand (1) the relationship between B12 and the processes involved in inflammation in *in vitro* human adipocyte cell; (2) endorse these findings in cultures of human primary pre-adipocytes taken from subjects with different degrees of BMI; (3) endorse these findings in mature AT from subjects with low and normal levels of B12 indifferent adipose depots such as ScWAT and OmWAT; (4) evaluate serum cytokine expression in patients with low and normal levels of B12.

6.2 Results

6.2.1 Effect of B12 on gene expression of inflammatory cytokines in Chubs-S7

Methods

To investigate the gene expression of inflammatory cytokines in Chubs-S7 pre-adipocytes under B12 deficiency conditions, were seeded in 6-well plates and differentiated as described in the methods section. Changes in mRNA expression of IL-1 β , IL-6, IL-8, IL-18, MCP-1, TGF- β and TNF- α in the four different condition of B12 were observed. Different cytokine mRNA levels measured were compared with the control (500 nM).

Results

As shown in figure 54, pre-adipocytes cultured in low B12 conditions (25 pM) showed significantly increased gene expression of pro-inflammatory cytokines such as IL-1 β , IL-6, IL-8, IL-18, MCP-1, TGF- β and TNF- α compared to control.

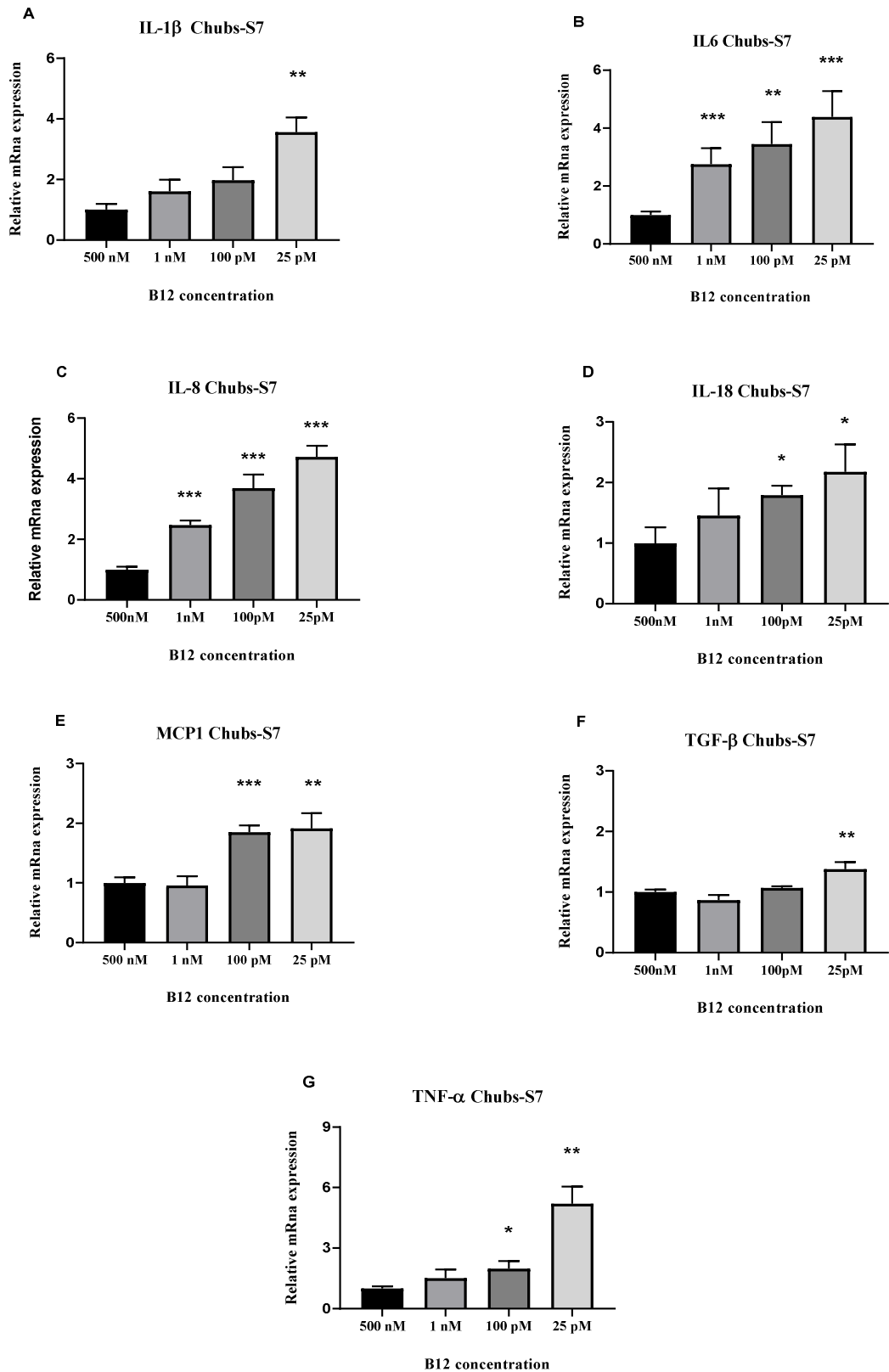


Figure 54: Gene expression of inflammatory markers altered in low B12 treated cells: The graphs show mRNA expression of IL-1 β , IL-6, IL-8, IL-18, MCP-1, TGF- β and TNF- α in Chubs-S7. The adipocytes were seeded and differentiated with four different concentrations of B12 and mRNA expression was measured by RT-qPCR. The mRNA levels of each gene were normalized to L19. Data are expressed as mean \pm SE and significance levels are indicated as follows; * p <0.05, ** P <0.01, *** P <0.001, according to Mann-Whitney U' test.

6.2.2 Effect of B12 on gene expression of inflammatory cytokines in human primary pre-adipocytes derived from different BMI groups.

Methods

To further assess the effect of B12 deficiency on the gene expression of inflammatory cytokines, and confirm the results observed in Chub-S7 cell line, we performed our experiments in human subcutaneous primary pre-adipocytes derived from subjects with different degrees of obesity. Primary preadipocytes were treated with two different concentrations of B12 such as 25 pM (low B12) and 500 nM (considered the control), Different cytokine mRNA levels were compared with the control (500 nM).

Results

Our findings revealed that adipocytes from all the categories of BMI showed a significant increment in relative mRNA expression of inflammatory cytokines IL-1 β , IL-6, IL-8, IL-18, MCP-1, TGF- β and TNF- α when treated in B12 deficiency condition. Interestingly, morbidly obese and overweight derived adipocytes showed higher gene expression of cytokines in low B12. In particular, overweight derived adipocytes showed a 20-fold increase in IL-8 and MCP-1 mRNA expression. Morbid obese derived adipocytes showed 30-fold increase in IL-8 mRNA levels (figure 55).

■ 500 nM B12 (control)
 ■ 25 pM B12

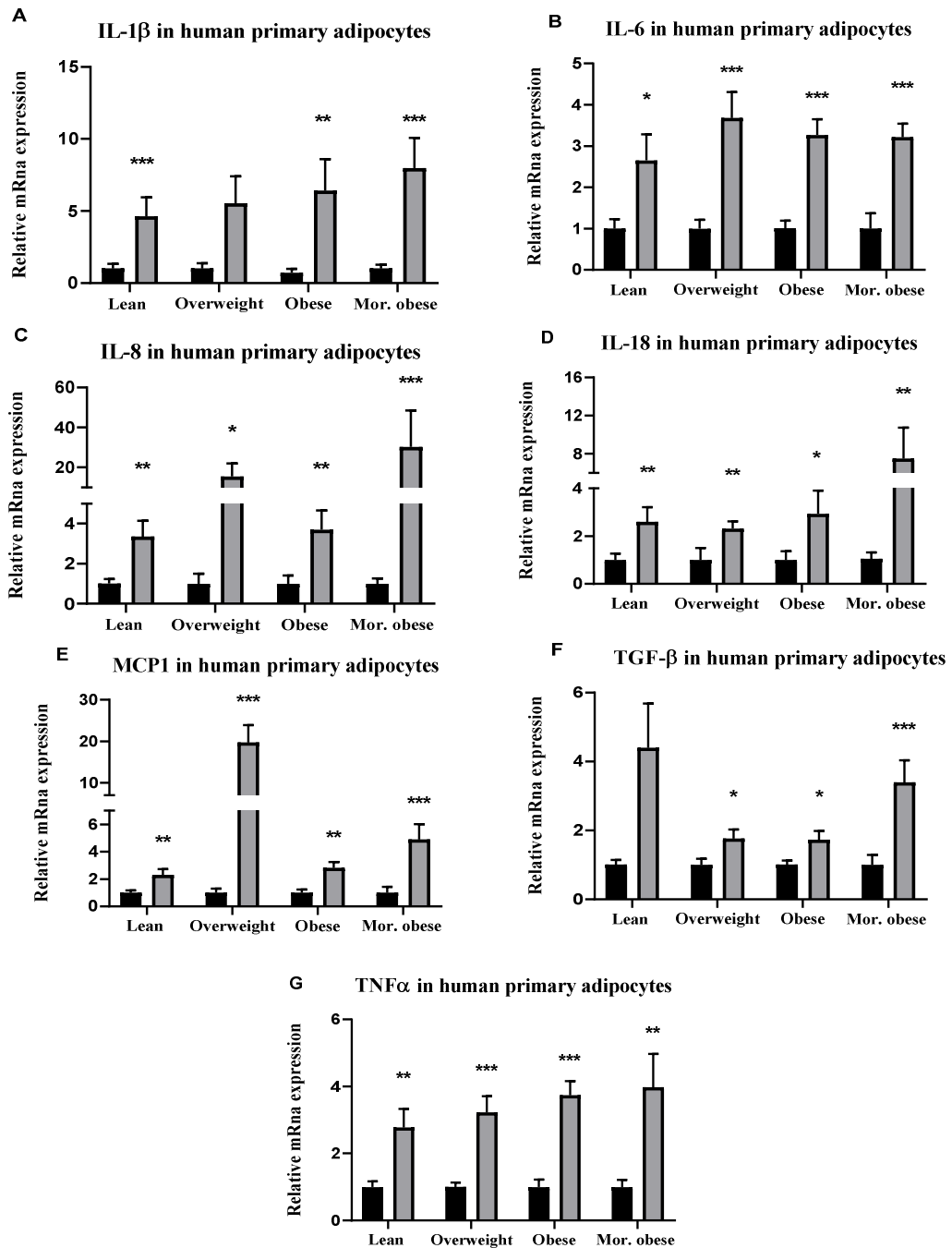


Figure 55: Effect of B12 deficiency on the gene expression of inflammatory cytokines in primary adipocytes derived from four categories of BMI. Primary subcutaneous derived adipocytes from lean, overweight, obese and morbidly obese patients (3 cultures per group), were seeded and differentiated with two different concentrations of B12 and mRNA expression was measured by RT-qPCR. Data are expressed as mean \pm SEM and significance levels are indicated as follows: *p<0.05, **p<0.01, ***p<0.001, compared to control (500nM), according to Mann-Whitney U's test.

6.2.4 Experiments in human AT:

Effect of B12 on gene expression of inflammatory cytokines in mature human AT from different depots

Methods

In order to investigate the extent of the data obtained in Chubs-S7 cell line and human primary adipocytes, we performed the study on human OmWAT and ScWAT taken from pregnant women. Different cytokine mRNA levels measured in different depots in low B12 AT and compared with the normal B12.

Results

Our findings revealed, as confirmed in cell line and primary adipocytes, a significant increase of mRNA expression of inflammatory cytokines IL-1, IL-6, IL-8, IL-18, MCP-1, TGF- β , TNF- α in both OmWAT and ScWAT of B12 deficient patients compared with subjects with adequate levels of B12 (figure 56) except TGF- β in OmWAT (figure 56F).

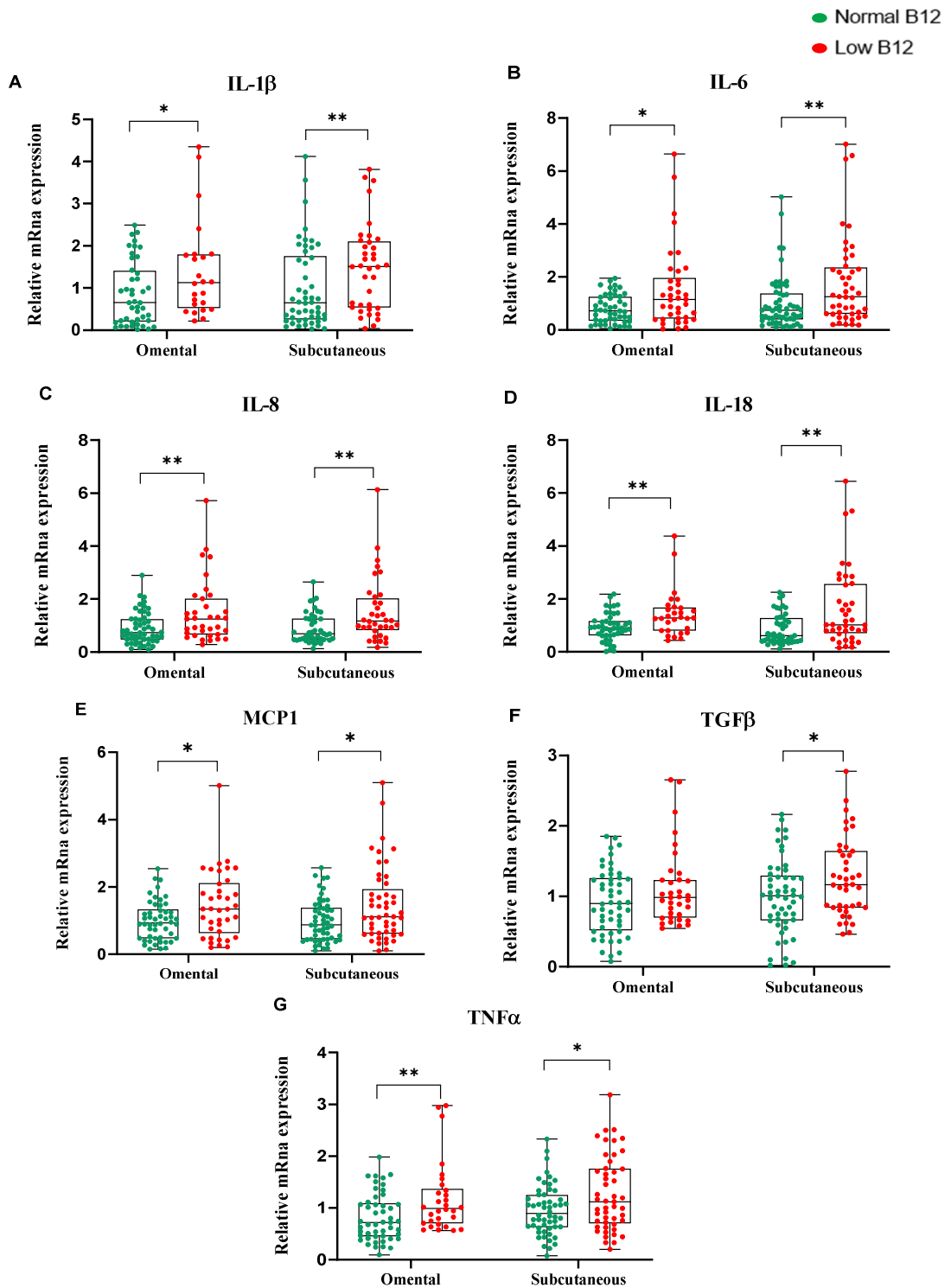


Figure 56 Effect of B12 deficiency on inflammatory cytokine gene expression in omental and subcutaneous adipose tissue. Omental and subcutaneous adipose tissue obtained from 106 pregnant women were used to assess gene expression of inflammatory cytokines by RT-qPCR. Patients were divided into two groups according to their vitamin B12 levels (Normal B12= 60 (green) and LowB12=46 (red)) and gene expression assay was assessed. Data are not-normally distributed and reported as box charts. *P*-value derived from Mann-Whitney U-test is indicated as * $p < 0.05$, ** $p < 0.01$. Normal B12 (> 200pg/ml) low B12 (<200pg/ml).

6.2.5 Serum inflammatory cytokines levels detection in the cohort study

To further validate the results obtained from the gene expression assay on the AT, we examined the serum levels of MCP1 and IL-8 pro-inflammatory cytokines. Elisa technique as described in section 2.3.3. As seen in figure 57, we found a significant increase of MCP1 and IL-8 in the serum of B12 deficient patients than the normal B12 level. This indicates that micronutrient B12 deficiency affects circulatory cytokines. On the other hand, current evidence demonstrated that B12 deficiency is linked with increased risk in the development of CVD and IR through hyperhomocysteinemia (Lai & Kan, 2015).

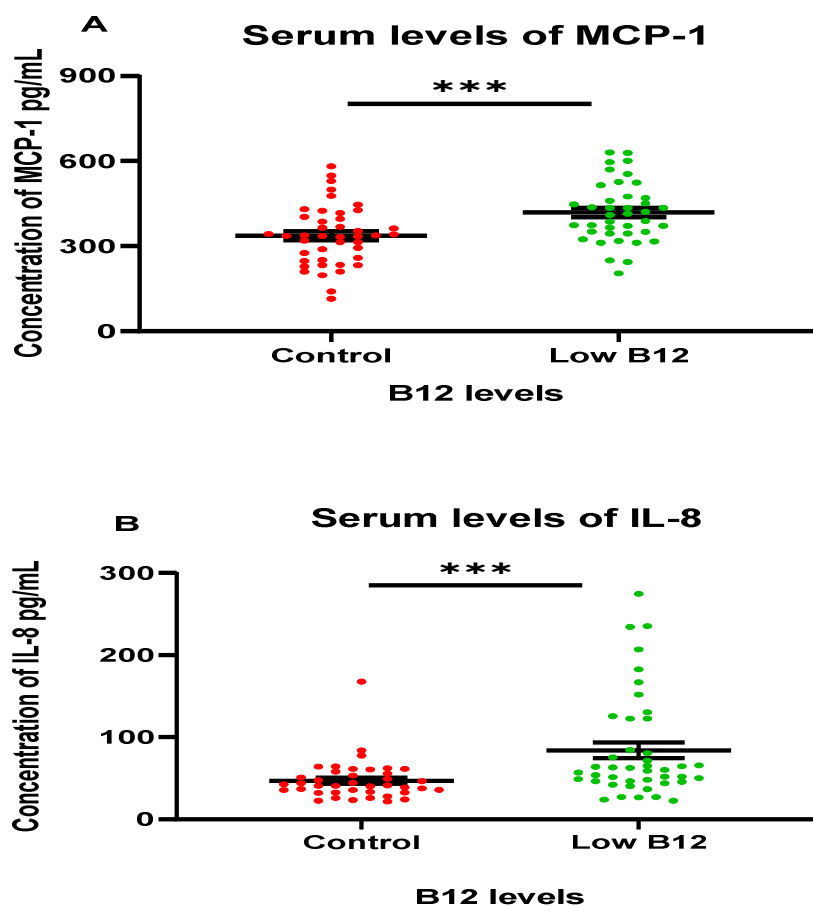


Figure 57: Inflammatory cytokines serum levels are increased in B12 deficient patients

(A) MCP1 and (B) IL-8 serum concentrations. Patients were divided into two groups according to their vitamin B12 levels (Normal B12 (control)=63 (red) and LowB12=52 (green)) serum cytokines concentrations were assessed. Data are not-normally distributed. Dots depict individual study subjects, the line represents the mean and the whiskers depict the standard error mean. *P*-value derived from Mann-Whitney U-test is indicated as ****p* < 0.001. Normal B12 (> 200pg/ml) low B12 (<200pg/ml).

6.2.6 Association between inflammatory cytokines in AT, circulating B12 and biochemical values

Next in order to investigate the relation between the gene expression of inflammatory cytokines, specific to mature AT and the serum levels of B12 and the biochemical parameters, we performed Spearman' correlation analysis.

Subcutaneous Adipose Tissue:

In ScWAT the correlation analysis showed a negative association between B12 and mRNAs of IL-1 β , IL-6, IL-18, MCP-1 and TNF- α but not IL-8 and TNF- β (Figure 58). A positive correlation has been found between ScIL-1 β and LDL as well as circulating MCP-1 and IL-8. A positive correlation has been ScIL-8 positively correlated with glucose and triglycerides and negatively with HDL. ScMCP-1 negatively correlated with serum HDL (Table 7).

Omental adipose tissue:

Figure 59 shows a negative association between B12 and mRNAs of IL-1 β , IL-18, and MCP-1 but not with IL-6, IL-8, TGF- β and TNF- α . In addition, OmIL-6 positively correlated with LDL and negatively with serum IL-8. OmIL-8 negatively correlated with glucose and HDL. OmIL-18 negatively correlated with HDL and positively correlated with TAGs and serum IL-8. OmTGF- β negatively correlated with cholesterol and LDL. OmTNF α positively correlated with glucose and HDL (Table 8).

Relative Sub. mRNA expression	Serum biochemical values						
	Glucose (mmol/l)	Chol. (mmol/l)	HDL (mmol/l)	LDL (mmol/l)	Triglycerides (mmol/l)	MCP-1 (pg/ml)	IL-8 (pg/ml)
IL-1β	0.080	0.136	0.056	0.263**	-0.063	0.325**	0.234*
IL-6	0.097	0.025	-0.029	0.026	0.026	-0.000	0.132
IL-8	-0.206*	-0.109	-0.203*	0.012	0.201*	0.138	0.121
IL-18	0.120	-0.005	-0.022	0.028	-0.030	0.009	0.122
MCP-1	-0.002	-0.063	-0.255**	0.011	0.134	-0.015	0.054
TGF-β	-0.087	-0.026	0.082	-0.060	-0.073	0.120	0.135
TNF-α	-0.031	0.156	0.110	0.138	0.030	0.006	0.0061

Table 7: Subcutaneous fatty acid β -oxidation gene expression correlates with serum biochemical metabolic related values

Correlation between subcutaneous inflammatory cytokines mRNA expression and serum metabolic related biochemical values. Data are represented as Spearman's R values. Abbreviation: IL-1 β interleukin-1 beta, IL-6 interleukin-6 IL-8, interleukin-8; IL-18, interleukin-18 MCP-1, monocyte chemoattractant protein-1, TGF- β transforming growth factor beta, TNF- α tumour necrosis factor alpha, HDL high-Density lipoprotein, LDL low-Density lipoprotein.

Relative Omental mRNA expression	Serum biochemical values						
	Glucose (mmol/L)	Cholesterol (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	Triglycerides (mmol/L)	MCP-1 (pg/ml)	IL-8 (pg/ml)
IL-1β	-0.060	0.196*	0.027	0.212*	-0.040	-0.250*	-0.013
IL-6	-0.083	0.077	0.070	0.203*	-0.044	-0.083	-0.257*
IL-8	-0.206*	-0.109	-0.203*	0.012	0.020	0.138	0.121
IL-18	0.006	0.029	- 0.333***	0.122	0.361**	0.160	0.232*
MCP-1	0.113	-0.008	-0.084	0.090	-0.033	0.145	0.030
TGF-β	0.049	-0.232*	-0.091	-0.215*	-0.061	0.140	0.140
TNF-α	0.203*	-0.037	0.198*	-0.070	-0.029	0.077	0.069

Table 8: Omental fatty acid β -oxidation gene expression correlates with serum biochemical metabolic related values

Correlation between omental inflammatory cytokines mRNA expression and serum metabolic related biochemical values. Data are represented as Spearman's R values. Abbreviation: IL-1 β interleukin-1 beta, IL-6 interleukin-6 IL-8, interleukin-8; IL-18, interleukin-18 MCP-1, monocyte chemoattractant protein-1, TGF- β transforming growth factor beta, TNF- α tumour necrosis factor alpha. , HDL high-Density lipoprotein, LDL low-Density lipoprotein.

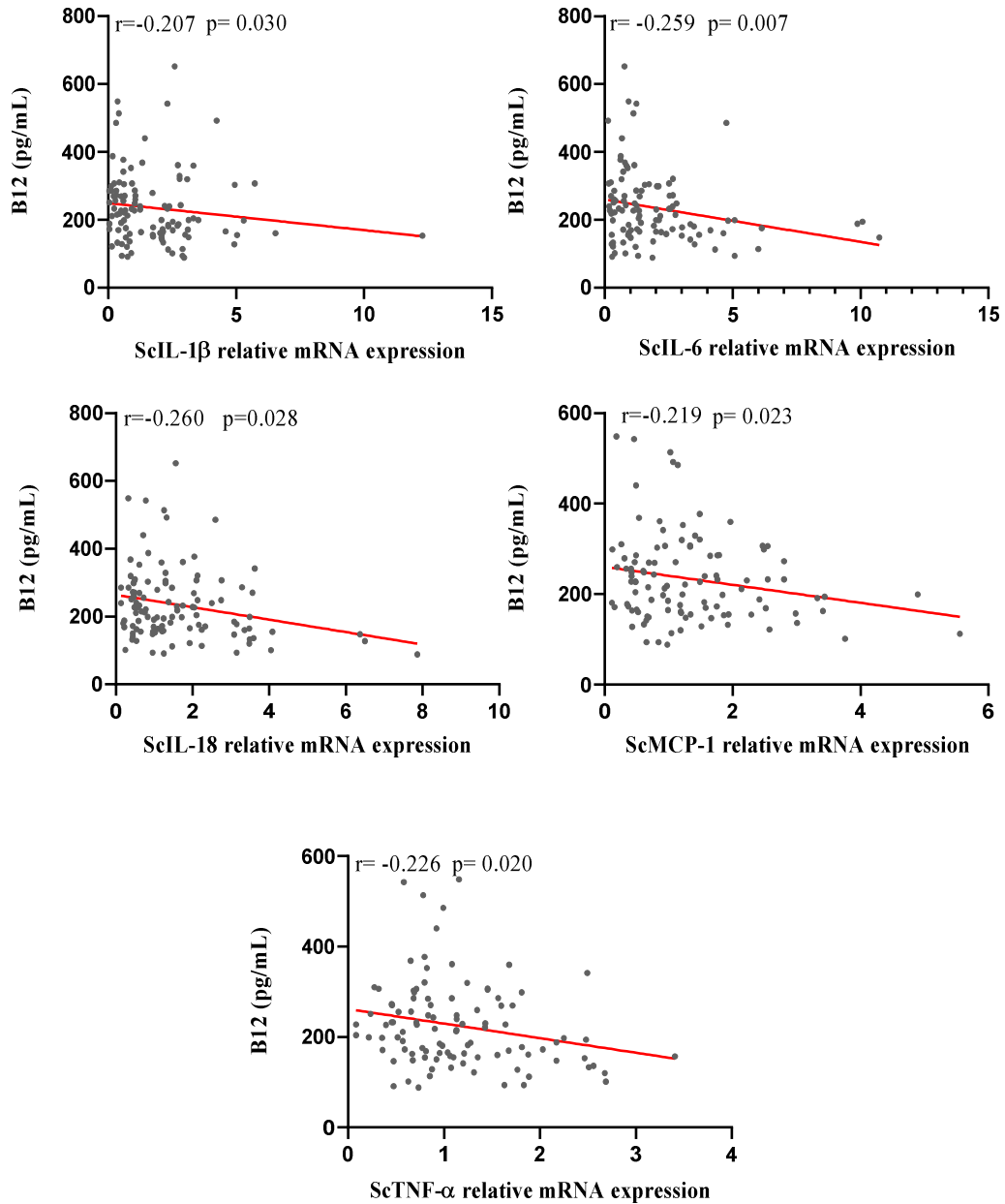


Figure 58: Subcutaneous inflammatory cytokines mRNA expression correlate with serum B12

Correlation between subcutaneous IL-1 β , IL-6, IL-18, MCP-1, TNF- α and serum vitamin B12. Dots depict individual study subjects and data are presented as Spearman's R values: IL-1 β interleukin-1 beta, IL-6 interleukin-6 IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1, TNF- α tumour necrosis factor alpha.

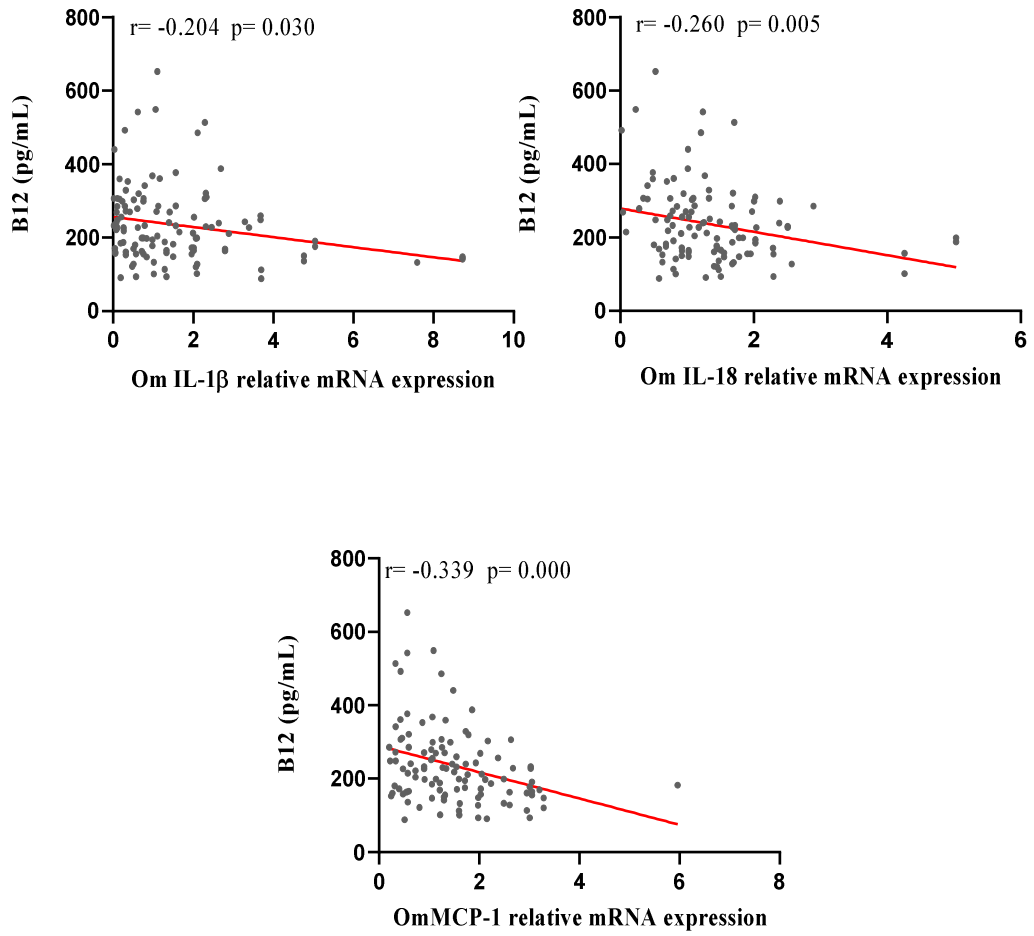


Figure 59: Omental inflammatory cytokines mRNA expression correlate with serum B12

Correlation between omental IL-1 β , IL-18, MCP-1 and serum vitamin B12. Dots depict individual study subjects and data are presented as Spearman's R values: IL-1 β interleukin-1 beta, IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1.

To understand the clinical and cardiometabolic variables in low and normal B12 levels, Table 9 shows these characteristics based on these circulating B12 status (This is also shown in chapter 3). HDL were lower TAGs were higher in the deficient group with similar levels of age, BMI and glucose levels. Serum folate was significantly higher in the normal B12 group (Table 9).

Interestingly, as shown in figure 60, serum MCP-1 and IL-8 were highly negatively correlated with B12 and a positive correlation has been found with glucose suggesting, therefore, a possible association of these parameters. Moreover, the inflammatory markers were significantly correlated with each other. No correlation has been found with other biochemical values.

Variables	B12 non-deficient group (>200pg/ml) n=63	B12 deficient group (<200pg/ml) n=52	P-value
Age (yrs.) [mean±SD]	32.1±5.7	31.5±6.5	0.296
Weight (kg) [mean±SD]	73.2±14.6	75.6±19.4	0.708
BMI (kg/m ²) [mean±SD]	26.5±3.9	28.5±6.37	0.041
Glucose (mmol/l) [mean±SD]	3.68±0.6	3.71±0.6	0.787
Cholesterol (mmol/l) [mean±SD]	6.6±1.4	6.7±1.5	0.947
HDL (mmol/l) [mean±SD]	1.7±0.4	1.4±0.2	0.0002
LDL (mmol/l) [mean±SD]	4.3±1.2	4.8±1.6	0.722
Triglycerides (mmol/l) [mean±SD]	2.9±0.9	3.5±1.0	0.0094
Folate (ng/ml) [mean±SD]	9.9±5.4	6.6±4.3	0.0009

Table 9: Clinical and biochemical characteristics according to vitamin B12 values. P-value from Mann-Whitney U-test. Abbreviations: SD, standard deviation; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

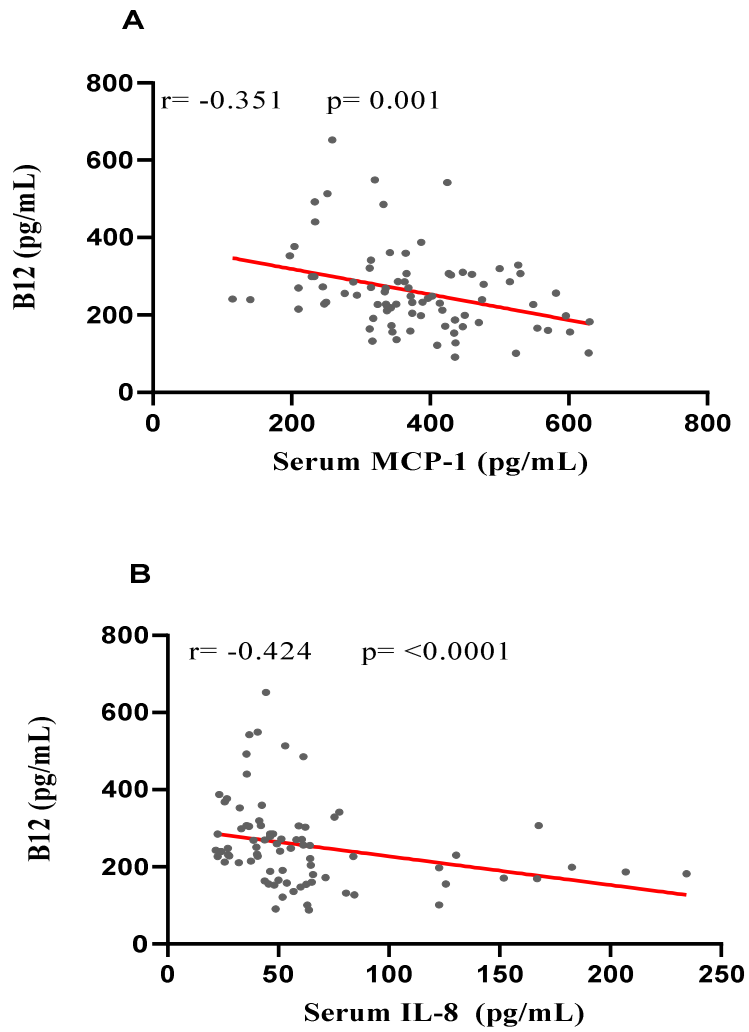


Figure 60: Correlation between serum inflammatory cytokine MCP-1 and IL-8 and serum vitamin B12

Correlation between serum levels of MCP-1 and IL-8 and serum B12. Dots depict individual study subjects and data are presented as Spearman's R values. IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1.

6.3 Discussion

The purpose of this study was to examine the effects of B12 deficiency on inflammatory genes in the AT and circulating levels. In this study, we found increased gene expression of inflammatory cytokines in human pre-adipocyte cell line, pre-adipocytes derived from human mature AT from individuals with different degrees of obesity and exposed to low levels of B12 as well as in human mature AT from different depots such as OmWAT and ScWAT. In addition, the circulatory cytokines levels were higher in subjects with B12 deficiency.

There is a well-organized interaction between the metabolic and immune system and an unbalanced nutritional status can bring instability in this well tight synergy. Over and under-nutrition status can, in fact, influence the proper immune functions by activating a low-grade stress condition that would lead to an activation of the immune system, or on the contrary a suppression (under-nutrition status). The activation of AT inflammation induces, therefore, a low-grade systemic inflammation leading to IR and development of metabolic related diseases. In this scenario, micronutrients seem to play a key role with several studies demonstrating the possible influence of an unbalanced micronutrient diet on health. For this reason, we investigated the role of B12 deficiency on key cytokine gene expression.

Interleukin 1 beta (IL-1 β) has been the first target of investigation, it's a cytokine expressed mainly by ScWAT with an autocrine and/or paracrine role. It has also been suggested that IL-1 β could have important role in the regulation of lipogenesis in the macrophage cell membrane giving to it a central role in the link between lipid metabolism and innate immunity (Speaker & Fleshner, 2012). In this study, we observed that B12 deficiency, in pre-adipocytes cultures (cell lines and primaries derived from different metabolic phenotypes), has induced a significant upregulation of IL-1 β . These observations were confirmed in the two compartments of mature AT derived from B12 deficient patients, where the ScWAT resulted the most affected compartment, confirming the literature. This study is in line with an in vitro study conducted on non-visceral AT in which lipid retain, hyperplasia and IL- 1 β has been observed after acute stress induction (Ha *et al.*, 2013). We then focused our attention on the study of interleukin 6 (IL-6) gene expression, cytokine expressed by cells of the stromal vascular fraction like pre-

adipocytes, endothelial cells and monocyte-macrophages and then secreted in the circulation. It is thought that 15 to 30% of circulating IL-6 levels derives from AT production without an acute inflammation (Trayhurn & Wood, 2004). In this study, we observed that B12 deficiency, in pre-adipocytes cultures (cell lines and primaries derived from different metabolic phenotypes), has induced a significant upregulation of IL-6. These observations were also confirmed in the two compartments of mature AT derived from B12 deficient patients where the ScWAT resulted the most affected. This study is in line with studies conducted both in mice and humans where a high expression of this molecule in AT of obese and insulin resistant subjects has been shown and hyperglycemia results in increased IL-6 levels as well (Bastard *et al.*, 2006).

This study observed also effect of B12 deficiency on Interleukin 8 (IL-8), a pro-inflammatory cytokine produced mainly by macrophages and monocytes and induce adhesion of monocytes to endothelium causing atherosclerosis. An upregulation of the gene expression has been shown in both B12 deficient adipocytes (Chubs-S7 and primary adipocytes) and on both human AT compartments derived from B12 deficient patients. These findings are in accordance with animal and human studies reporting that IL-8 seems to strongly correlate with visceral fat mass in obese subjects (Ha *et al.*, 2013; Strackowski *et al.*, 2002).

Interleukin 18 (IL-18) gene expression has been observed in B12 deficiency showing an increase in B12 deficient chubs-S7 and primary adipocyte derived from different metabolic phenotypes. These results were also confirmed in both ScWAT and OmWAT derived from B12 deficient patients. IL-18 is pro-inflammatory cytokine detected in both ScWAT and OmWAT and it has been positively correlated with obesity and IR. Studies conducted on mice in the past decade demonstrated a high positive correlation between circulating IL-18 levels and obesity and IR and a decrease of its levels after weight loss, in accordance with this study (Netea *et al.*, 2016; Wood *et al.*, 2005)

We further analysed the effect of B12 deficiency on Monocyte Chemoattractant Protein-1 (MCP-1) a chemokine highly expressed in OmWat and ScWAT of obese subjects and involved in recruiting monocytes, neutrophils and lymphocytes (Shimobayashi *et al.*, 2018). An upregulation of the gene expression in both B12 deficient adipocytes (Chubs-S7 and primary adipocytes, in which a strong upregulation has been seen in adipocytes derived from overweight patients probably due to an increased susceptibility of this category of BMI to B12 deficiency) and on both human AT compartments derived from

B12 deficient patients has been shown. This study is in accordance with *in vitro* and clinical studies in which an increase of MCP1 has been seen in T2DM subjects (Meijer *et al.*, 2011; Raina *et al.*, 2015).

This study observed also the effect of B12 deficiency on TGF- β gene expression, cytokine which expression is upregulated in obesity and its associated complications (Yadav *et al.*, 2011). It has been observed that B12 deficiency, in pre-adipocytes cultures (cell lines and primaries derived from different metabolic phenotypes), has induced a significant upregulation of TGF- β . These observations were confirmed in the ScWAT derived from B12 deficient patients. This finding is in line with studies on animal models in which an increase in relative mRNA expression of TGF- β in obese mice compared the lean group has been observed (Trayhurn & Wood, 2004).

We finally analysed the effect of B12 deficiency on tumour necrosis factor alpha TNF- α showing a significant increased gene expression of this cytokine in Chubs-S7 and adipocytes derived from different BMI categories cultured in low B12 conditions. Similar results were found in both of the AT compartments derived from B12 deficient patients with the OmWAT resulted more affected.

This study is in line with studies conducted on mice confirming the increase of TNF- α expression in obesity and has been proposed to be linked with IR through the inhibition of the insulin receptor (Hotamisligil, 2003a), whereas in humans the expression of TNF- α is increased in obesity and IR (Jung & Choi, 2014).

Our findings are in line with *in vitro* experiments conducted on SK-HEP-1, human hepatic adenocarcinoma cell line, which showed the B12 protective anti-oxidant role against exposure of high dosage of the pro-oxidants homocysteine and hydrogen peroxide (Birch *et al.*, 2009). Moreover, our results are in line with an earlier study conducted on rats showing that myelinolytic lesions in the spinal cord of rats made totally deficient of B12 by gastrectomy, is associated with increase in TNF- α (Scalabrino *et al.*, 1999). Studies have also shown an increase of serum transcobalamin levels in inflammatory diseases and an upregulation of CD320 receptor by pro-inflammatory cytokines (Kalra *et al.*, 2007; S. *et al.*, 1999). Experiments conducted on liver and spinal cord of B12 deficient rats confirmed the upregulation of TNF- α which induce an increase in nuclear NF- κ B (Veber *et al.*, 2008). Interestingly, the results obtained in cell line adipocytes were also confirmed in the adipocytes derived from all the four main categories of BMI. Since B12 deficiency causes an increase of inflammation in all categories of BMI, leads us to think

about its peculiar anti-inflammatory effect and the importance of this micronutrient in the prevention of metabolic related diseases.

Furthermore, the pro-inflammatory cytokines secretion in the peripheral blood has been examined showing a significant increase of MCP1 and IL-8 in the serum of B12 deficient patients, in accordance with the study on C57BL/6 mice with severe deficiency of B12 where an increase of inflammatory cytokines has been shown (Ghosh *et al.*, 2016). In support to our results, clinical findings on diabetic patient with B12 deficiency showed an increase in higher levels of glucose, inflammation markers and lower antioxidant enzymes activity than those with higher B12 status, thus suggesting that a deficiency of this micronutrient would increase the risk of having lower antioxidant capacity and higher inflammatory status (Lee *et al.*, 2016). Our findings are consistent with a series of interesting investigations conducted by Solini and colleagues, showing that a supplementation of folic acid (a methyl donor) in overweight patients contributes to a reduction of inflammatory cytokines (Solini *et al.*, 2006).

During pregnancy, the increased concentrations of progesterone and oestrogen and the increased hepatic lipase activity, contribute to the rise in total cholesterol, LDL cholesterol and TAGs (Bartels & O'Donoghue, 2011; Brizzi *et al.*, 1999).

Recent animal and human studies revealed that maternal hypercholesterolemia is linked with increased risks of preterm delivery, GDM, and preeclampsia, as well as the later development of atherosclerosis in offspring giving the idea of how an unbalanced nutritional status could be harmful to both maternal and foetal health. Moreover, during pregnancy, the maternal immune system undergoes significant changes to maintain a healthy body. Pregnancy is in fact characterized by an increase, depending on the stage, in both pro and anti-inflammatory cytokines levels which may lead to the activation of the immune system (Christian & Porter, 2014). Studies have been suggested that increased production of pro-inflammatory cytokines is associated with recruitment of immune cells to AT inducing an elevated risk of metabolic diseases in the mother and increase the risk of obesity in early childhood (Polari *et al.*, 2018). Nutrition plays an important role in the development of the foetus and on maternal health. Recent studies have in fact demonstrated that unbalanced micronutrient factors and dyslipidaemia leads to maternal and foetal metabolic related diseases (Adaikalakoteswari *et al.*, 2017; Baker *et al.*, 2018). Among maternal micronutrient deficiencies, B12 is known to be associated

with poor pregnancy outcomes associated with a higher risk of T2DM, obesity and IR in babies.

In line with these notions, we aimed to examine, in our cohort study, the association between local AT pro-inflammatory gene expression, lipid profile, serum pro-inflammatory cytokines and metabolic values in our cohort study. To determine associations between biomarkers, Spearman's correlations were conducted.

In the OmWAT a positive association between the gene expression of most of the pro-inflammatory cytokines and cholesterol, LDL and TAGs has been found, whereas in the ScWAT compartment only IL-1 and MCP-1 showed a correlation with lipid profile values. Our results may suggest a major involvement of the omental compartment in the increase of susceptibility in the development of dyslipidemia as suggested by the literature. A study by Adaikalakoteswari and colleagues demonstrated that B12 deficiency in type 2 diabetes is associated with adverse lipid parameters and higher risk of CVD (Adaikalakoteswari *et al.*, 2014) but no correlation with pro-inflammatory cytokines was evaluated. Similar results were found in a study conducted on a population with coronary heart disease in which B12 result to be associated with an increase of inflammatory cytokines and cardiovascular risk factors (Mahalle *et al.*, 2013). These findings are in line with various studies confirming the presence of altered lipid metabolism in some chronic inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, psoriasis and inflammatory bowel disease increasing therefore the risk of CVD (Feingold K, 2017 Feb 24).

In order to understand a possible relationship between metabolic disorders and B12 deficiency, we have further explored the clinical and biochemical characteristics of our cohort study based on B12 status and correlated serum laboratory values with serum pro-inflammatory cytokines. A significant difference in variables related to energy metabolism and folate in the blood has been observed in our population study stratified by B12 in accordance with a study comparing non-vegetarians population with vegetarians that shows an improved profile of CVD factors (Pawlak, 2015).

The results of the present study provide novel evidence that vitamin B₁₂ deficiency may be involved in the increased local inflammation. Specifically, we have demonstrated that low vitamin B₁₂ (a) induce an increased expression of pro-inflammatory genes in Chubs-S7; (b) induce an increased expression of pro-inflammatory genes in human primary

adipocytes derived from different categories of BMI with a prominent increment in adipocytes derived from the overweight group (maybe due to an increased susceptibility of this category of BMI to B12 deficiency); (c) increased expression of pro-inflammatory genes in both compartments of the human adipose tissue studied (ScWAT and OmWAT) ; (d) increased serum secretion of MCP-1 and IL-8 in B12 deficient patients.

The exact biochemical process by which cellular B12 deficiency leads to an increment in local inflammation is not clearly understood. A hypothetical explanation would be that the increased lipid accumulation due to an increased triglyceride biosynthesis and a decreased fatty acid oxidation caused by a lack of B12 would lead to increase in pro-inflammatory cytokines biosynthesis with a local activation of immune system. A lack of B12 (an anti-inflammatory molecule) would indeed lead to an upregulation of the transcription factor nuclear factor-kappa B (NF- κ B), of nitric oxide synthase, and downregulation of oxidative phosphorylation (Veber *et al.*, 2008).

In conclusion, the data obtained from the study confirm the hypothesis of a possible role of B12 on inflammatory adipokines secretion which might lead to adipocyte dysfunction and therefore suggesting a possible role of this molecule in obesity. The exact mechanism of such dysfunction needs to be studied which could open new insights into the pathogenesis of maternal obesity and the relevance of micronutrient supplementation for pregnant mothers.

Chapter 7: Serum and seminal correlation of B12 and holotranscobalamin levels with seminal quality in morbid obesity

7.1 Introduction

B12 and Obesity:

Vitamin B12 is crucial for homeostasis and growth as it acts as a coenzyme for the synthesis of methionine, a precursor of S-adenosyl methionine which provides the methyl groups for the methylation processes of macromolecules such as DNA, lipids, proteins, and neurotransmitters acting in the one-carbon-metabolism (Banerjee & Matthews, 1990; Gherasim *et al.*, 2013). Around 10%–30% of circulating B12 is bound to the carrier transcobalamin in a complex named holotranscobalamin (holoTC), which is considered the bioactive B12 and it has been proposed to represent a good marker vitamin deficiency status (Heil *et al.*, 2012; Nexo & Hoffmann-Lücke, 2011). Various studies have supported an association between B12 and metabolic pathologies, including obesity. Morbidly obese subjects and patients with IR present low concentrations of serum B12 (Knight *et al.*, 2015; Krzizek *et al.*, 2018). Moreover, the recent analysis of the data from the nationwide population-based study in the U.S (National Health and Nutrition Examination Survey, NHANES), demonstrated the association of serum B12 with obesity by showing that those individuals with higher serum B12 levels were less likely to be obese (Sun *et al.*, 2019).

Obesity and Sub-fertility:

It is known that morbid obesity is associated with sub-fertility (Belloc *et al.*, 2014; Bieniek *et al.*, 2016; Luconi *et al.*, 2013) reduced semen quality (Ramaraju *et al.*, 2018; Samavat *et al.*, 2018) impaired sperm acrosome reaction (Samavat *et al.*, 2014b), and sperm fatty acid composition and functions (Andersen *et al.*, 2016) Scientists have also linked the obesity related increased inflammation to impaired sperm production and function (Fan *et al.*, 2018; Kahn & Brannigan, 2017; Pearce *et al.*, 2019; Wellen & Hotamisligil, 2005)

In the past four decades various studies have examined the effect of B12 on semen quality (Boxmeer *et al.*, 2007; Isoyama *et al.*, 1984; Watson, 1962). It has been found that B12 is transferred from the blood to the male reproductive organs which gives a clear idea of the role of this micronutrient on spermatogenesis (Banihani, 2017). However conflicting data is still present in the literature about the relation between low circulating B12 and male infertility (Dhillon *et al.*, 2007; Murphy *et al.*, 2011), a strong correlation was found

between seminal B12 and sperm concentrations in a cohort of couples undergoing assisted reproductive techniques for infertility (Boxmeer *et al.*, 2007).

Altered absorption of B12 has been described in morbid obesity (Li *et al.*, 2018), however B12 and holoTC in serum and semen of these subjects have never been investigated and related to semen quality.

We aimed to explore the combined effect of B12 deficiency on male infertility as the effect of infertility in obesity is thought to be mediated through inflammation (Setayesh *et al.*, 2018). In the previous chapters we showed that low B12 is adversely associated with lipogenesis as well as excess circulatory inflammatory cytokines in the pregnant mothers. It is conceivable that similar pattern may exist in the fathers too. So we hypothesises that paternal circulating B12 levels affect the seminal B12 levels and in B12 deficiency states it reduces seminal quality and function. Therefore, the aim of the present study is to test this hypothesis in a cross-sectional observational study to determine the relationship between B12 status and semen quality in a cohort of 47 morbidly obese versus 21 lean men. We studied holoTC, functional part of the circulating B12.

7.2 Methods

7.2.1 Patients

Morbidly obese men who were on the waiting list for bariatric surgery at the Bariatric Unit of Careggi University Hospital in Florence (AOUC) were recruited to the study. Any bias with infertility was avoided as the morbidly obese patients were not recruited in andrology centres. Inclusion criteria: BMI ≥ 38 Kg/m², age between 20 and 65 years. Exclusion criteria: restrictive diet regimen, gastric balloon insertion, previous bariatric intervention, vitamin B12 supplementation, and tumour pathologies. A standard non-restrictive balanced diet had been prescribed to all patients by the Unit's dieticians. Lean men were enrolled among subjects undergoing routine semen analysis for couple infertility at the Andrology Unit at AOUC. Inclusion criteria: $18.5 \text{ Kg/m}^2 \leq \text{BMI} < 25 \text{ Kg/m}^2$, age between 20 and 65 years. Exclusion criteria: couple infertility due to male factor, restrictive diet regimen, vitamin B12 supplementation, and tumour pathologies. The study was approved by the Local Ethical Committee and Institutional Review Board (approval protocol number 83/13 of 10.25.2003). All patients provided signed informed consent after receiving written and oral information on the study.

7.2.2 Biochemical and Anthropometric Measurements

1) Anthropometric measures: Height, weight, and waist circumference were measured in each subject and blood was drawn in the morning before seminal analysis.

2) Vitamin B12: CBL and holoTC concentrations were measured in frozen serum and semen samples using the respective competitive and chemiluminescent enzyme immunoassays (ADVIA Centaur B12 and ADVIA Centaur Active-B12 Assays, Siemens Healthcare, Milan, Italy) based on LOCI technology on the Dimension Vista System (Siemens). Before holoTC determination, semen samples were diluted (1:3) with Multi-Diluent 13 (Siemens), centrifuged at 2,700g for 10 minutes and the supernatant was analysed. CBL and holoTC concentrations were expressed in pmol/l.

3) Biochemical and sex steroid hormones: Glycated haemoglobin (HbA1c) was measured on the whole-blood samples by high-performance liquid chromatography ion exchange chromatography on a VARIANT II instrument (Biorad Laboratories, Milan, Italy). HbA1c values were used for the diagnosis of T2DM at the 6.5% threshold. Serum levels of total testosterone (TT), sex-hormone-binding-globulin (SHBG), oestradiol (E2), and the gonadotropin follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by immunoassay (Immulite 2000, M-Medical System, Italy); assay analytical sensitivity: 0.5 nM (TT), 55 pM (E2), 0.02 nM (SHBG), 20.1 mIU/ml (FSH) and 0.05 mIU/ml (LH). Free testosterone (cFT) was calculated on SHBG and TT, as previously described (Samavat *et al.*, 2019; Vermeulen *et al.*, 1999).

7.2.3 Seminal analysis

Human semen was obtained by masturbation according to the World Health Organization procedure (Cao *et al.*, 2011) together with a blood sample on the same day or within one week. Semen parameters were analysed by a routine procedure (Cao *et al.*, 2011; Samavat *et al.*, 2018). Briefly, sperm morphology and motility were assessed by optical microscopy. Sperm morphology was evaluated as the percentage of normal and abnormal forms with Diff-Quick staining, scoring at least 100 spermatozoa/slide. Sperm motility was reported as the percentage of progressive motile, non-progressive motile, and immotile spermatozoa on at least 200 sperm/slide, total sperm motility was defined as progressive and non-progressive motile (Cao *et al.*, 2011; Samavat *et al.*, 2019).

7.3 Results

7.3.1 Biochemical and anthropometric characteristics of the patients

As shown in table 10 a significant difference between the two group subjects has been shown in the anthropometric measurements such as BMI, weight and waist circumference ($p=0.0001$) whereas the prevalence of T2DM and hypogonadism was 28% and 66% respectively in the morbidly obese cohort, while in the lean cohort was 0% and 22% respectively.

	Morbidly Obese n=47	Lean n=21	p
Age (years)	42.0± 11.6	38.4 ±7.9	0.207
BMI (Kg/m²)	46.5±7.7	25.2 ±3.9	<i>0.0001</i>
Weight (Kg)	149.5±26.6	79.1 ±10.0	<i>0.0001</i>
WC (cm)	142.7±18.5	96.6± 4.0	<i>0.0001</i>
FSH (mIU/L)	4.47 ±2.88	4.10± 1.47	0.740
LH (mIU/L)	2.98 ±1.93	3.21 ±1.86	0.757
TT (nM)	9.19 ±4.31	14.44±6.39	<i>0.005</i>
fT (nM)	0.219±0.104	0.319±0.104	<i>0.015</i>
E2 (pM)	139.2±53.4	101.8±35.2	<i>0.024</i>
SHBG (nM)	23.0±9.8	28.3 ±10.5	0.168
HbA1c (%)	6.2± 1.2	4.8± 0.3	<i>0.0001</i>
Hypogonadism (%)	66	22	<i>0.029</i>
T2D (%)	28	0	<i>0.045</i>
Smokers (% ,)	16	19	0.786
Alcohol consumers (%)	21	15	0.567

Table 10: Biochemical and anthropometric characteristics of the patients: Anthropometric data and circulating levels of sex steroid hormones, SHBG and HbA1c are reported along with the prevalence of hypogonadism (TT < 12 nM), T2DM (HbA1c \geq 6.5), smokers (at least 1 cigarette/day), and alcohol consumers (at least 2 drinks/day) in each cohort. Data was normally distributed and reported as mean \pm SD. Two-tailed Student's t-test p values for comparison between obese and lean subjects are shown; χ^2 was applied for comparison of non-continuous parameters (hypogonadism, T2DM, smokers, alcohol consumers). Significant p values are indicated in italics. Abbreviations: BMI: body mass index; WC: waist circumference; FSH: follicle stimulating hormone; LH: luteinizing hormone; TT: testosterone; fT: free-testosterone; T2DM: type 2 diabetes mellitus.

7.3.2 Vitamin B12 and Holotranscobalamin measurements and correlation in serum and seminal fluid

Vitamin B12 and holoTC concentrations were further measured in the serum and semen of morbidly obese and lean subjects. As shown in Table 11, in both cohorts, B12 and holoTC concentrations were significantly higher in semen versus serum (B12: $p < 0.001$, and holoTC: $p < 0.005$).

In the seminal fluid Morbidly obese subjects presented significantly lower concentrations of both B12 ($p = 0.006$) and holoTC ($p = 0.025$) in semen compared to lean subjects (table 11, figure 61), while in the serum the differences between the two cohorts reached a statistical significance for B12 only ($p = 0.012$, Table 11, Figure 61). Moreover B12 deficiency, defined as <148 pmol/L serum concentrations (Hannibal *et al.*, 2016), was present in 8% of the morbidly obese men and not in the lean cohort. Next, in order to investigate the relation between HoloTC and B12 Spearman' correlation analysis was performed. As shown in figure 62 in both semen and serum HoloTC strongly correlated with B12 in the whole population.

Not-statistically significant correlations were found between holoTC/B12 and BMI, weight, waist circumference (WC), sex steroid hormones and HbA1c.

	Morbidly Obese n=47	Lean n=21	p Mann Whitney U
<i>Serum concentrations</i>			
holoTC (pmol/L)	64.5[51.7]	95.1[52.1]	0.780
B12 (pmol/L)	233.5[99.2]	328.0[259.0]	0.012
<i>Semen concentrations</i>			
holoTC (pmol/L)	83.9[95.1] *	148.3[67.0] *	0.025
B12 (pmol/L)	811.3[477.0] **	1074[456.0] **	0.006

Table 11: B12 and holoTC concentrations in serum and semen: HoloTC and B12 concentrations are reported for morbidly obese and lean subjects as median (IQR) for not-normally distributed parameters and the Mann–Whitney U test was applied for comparison between obese and lean subjects. Statistical significance between seminal and circulating concentrations of holoTC and B12: Significant p values are indicated in italics for comparison between morbidly obese and lean subjects. Abbreviations B12: Vitamin B12; holoTC: holotranscobalamin; * p < 0.005 and ** p < 0.001.

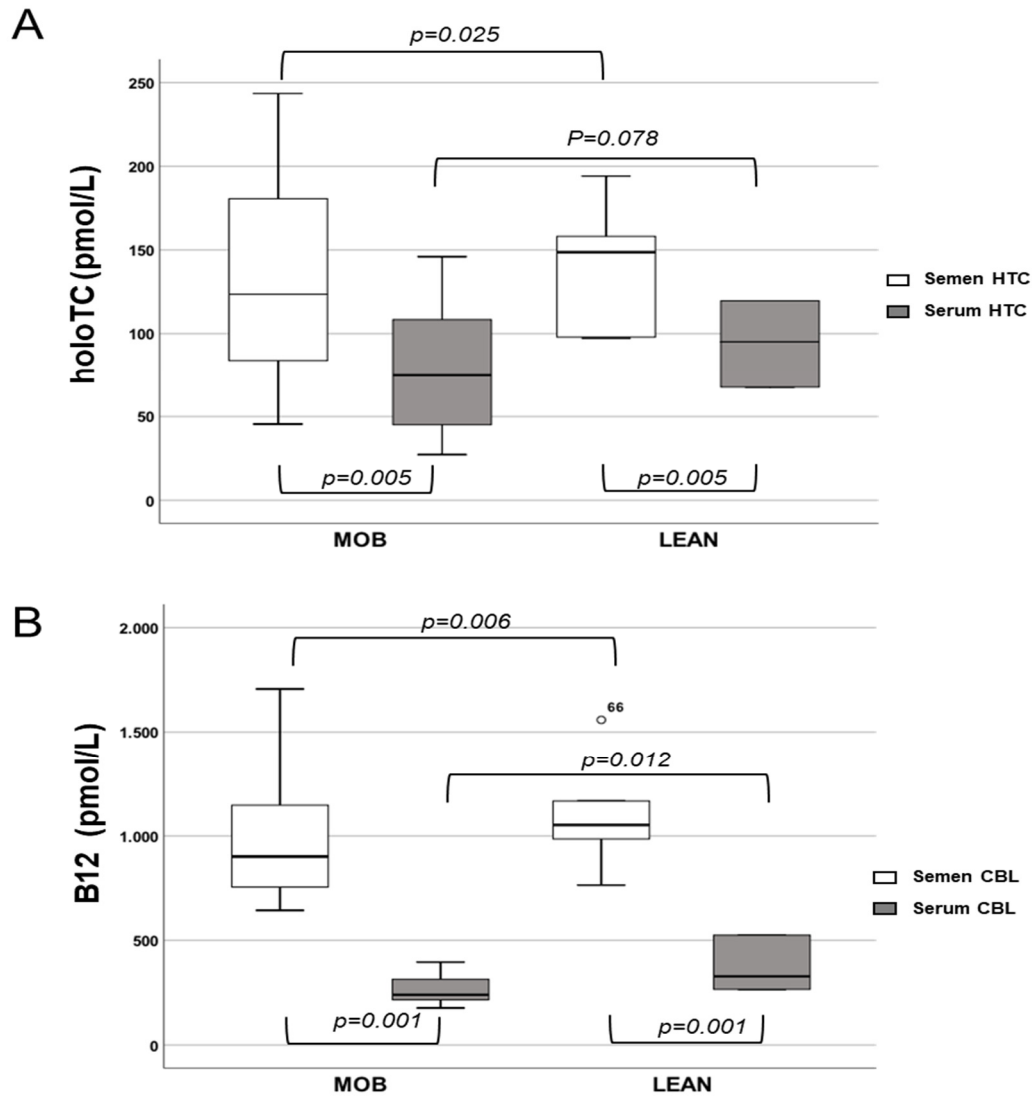
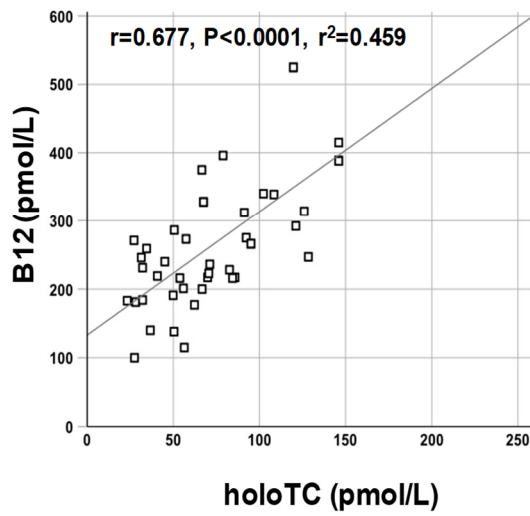


Figure 61: B12 and holoTC concentrations in serum and seminal samples from morbidly obese and lean subjects. Data is not-normally distributed and reported as box charts for holoTC (A) and B12 (B) serum and semen concentrations; p derived from the Mann-Whitney U test analysis is indicated. MOB: morbidly obese subjects; LEAN: lean subjects.

A. Seminal levels



B. Circulating levels

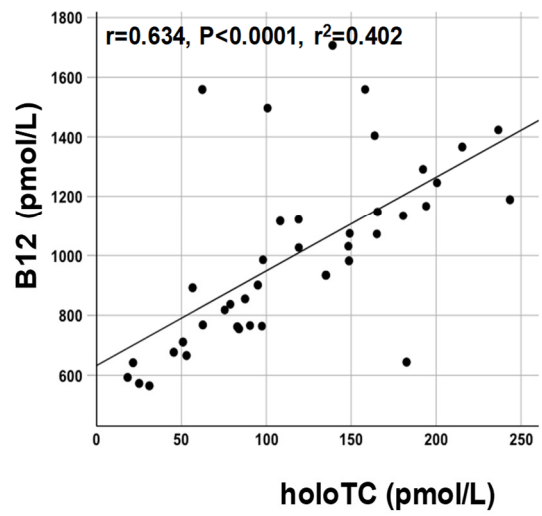


Figure 62: Positive correlations between B12 and holoTC concentrations in semen (A) and serum (B) in all subjects. Correlations were evaluated by Spearman's test for not-normally distributed data in a univariate analysis: r , r^2 , and p from linear regression are indicated. B12: vitamin B12; holoTC: holotranscobalamin.

7.3.3 Association between seminal parameters and seminal HoloTC and B12

As shown in table 12 the quality of seminal parameters was significantly reduced in the morbidly obese cohort compared to the lean subjects, in particular when considering total sperm motility, vitality, concentration, and total number, as well as semen volume and pH (Samavat *et al.*, 2019).

Moreover, in order to investigate the relationship between HoloTC, B12 and semen quality, seminal characteristics and sperm parameters were evaluated in the whole population (table 13). Both seminal HoloTC and B12 positively correlated with total sperm motility (HoloTC: $p=0.012$; B12: $p=0.027$) total sperm number (HoloTC: $p=0.009$; B12: $p=0.001$) and sperm concentration (HoloTC: $p=0.028$; B12: $p=0.002$). A significant negative correlation has been found between seminal HoloTC ($p=0.0001$), seminal B12 ($p=0.001$) and pH.

Subsequently, the whole cohort of morbidly obese and lean subjects were stratified in two classes according to the seminal holoTC (118 pmol/L) and seminal B12 median value (1,069 pmol/L). According to the median value of holoTC derived from the seminal distribution of the whole cohort, total sperm number and seminal volume were significantly higher and pH significantly lower in those subjects with high seminal holoTC (Figure 63A–C). Similar results were obtained for total sperm number and pH (Figure 3D, E) but not for semen volume (Figure 63F) when subjects were stratified according to the median seminal B12 levels.

Finally, a ROC analysis was performed in order to compare the sensitivity and specificity of seminal and serum holoTC and CBL in predicting semen quality. As seen in figure 64A–D seminal holoTC and B12 shown a statistically significant accuracy in predicting sperm number and concentration. On the contrary, serum values of holoTC and B12 were not able to predict semen quality. In addition, the cut-off values that identify the best combination of sensitivity and specificity were similar to the median values of holoTC and B12 in semen.

Table 14 reports the mean values of the different parameters when all subjects were stratified by the best cut-off values of seminal holoTC and B12, as identified in the ROC analysis.

Seminal Parameter	Morbidly Obese n=47	Lean n=21	p Mann Whitney U
Progressive Motility (%)	45.0[31.0]	55.0[19.5]	<i>0.017</i>
Total Motility (%)	52.0[25.0]	65.0[18.5]	<i>0.004</i>
Normal Morphology (%)	3.0[5.0]	5.0[6.0]	0.167
Vitality (%)	69.0[29.0]	80.0[6.0]	0.013
Total Sperm Number (millions)	34.5[161.8]	220.0[328.5]	<i>0.001</i>
Sperm Concentration (million/ml)	10.0[52.6]	52.5[75.0]	<i>0.013</i>
Volume (ml)	3.1± 1.7	4.2± 1.4	<i>0.019</i>
pH	7.6± 0.3	7.4± 0.2	<i>0.001</i>
Abstinence (days)	4.5 ±3.8	3.8± 2.0	0.707

Table 12: Seminal parameters: Seminal parameters of morbidly obese and lean subjects are reported as mean ± SD for normally distributed and median (IQR) for not-normally distributed parameters and analysed with the Mann–Whitney U test for comparison between obese and lean subjects. Significant p values are indicated in italics.

	Seminal holoTC	Seminal B12
Total Sperm Motility (%)	r=0.394 <i>p=0.012</i>	r=0.345 <i>p=0.027</i>
Total Sperm Number (millions)	r=0.401 <i>p=0.009</i>	r=0.482 <i>p=0.001</i>
Sperm Concentration (mil/ml)	r=0.343 <i>p=0.028</i>	r=0.465 <i>0.002</i>
pH	r=-0.535 <i>p=0.0001</i>	r=-0.481 <i>p=0.001</i>

Table 13: Correlations between seminal holoTC or B12 and seminal parameters. r and p values of Spearman’s correlation are indicated. The analysis was performed on the whole population.

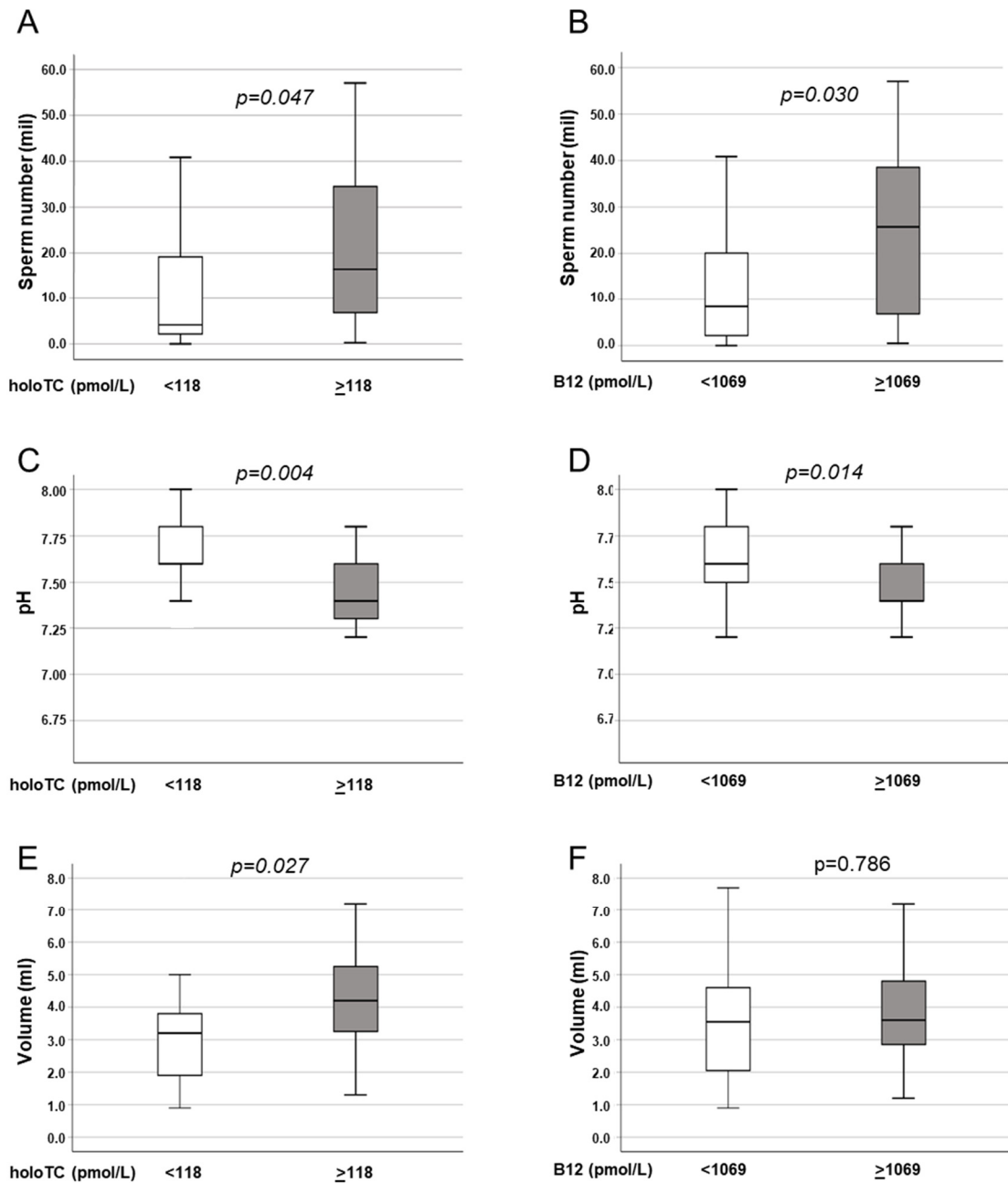


Figure 63 Seminal parameters stratified for seminal holoTC and B12 in the total population of obese and lean subjects. Data reported as box charts indicates sperm number (A,B), seminal pH (C,D), and volume (E,F) in all subjects stratified for low and high seminal holoTC (A,C,E) and B12 (B,D,F), respectively. Cut-off values were chosen as the median value of holoTC (118 pmol/L) and B12 (1,069 pmol/L) distribution in the whole population; p values were calculated with the Mann-Whitney U test.

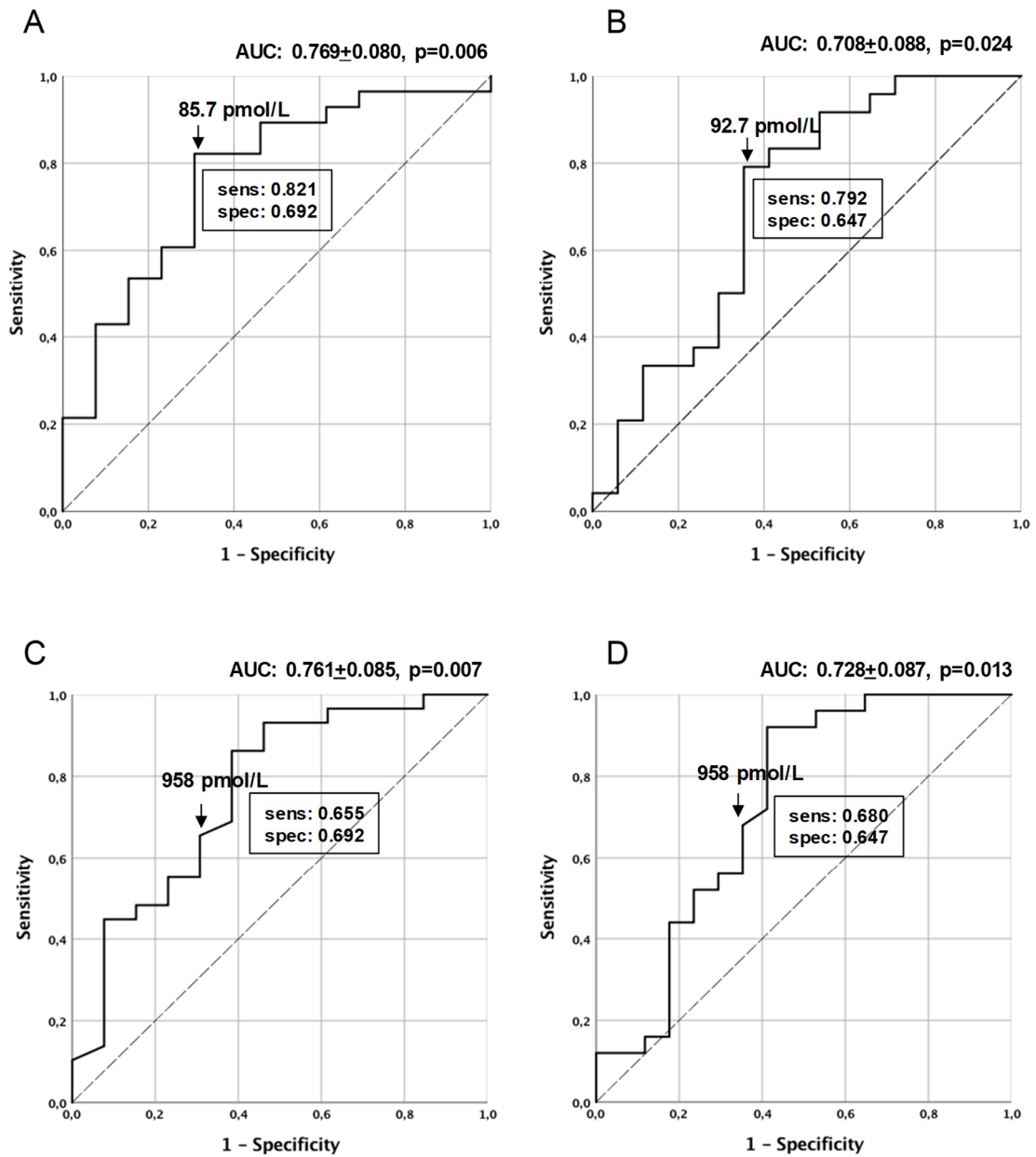


Figure 64: ROC analysis of seminal holoTC and B12 potency in predicting sperm number and concentration. ROC analysis of seminal holoTC predictive power for sperm number (A) and sperm concentration (B), and for seminal B12 predictive power for sperm number (C) and sperm concentration (D). Accuracy±SD (AUC) and p value are indicated for each analysis, along with the cut-off value of holoTC and B12 associated with the best value of sensitivity (sens) and specificity (spec) of the method.

	Seminal holoTC			Seminal B12		
	≥85.7 (pmol/L)	<85.7 (pmol/L)	p	≥958 (pmol/L)	<958 (pmol/L)	p
BMI (Kg/m²)	40.1[39.0]	42.6[15.8]	0.120	30.8[27.1]	43.2[36.1]	0.005
WC (cm)	120.0[83.5]	142.0[58.0]	0.032	120.0[67.5]	144.0[81.8]	0.018
Total Motility (%)	60.0[79.0]	52.0[75.0]	0.034	65.[61.0]	54.0[75.0]	0.066
Total Sperm Number (millions)	166.5[567.7]	30.8[281.0]	0.001	162.4[567.6]	41.6[408.0]	0.018
Sperm Concentration (million/ml)	44.0[306.0]	9.5[70.0]	0.001	44.0[178.1]	10.0[213.0]	0.03
pH	7.4[1.2]	7.8[0.6]	0.0001	7.4[1.2]	7.6[0.8]	0.005

Table 14: Stratification of the whole subject cohort according to seminal holoTC and B12 cut-off values identified by ROC analysis. Data is reported as median (IQR) for each parameter when all subjects were stratified in two groups according to the indicated cut-off values of seminal holoTC and B12 identified by ROC analysis. Significant p values after the Mann–Whitney U test are indicated.

7.4 Discussion

This study showed interesting and novel findings. In line with the higher holoTC and B12 concentrations found in semen compared to serum in the whole population of subjects studied, it is likely that B12 may actively transported from the circulation to the seminal compartment. The higher concentrations found for holoTC and B12 in semen might support a role of B12 in controlling semen quality. Accordingly, a recent review showed that B12 and antioxidant supplementation can improve semen quality, in particular enhancing sperm count and motility (Banihani, 2017).

Secondly, we found that seminal B12 and holoTC concentrations were significantly lower than serum levels in morbidly obese compared to lean subjects. This suggests that alterations associated with high grades of obesity may somehow affect the process of local concentration in the reproductive tract, at least in the ejaculate, thus contributing to the reduced semen quality observed in morbidly obese men (Belloc *et al.*, 2014; Luque *et al.*, 2017; Samavat *et al.*, 2018). Regarding this, a significant positive correlation between seminal holoTC/B12 and some seminal parameters has been found, which lost statistical significance when serum holoTC/B12 concentrations were considered. In particular, this was found for both holoTC and B12 and total sperm motility, total number, concentration, and seminal pH. Conversely, a not-statistically significant correlation with normal sperm morphology was found. These findings indicate an active role of seminal B12 in controlling semen quality at the post-testicular level, such as in the epididymis, where spermatozoa are concentrated and acquire active motility (Luconi *et al.*, 2006) ,as well as in semen production, acting on the prostate and seminal vesicles. Interestingly, alterations at the post-testicular level in the control of sperm maturation have also been hypothesized to contribute to the reduced semen quality found in morbid obesity (Belloc *et al.*, 2014; Luque *et al.*, 2017; Samavat *et al.*, 2018).

The extreme obesity may affect sperm quality at a post-testicular level, which was confirmed by the absence of any effect on the normal morphology parameter and conversely related to testicular maturation as previously shown (Belloc *et al.*, 2014; Rosenblatt *et al.*, 2015). The correlation between morbid obesity and hypogonadism has been extensively validated (Li *et al.*, 1994; Rosenblatt *et al.*, 2015; Samavat *et al.*, 2014a) and the high prevalence of hypogonadism in this type of obesity has been further confirmed. However, in our study no significant correlations were found between seminal holoTC or B12 and sex steroid hormones, and no influence of hypogonadism on B12 was

evident, suggesting that hypogonadism does not contribute to regulate holoTC and B12 concentrations. The role of seminal B12 in the post-testicular control of semen quality was further suggested by the significant difference observed in total sperm number and semen pH between subjects stratified according to low and high holoTC and B12. In particular, seminal holoTC, but not CBL concentrations, significantly differentiated patients for semen volume, which was substantially lower in subjects with low seminal holoTC. Interestingly, semen volume was one of the parameters strictly associated with prostate and seminal vesicle functionality (Lotti *et al.*, 2012). Finally, according to the higher level of accuracy and best sensitivity/specificity associated with seminal holoTC evaluation compared to B12, seminal holoTC turned out to be the best predictor of semen quality (sperm number and concentration), suggesting that this parameter could be considered as a potential useful marker of patients' fertility. In conclusion, our findings suggest that seminal rather than serum holoTC might represent a good marker of semen quality in morbidly obese subjects, whereby, despite the normal serum levels, low B12 and holoTC were found. Further studies investigating the local levels of holoTC on large populations including mildly obese/overweight subjects and elucidating the mechanisms which could affect semen quality are warranted.

Chapter 8: General discussion

8.1 Discussion

Obesity is a chronic relapsing disorder according to a recent statement of the World Obesity Federation and the increasing prevalence, variable pathogenesis, progressive natural history, and complications related to this disease emphasise the urgent need for new treatment strategies. According to estimates by the World Health Organization in 2016, overweight adults (considering a body mass index or BMI ≥ 25 kg / m²) were more than 1.9 billion adults, of these over 650 million adults were clinically obese [WHO, 2016]. All this translates into an enormous social impact, as obesity and related metabolic diseases, cause a worsening of the quality of life and increasing therefore health and social costs. Growing evidence have also shown the association of B12 deficiency with obesity (Allin *et al.*, 2017; Li *et al.*, 2018), dyslipidaemia (Adaikalakoteswari *et al.*, 2015b; Ghosh *et al.*, 2016) GDM (Sukumar *et al.*, 2016b; Yajnik *et al.*, 2008) and T2DM (Jayabalan & Low, 2016). In particular, earlier experimental studies conducted on adipocytes have shown an association between B12 deficiency and altered lipid handling, resulting in increased cholesterol accumulation as well as hypomethylation of regulatory genes of lipid biosynthesis (Adaikalakoteswari *et al.*, 2017). Moreover, a clinical study conducted on British and South Asian populations showed high TAG levels in B12 deficient patients (Adaikalakoteswari *et al.*, 2014b). Furthermore, various studies have also explored the effect of B12 on semen quality (Banihani, 2017), with conflicting data still present in the literature about the relation between low circulating B12 and male infertility (Dhillon *et al.*, 2007; Murphy *et al.*, 2011). Adipocytes play a pivotal role in energy storage as well as in the energy metabolism through lipid handling, therefore the current study was designed to explore cellular and tissue expression of B12 transporter and receptors, lipid metabolism and cytokine gene expression under B12 deficient status. Moreover, in this study, it has also been questioned whether B12 deficiency had any systemic effect in the circulation such as circulating inflammatory markers. In addition, its effect on other tissues such as seminal fluid was also explored.

B12 receptor and transporter

In pregnancy, maternal B12 levels tend to drop partly due to hemodilution, increased utilisation by the mother as well as partly due to high foetal demand. However, several studies consistently showed that cord blood B12 levels are higher than maternal levels, presumably to protect the growing foetus. It is likely this is due to upregulation of placental B12 transporter although this has been studied in detail. It is also likely that increased expression of B12 receptors in the target tissues also contribute to maintain such relatively higher levels in the cord blood. In deed, higher serum levels of soluble CD320 were observed in mothers which was suggested to have originated from the placenta (Abuyaman *et al.*, 2013). Similar observation was reported for TCN2 in the circulation of pregnant mothers with low B12, accounting for high cord blood and foetal B12 concentrations (Finkelstein *et al.*, 2019). Although several studies explored the expression of cellular B12 receptor (CD320) and transporters (TCN2) that may regulate cellular uptake and storage of B12 in several tissues like placenta, liver and brain, no study have been conducted on adipocytes so far. Therefore, we aimed to investigate the intracellular B12 levels in human adipocytes and the expression of B12 receptor and transporter in human adipocytes and in human ScWAT and OmWAT. Intracellular levels of B12 in adipocytes was assessed in relation to circulating B12 levels in adipocytes. Human pre-adipocyte cell line (Chubs-S7) in decreasing extracellular B12 media concentrations resulted in progressively increased intracellular B12 levels. In particular, intracellular levels of B12 increased up to three times in adipocytes cultured in B12 deficient media such as 100 pM- 25 pM, whereas increasing concentrations of B12 media to 100-500 nM resulted in a progressive reduction of intracellular levels. Similar evidence has also been seen in a recent study conducted on several tissues (but not in adipose tissue) in rats (Kornerup *et al.*, 2018) and in cord blood of pregnant women (Obeid *et al.*, 2006). An increased gene expression of B12 receptor CD320 and transporter TCN2 has been shown in adipocytes (both cell line and primaries) cultured with low concentrations of B12. Similar results were observed in ScWAT and OmWAT of B12 deficient pregnant women compared with B12 non-deficient patients. In this study it has also been found an association between Sc and Om CD320 gene expression and metabolic risk variables such as HDL, LDL, cholesterol and glucose. Altogether, evidence from this current study shows that intracellular levels of B12 in adipocytes is modulated by the expression of B12 transporters and receptors. Our findings suggest that 1) increased intracellular accumulation in B12 deficient adipocytes may be due to active transport of B12 through

these receptors and transporters 2) B12 receptor (CD320) and transporters (TCN2) are *de novo* synthesized in the adipocytes. This indicates that compensatory upregulation of B12 receptor and transporter occurs when low B12 is in circulation in order to maintain intracellular B12 levels. It also indicates that this transport mechanism is designed to protect extremely high levels entering adipocytes, probably to prevent cell death.

B12 and *de novo* lipid synthesis

We then went on to investigate, the effect of B12 deficiency on lipid metabolism, targeting *de novo* lipid synthesis and the fatty acid oxidation (FAO), in adipocytes. Low B12 in adipocytes, resulted in increased gene expression of enzymes involved in FA synthesis, elongation and TAG synthesis and higher levels of synthesized fatty acids, confirmed by increased synthesis of radio-labelled TAGs in a dynamic flux assay. This was also confirmed in human mature AT from different depots such as ScWAT and OmWAT showing increased lipogenesis in patients with B12 deficiency. Several pre-clinical and clinical studies have reported the association of B12 with obesity (Kaidar-Person *et al.*, 2008; Surendran *et al.*, 2019b) and dyslipidaemia (Kumar *et al.*, 2014). It has been shown recently that epigenetic modification such as hypo-methylation near the promoter regions of SREBF1 and LDLR genes (key genes in cholesterol and lipid synthesis) in low B12 adipocytes would be a justification for their upregulation leading to increased lipid synthesis (Adaikalakoteswari *et al.*, 2014a). It has been demonstrated that B12 deficiency affects the mitochondrial methyl malonylCoA mutase reaction leading to an increase of levels of methyl-malonylCoA and consequently enhancing increased lipogenesis (Ruderman *et al.*, 2003). Clinical and biochemical characteristics of our cohort study were further explored and correlated with *de novo* lipid synthesis genes. Interestingly, the gene expression in the ScWAT and OmWAT compartment of this metabolic pathway resulted significantly associated with lipid profile values such as total cholesterol, HDL and LDL, in line with a recent study conducted on British pregnant women in which B12 resulted to be positively correlated with HDL and negatively correlated with TAGs (Knight *et al.*, 2015). Therefore, our findings suggest that low B12 induce lipid accumulation by upregulating the *de novo* lipogenesis potentially leading to development of dyslipidaemia.

B12 and the fatty acid β -oxidation

We observed that low B12 impaired FAO by decreasing the expression of the rate limiting enzymes MCD, CPT1 β , CPT2 and downstream genes crucial in the FAO pathway. Similarly, human mature AT from different depots, such as ScWAT and OmWAT, showed a decrease in FAO in patients with B12 deficiency. Similar studies conducted on hepatic tissue (Dahlhoff *et al.*, 2014) and myocardial cell (Garcia *et al.*, 2011) have shown that methyl donor (B12 and folate) deficiency resulted in impaired FAO by decreasing the level of enzymes crucial in FAO the pathway. B12 is required for the conversion of MMA to succinyl-CoA through the action of methylmalonyl-CoA mutase. During a B12 deficiency state the enzyme function of methylmalonyl-CoA mutase is inhibited, causing therefore an accumulation of MMA which is, known to be a potent inhibitor of CPT1 (Brito *et al.*, 2017). Another hypothesis would be due to the impairment in the biosynthesis of carnitine, molecule that plays an important role in facilitating the entry of long-chain fatty acids into the mitochondria via the carnitine shuttle (Brito *et al.*, 2017). However, the precise mechanism of B12 regulation of downstream FAO enzymes requires further investigation. The increase in MMA due to a deficiency of B12 has been also hypothesized to affect mitochondrial respiration decreasing mitochondrial complexes II and I activity (Brito *et al.*, 2017; Kolker *et al.*, 2003; Preynat *et al.*, 2010; Wajner & Coelho, 1997).

In our experiments, we have demonstrated, that the mitochondrial functional integrity was compromised in low B12 accounting for impaired utilization of long chain FA (palmitate) for energy metabolism. Similar results were also obtained in studies conducted on rat liver (Toyoshima *et al.*, 1995). Correlation were found also between clinical and biochemical characteristics of our cohort study based on B12 and FAO gene expression in both depots of AT, validating the hypothesis of the potential importance of B12 on lipid metabolism management, especially early in the development of dyslipidaemia Altogether, the increased lipogenesis with altered FA and triglyceride profile, as well as impaired FAO and mitochondrial dysfunction in low B12, induce uncontrolled lipid accumulation in the AT. 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.

B12 and inflammation

In this part of the study, we hypothesised that if B12 deficiency induces lipogenesis and impairs FAO, this may result in adipocyte dysfunction causing inflammation. We found increased gene expression of inflammatory cytokines in human pre-adipocyte cell line, pre-adipocytes derived from different degrees of obesity exposed to low levels of B12 and in human mature AT from different depots such as ScWAT and OmWAT, in patients with B12 deficiency. In the AT the activation of immune system due to low-grade stress condition lead IR and development of metabolic syndrome. In this scenario, several studies have reported the association of B12 deficiency with increased pro-inflammatory cytokines (Kalra *et al.*, 2007; S. *et al.*, 1999). It has also been shown in pre-clinical studies on rat that a total deficiency of B12 is associated with increase in TNF- α (Scalabrino *et al.*, 1999). The results obtained in cell line adipocytes where also confirmed in the adipocytes derived from all the four main categories of BMI. In view of the fact that B12 deficiency causes an increase of inflammation in all categories of BMI, leads us to think about its peculiar anti-inflammatory effect and the importance of this micronutrient in the prevention of cardiovascular and metabolic diseases. In addition, the circulatory cytokines levels of MCP1 and IL-8 were higher in subjects with B12 deficiency compared with the control group. Several clinical studies (Lee *et al.*, 2016, (Solini *et al.*, 2006) support our hypothesis. In order to understand a possible relationship between metabolic disorders and B12 deficiency, we have further explored the clinical and biochemical characteristics of our cohort study based on B12 status and correlated serum laboratory values with serum pro-inflammatory cytokines. A possible explanation of the anti-inflammation action of B12 could be the down regulation of the transcription factor nuclear factor-kappa B (NF-kB), of nitric oxide synthase, and promotion of oxidative phosphorylation. These results thus suggest that the lipid accumulation, as a consequence of B12 deficiency, has induced an increased local inflammation in adipocytes, leading to adipocyte dysfunction.

Serum and seminal correlation of B12 and holotranscobalamin levels with seminal quality in morbid obesity

Lastly, we wanted to study the paternal influence of B12 deficiency. We hypothesised that if maternal levels of B12 is inversely associated with obesity (Knight *et al.*, 2015; Krzizek *et al.*, 2018), a similar process might present in the fathers. If that is the case such lower levels in paternal circulation might cause lower levels of B12 in seminal fluid, leading to poor seminal quality and function. We studied this in a cross-section study of morbidly obese patients presenting to bariatric surgery, who also had detailed assessment of their seminal function.

It is known that morbid obesity is associated with sub-fertility (Belloc *et al.*, 2014; Bieniek *et al.*, 2016; Luconi *et al.*, 2013) reduced semen quality (Ramaraju *et al.*, 2018; Samavat *et al.*, 2018) impaired sperm acrosome reaction (Samavat *et al.*, 2014b), and sperm fatty acid composition and functions (Andersen *et al.*, 2016). Scientist have also related the obesity related increased inflammation to impaired sperm production and function (Fan *et al.*, 2018; Kahn & Brannigan, 2017; Pearce *et al.*, 2019; Wellen & Hotamisligil, 2005). Clinical data have also shown a strong correlation between seminal B12 and sperm concentrations (Boxmeer *et al.*, 2007). In our study, we observed, firstly, higher holoTC and B12 concentrations found in semen compared to serum in the whole population. This indicates, similar to the observation seen on maternal – cord blood studies (Adaikalakoteswari *et al.*, 2015), B12 is actively transported from the circulation to the seminal compartment. Secondly, we found that seminal B12 and holoTC concentrations were significantly lower than serum levels in morbidly obese compared to lean subjects. This suggests that alterations associated with high grades of obesity may somehow affect the process of local concentration in the reproductive tract, at least in the ejaculate, thus contributing to the reduced semen quality observed in morbidly obese men. These findings suggest that seminal rather than serum holoTC might represent a good marker of semen quality in morbidly obese subjects.

The findings in adipocytes and adipose tissue in this study support the hypothesis that an overexpression of fatty acids and triglycerides biosynthesis and repression of fatty acid oxidation might exist during B12 deficiency leading to an upregulated local pro-inflammatory response.

The potential mechanistic pathway triggered by a B12 deficient status suggested by the results obtained in this project might be the following:

- Increased gene expression of Acetyl-CoA carboxylase and decreased gene expression of Malonyl-CoA decarboxylase leads to an increment in malonyl-CoA levels translating to (1) aberrant FA and TAG biosynthesis (2) downregulation of carnitine palmitoyl transferase (CPT-1 β) in the mitochondria leading to a decrease in fatty acid oxidation and therefore incapacity of the cell to burn FA to produce energy in form of ATP, as suggested also by Ruderman and colleagues (Ruderman *et al.*, 2003);
- Downregulation of the mitochondrial respiratory chain complex activities due to increased MMA levels due to lack of B12 translating therefore in a decreased respiratory capacity and mitochondrial health, as suggested also by Kolker and colleagues (Kolker *et al.*, 2003).

Moreover, increased gene expression of pro-inflammatory cytokines might be due not only by harmful accumulation of lipids accumulation that would lead to with a local activation of immune system but also by the lack of B12. A lack of B12, an anti-inflammatory molecule, leading to an upregulation of the transcription factor nuclear factor-kappa B (NF- κ B), of nitric oxide synthase, and downregulation of oxidative phosphorylation (Veber *et al.*, 2008). The potential mechanistic pathway is summarised in figure 65.

Our findings support that optimal physiological levels of B12 are required for the maintenance of a metabolically healthy adipocyte. B12 is involved in essential reactions as nucleic acid synthesis, erythropoiesis in bone marrow and myelination of central nervous system. In adipocytes, through its active forms, methylcobalamin (Me-Cbl) and adenosylcobalamin (Ado-Cbl), serve as coenzymes in two significant pathways regulating energy metabolism. Intracellular levels of B12 in adipocytes and the adipose tissue is however less explored, therefore, our study is the first to characterize the cellular and tissue levels of B12 with respect to varying circulating B12 levels. Moreover, we showed an active role of seminal B12 in controlling semen quality and seminal levels of holoTC, might representing a good marker of semen quality in morbidly obese subjects.

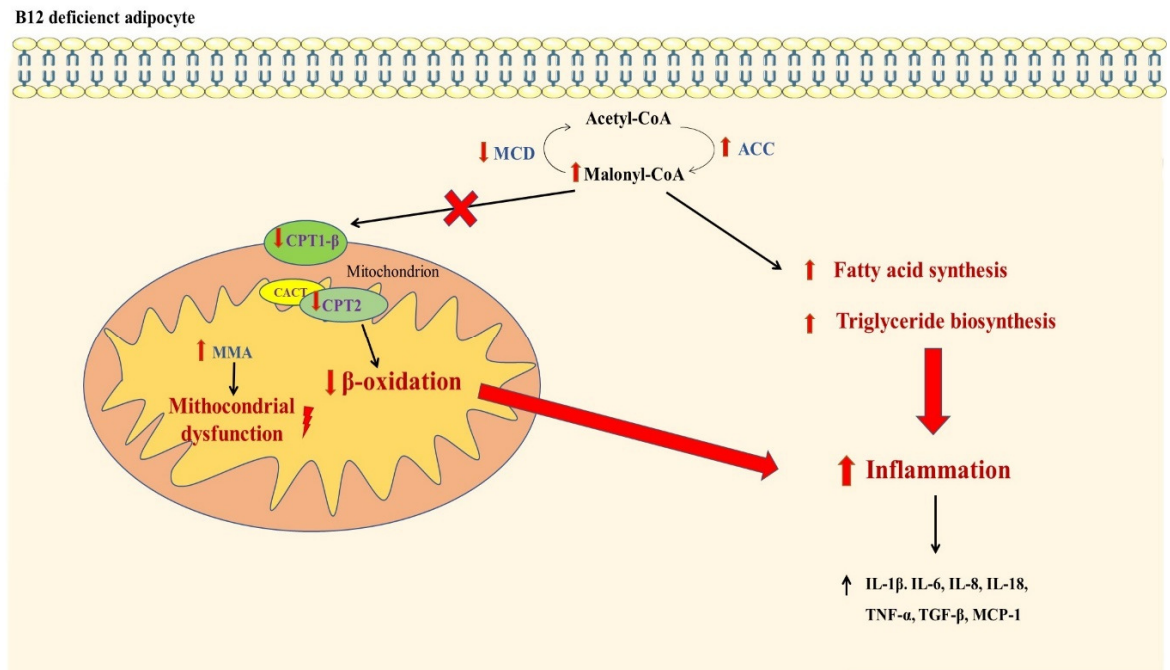


Figure 65: Potential mechanistic pathway in B12 deficient adipocyte

B12 deficiency leads to an increased gene expression of Acetyl-CoA carboxylase (ACC) and a decreased gene expression of Malonyl-CoA carboxylase (MCD) translating in an increased synthesis of Malonyl-CoA. The increased levels of Malonyl-CoA induce either a downregulation of CPT-1 β which translate in decreased fatty acid oxidation and to increased fatty acid and triglyceride biosynthesis. The decreased levels of B12 cause also an accumulation of MMA in the mitochondria causing downregulation of the mitochondrial respiratory chain complex capacity leading to a local damage. The overload of fatty acids and triglycerides in adipocytes leads to a hypertrophic and hyperplastic status bringing to activation of local immune system with increased expression of pro-inflammatory cytokines.

8.2 Conclusion and future directions

This study provides novel evidence of the possible role of B12 deficiency in the expression of B12 receptors and transporters in adipocytes, disruption of adipose lipid metabolism leading to inflammation which could contribute to the development of metabolic disorder such as obesity, T2DM and other metabolic related diseases. This project also allowed us also to conclude that holoTC can be considered as good marker of semen quality in morbidly obese subjects. Clinical and pre-clinical studies have been shown the association of B12 deficiency and the manifestation of metabolic risk. Moreover, studies conducted on human adipocytes have demonstrated a possible role of epigenetic mechanisms due to B12 deficiency which may dysregulate lipid metabolism (Adaikalakoteswari *et al.*, 2015).

The current study provide novel evidence of intracellular adipose B12 levels and storage *via* modulation of B12 transporters and receptors with respect to various circulatory levels including low and high B12. Low B12 accounted for increased adipose lipogenesis with increased evidence of higher total intracellular TAG level and utilization of radiolabelled-FA for TAG synthesis. Alongside, oxidation of FAs (FAO) and mitochondrial functional efficiency were impaired in low B12 in adipocytes and both omental and subcutaneous adipose tissue of B12 deficient subjects. Moreover, as a consequence of an enlarged adipocyte with an increase lipid synthesis and a disrupted FAO, increased adipose inflammation has been seen. The current study also provides novel evidence that seminal levels of both B12 and holoTC result to be higher compared to serum levels. Furthermore, it also indicate holoTC as a predictor of semen quality in subjects characterized by morbid obesity.

In conclusion, given the pivotal role of AT in total body energy metabolism, the current evidence highlights that low B12 induces a dysregulation of fatty acid metabolism, leading to adipocyte dysfunction, opening new insights into the pathogenesis of maternal obesity higher adiposity and T2DM. Therefore, this study will provide novel evidence about the link between B12 deficiency and metabolic inflammation. opening new avenues into the pathogenesis of maternal obesity and the relevance of micronutrient supplementation and strategies for therapeutics for pregnant mothers and patients who undergo bariatric surgery. Furthermore, our findings suggest that paternal B12 levels (through the seminal quality and function) may also play a crucial role in the adverse programming of the offspring. However, to quantify this we may have to measure the seminal rather than serum holoTC. Further studies investigating the levels of holoTC on large populations including mildly obese/overweight subjects and elucidating the mechanisms which could affect semen quality are warranted.

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 for;

1. Elucidation of underlying epigenetic mechanisms induced by B12 deficiency in regulation of adipocyte lipid metabolism
2. Exploration of exact mechanisms induced by B12 deficiency in regulation of adipocyte inflammation

3. Evaluation of the epigenetic mechanisms of maternal B12 on offspring metabolic health mediating through inflammatory cytokines (such as DNA methylation and miRNA).
4. Exploring the potential benefits of optimizing B12 levels in pregnancy on the metabolic health of pregnant women and their offspring
5. Investigation of the role of vitamin B12 deficiency on brown adipose tissue as well as in other tissues such as hepatocytes and myocytes, tissues that are metabolically more active than white adipose tissues.

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Publications

Article

Seminal but not Serum Levels of Holotranscobalamin are Altered in Morbid Obesity and Correlate with Semen Quality: A Pilot Single Centre Study

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Abstract: Vitamin B12 (cobalamin) is an essential cofactor in the one-carbon metabolism. One-carbon metabolism is a set of complex biochemical reactions, through which methyl groups are utilised or generated, and thus plays a vital role to many cellular functions in humans. Low levels of cobalamin have been associated to metabolic/reproductive pathologies. However, cobalamin status has never been investigated in morbid obesity in relation with the reduced semen quality. We analysed the cross-sectional data of 47-morbidly-obese and 21 lean men at Careggi University Hospital and evaluated total cobalamin (CBL) and holotranscobalamin (the active form of B12; holoTC) levels in serum and semen.

1. Introduction

B12 (cobalamin, CBL) is a water-soluble vitamin synthesized only in bacteria [1]. Humans require an external source of this vitamin, primarily through dietary intake or from pharmacological supplementation, as it cannot be absorbed from the intestinal microbiota production [2]. This

Both seminal and serum concentrations of holoTC and CBL were lower in morbidly obese compared to lean men, although the difference did not reach any statistical significance for serum holoTC. Seminal CBL and holoTC were significantly higher than serum levels in both groups.

Significant positive correlations were observed between seminal holoTC and total sperm motility ($r = 0.394$, $p = 0.012$), sperm concentration ($r = 0.401$, $p = 0.009$), total sperm number ($r = 0.343$, $p = 0.028$), and negative correlation with semen pH ($r = -0.535$, $p = 0.0001$). ROC analysis supported seminal holoTC as the best predictor of sperm number (AUC = 0.769 ± 0.08 , $p = 0.006$). Our findings suggest that seminal rather than serum levels of holoTC may represent a good marker of semen quality in morbidly obese subjects.

Keywords: active vitamin B12; cobalamin; morbid obesity; body mass index; sperm; seminal plasma
vitamin is crucial for homeostasis and growth as it acts as a coenzyme for the synthesis of methionine, a precursor of S-adenosyl methionine which provides the methyl groups for the methylation processes of macromolecules such as DNA, lipids, proteins, and neurotransmitters acting in the one-carbon-metabolism [3,4]. Around 10%–30% of circulating CBL is bound to the carrier transcobalamin in a complex named holotranscobalamin (holoTC), which is considered the bioactive vitamin B12 [5]. HoloTC has been proposed to represent a better marker than CBL of the vitamin deficiency status [6–8]. Another metabolic marker inversely associated with cobalamin is methylmalonic acid used to detect CBL deficiency at tissue levels [6], however, it is not routinely available for clinical testing.

Data present in the literature supports an association between CBL serum concentrations and metabolic pathologies, including obesity. Morbidly obese subjects [9–11] and patients with insulin resistance [12] present low concentrations of serum CBL. A positive association between CBL and dyslipidemia was found in two ethnic groups with type 2 diabetes (T2D) [13]. Similarly, an association was described between CBL and lower cord blood HDL in White Europeans [14]. A recent systemic review failed to establish any inverse association between circulating CBL and body mass index (BMI) when considering studies including not only obese but also underweight subjects [12]: this relation resulted in a J-shaped association. However, the very large heterogeneity in the meta-analyses considered in that study might have significantly affected the results [12]. Moreover, folate and age but not circulating CBL remained independently associated with the number of metabolic syndrome components in a large cohort of morbidly obese subjects following multivariate analysis [12]. Finally, the very recent analysis of the data from the nationwide population-based study in the U.S (National Health and Nutrition Examination Survey, NHANES), demonstrated the association of serum cobalamin with obesity by showing that those individuals with higher serum cobalamin levels were less likely to be obese [15].

It is known that morbid obesity is associated with sub-fertility [16–19], reduced semen quality [19–22], impaired sperm acrosome reaction [23], and sperm fatty acid composition and functions [24,25]. In particular, a large population study performed in men ($n = 4,860$), classified according to their BMI (underweight: $n = 45$, normal weight: $n = 1,330$, overweight: $n = 2,493$, obese: $n = 926$, and morbidly obese: $n = 57$), has shown that at both extremes of BMI (underweight and morbidly obese subjects), semen parameters were significantly worse and associated with lower sperm epididymal maturation compared to the other BMI classes [22]. Similar results have been found in a recent observational study conducted on 3,966 sperm donors in China for the underweight and overweight classes, but not for the obese class [26]. This may be due to enrolment bias (sperm donor bank) and the involvement of a small percentage of obese men (0.9%) (see also [19]).

Conflicting data is still present in the literature about the relation between low circulating CBL and male infertility [27–29]. However, a strong correlation was found between seminal CBL and sperm concentrations in a cohort of couples undergoing assisted reproductive techniques for infertility [30].

Altered absorption of CBL has been described in morbid obesity [9], however CBL and holoTC in serum and semen of these subjects have never been investigated and related to semen quality.

Therefore, the aim of the present cross-sectional observational study was to investigate the possible relationship between holoTC/CBL status and semen quality in a cohort of 47 morbidly obese versus 21 lean men. In particular, we compared holoTC versus CBL concentrations in serum and semen, and

analysed their relationship with seminal parameters with no aim to use them for the diagnosis of vitamin deficiency.

2. Materials and Methods

2.1. Patients

Morbidly obese men who were on the waiting list for bariatric surgery at the Bariatric Unit of Careggi University Hospital in Florence (AOUC) were recruited to the proposed study. Any bias with infertility was avoided as the morbidly obese patients were not recruited in andrology centres. Inclusion criteria: BMI ≥ 38 Kg/m², age between 20 and 65 years. Exclusion criteria: restrictive diet regimen, gastric balloon insertion, previous bariatric intervention, vitamin B12 supplementation, and tumour pathologies. A standard non-restrictive balanced diet had been prescribed to all patients by the Unit's dietitians. Lean men were enrolled among subjects undergoing routine semen analysis for couple infertility at the Andrology Unit at AOUC. Inclusion criteria: $18.5 \text{ Kg/m}^2 \leq \text{BMI} < 25 \text{ Kg/m}^2$, age between 20 and 65 years. Exclusion criteria: couple infertility due to male factor, restrictive diet regimen, vitamin B12 supplementation, and tumour pathologies. The study was approved by the Local Ethical Committee and Institutional Review Board (approval protocol number 83/13 of 10.25.2003). All patients provided signed informed consent after receiving written and oral information on the study.

2.2. Biochemical and Anthropometric Measurements

- 1) Anthropometric measures: Height, weight, and waist circumference were measured in each subject and blood was drawn in the morning before seminal analysis.
- 2) Vitamin B12: CBL and holoTC concentrations were measured in frozen serum and semen samples using the respective competitive and chemiluminescent enzyme immunoassays (ADVIA Centaur B12 and ADVIA Centaur Active-B12 Assays, Siemens Healthcare, Milan, Italy) based on LOCI technology on the Dimension Vista System (Siemens). Before holoTC determination, semen samples were diluted (1:3) with Multi-Diluent 13 (Siemens), centrifuged at 2,700g for 10 minutes and the supernatant was analysed. CBL and holoTC concentrations were expressed in pmol/L.
- 3) Biochemical and sex steroid hormones: Glycated haemoglobin (HbA1c) was measured on the whole-blood samples by high-performance liquid chromatography ion exchange chromatography on a VARIANT II instrument (Biorad Laboratories, Milan, Italy). HbA1c values were used for the diagnosis of T2D at the 6.5% threshold. Serum levels of total testosterone (TT), sex-hormone-binding-globulin (SHBG), estradiol (E2), and the gonadotropin follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by immunoassay (Immulite 2000, M-Medical System, Italy); assay analytical sensitivity: 0.5 nM (TT), 55 pM (E2), 0.02 nM (SHBG), 20.1 mIU/ml (FSH) and 0.05 mIU/ml (LH). Free testosterone (cFT) was calculated on SHBG and TT, as previously described [31].

2.3. Semen Analysis

Human semen was obtained by masturbation according to the World Health Organization procedure [32] together with a blood sample on the same day or within one week. Semen parameters were analysed by a routine procedure [21,32]. Briefly, sperm morphology and motility were assessed by optical microscopy. Sperm morphology was evaluated as the percentage of normal and abnormal forms with Diff-Quick staining, scoring at least 100 spermatozoa/slide. Sperm motility was reported as the percentage of progressive motile, non-progressive motile, and immotile spermatozoa on at least 200 sperm/slide, total sperm motility was defined as progressive and non-progressive motile [21].

2.4. Statistical Analysis

Data was expressed as mean \pm SD and as median (interquartile range, IQR) for normally and non-normally distributed parameters, respectively. All statistical analyses were performed on SPSS 24.0 for Windows (Statistical Package for the Social Sciences, Chicago, USA). Kolmogoroff-Smirnov's test was used to determine the parametric distribution of data. CBL and holoTC data and most of the seminal

parameters had a not-normally distributed distribution, while BMI, weight, and waist circumference were normally distributed. The Mann–Whitney U test was used for comparing two groups of not-normally distributed data, while the two-tailed Student’s t-test was applied for comparison of normally distributed data. Correlations were assessed using Spearman’s method for not-normally distributed parameters. ROC analysis was performed to evaluate accuracy, as well as sensitivity and specificity of the assessment of semen and serum holoTC and CBL concentrations in predicting semen quality. A $p < 0.05$ value was used for statistical significance.

3. Results

Anthropometric characteristics of morbidly obese and lean patients are reported in Table 1, along with the circulating levels of sex steroid hormones (TT, fT, E2, FSH, and LH), SHBG, and HbA1c. The prevalence of hypogonadism (TT < 12 nM) [33] and T2D (HbA1c \geq 6.5%) was 66% and 28% in the morbidly obese cohort, while 22% and 0% in the lean cohort, respectively. Smokers and alcohol consumers are reported as percentages (Table 1).

Table 1. Biochemical and anthropometric characteristics of the patients: Anthropometric data and circulating levels of sex steroid hormones, SHBG and HbA1c are reported along with the prevalence of hypogonadism (TT < 12 nM), T2D (HbA1c \geq 6.5), smokers (at least 1 cigarette/day), and alcohol consumers (at least 2 drinks/day) in each cohort. Data was normally distributed and reported as mean \pm SD. Two-tailed Student’s t-test p values for comparison between obese and lean subjects are shown; χ^2 was applied for comparison of non-continuous parameters (hypogonadism, T2D, smokers, alcohol consumers). Significant p values are indicated in italics.

	Morbidly Obese $n=$ 47	Lean $n=$ 21	p
Age (years)	42.0 \pm 11.6	38.4 \pm 7.9	0.207
BMI (Kg/m ²)	46.5 \pm 7.7	25.2 \pm 3.9	0.0001
Weight (Kg)	149.5 \pm 26.6	79.1 \pm 10.0	0.0001
WC (cm)	142.7 \pm 18.5	96.6 \pm 4.0	0.0001
FSH (mIU/L)	4.47 \pm 2.88	4.10 \pm 1.47	0.740
LH (mIU/L)	2.98 \pm 1.93	3.21 \pm 1.86	0.757
TT (nM)	9.19 \pm 4.31	14.44 \pm 6.39	0.005
fT (nM)	0.219 \pm 0.104	0.319 \pm 0.104	0.015
E2 (pM)	139.2 \pm 53.4	101.8 \pm 35.2	0.024
SHBG (nM)	23.0 \pm 9.8	28.3 \pm 10.5	0.168
HbA1c (%)	6.2 \pm 1.2	4.8 \pm 0.3	0.0001

Hypogonadism (%)	66	22	0.029
T2D (%)	28	0	0.045
Smokers (%)	16	19	0.786
Alcohol consumers (%)	21	15	0.567

BMI: body mass index; WC: waist circumference; FSH: follicle stimulating hormone; LH: luteinizing hormone; TT: testosterone; FT: free-testosterone; T2D: type 2 diabetes.

CBL and holoTC concentrations were measured in the serum and semen of morbidly obese and lean subjects as shown in Table 2. CBL immunoassay had an analytical sensitivity of 37 pmol/L [34]. According to our measurements, the intra-assay and inter-assay coefficients of variation (CV%) in serum were 3.38 and 4.37 respectively, while in semen the values were 1.73 and 2.15, respectively. The values of analytical accuracy were evaluated according to CLSI EP05-A3 [35].

Table 2. CBL and holoTC concentrations in serum and semen: HoloTC and CBL concentrations are reported for morbidly obese and lean subjects as median (IQR) for not-normally distributed parameters and the Mann–Whitney U test was applied for comparison between obese and lean subjects. Statistical significance between seminal and circulating concentrations of holoTC and CBL: Significant *p* values are indicated in italics for comparison between morbidly obese and lean subjects.

	Morbidly Obese n= 47	Lean n= 21	<i>p</i> Mann–Whitney U
<i>Serum concentrations</i>			
holoTC (pmol/L)	64.5 (51.7)	95.1 (52.1)	0.780
CBL (pmol/L)	233.5 (99.2)	328.0 (259.0)	0.012
<i>Semen concentrations</i>			
holoTC (pmol/L)	83.9 (95.1) *	148.3 (67.0) *	0.025
CBL (pmol/L)	811.3 (477.0) **	1074 (456.0) **	0.006

CBL: cobalamin; holoTC: holotranscobalamin; * *p* < 0.005 and ** *p* < 0.001.

The ADVIA Centaur AB12 assay for holoTC in serum had the Limit of Detection (LOD) of 1.08 pmol/L and the Limit of Quantification (LOQ) of 5.00 pmol/L, determined as described in the CLSI documentation EP17-A2 [36]. The linearity was maintained for values 5.00–146.00 pmol/L, evaluated according to the protocol CLSI EP6-A [37]. According to our measurements, the intra-assay and inter-assay repeatability CVs% obtained in serum were 2.38 and 4.36 respectively, and in semen, 3.11 and 4.36, respectively.

CBL deficiency, defined as <148 pmol/L serum concentrations [38], was present in 8% of the morbidly obese men and not in the lean cohort.

HoloTC strongly correlated with CBL in both semen and serum in the whole population (Figure 1A,B). Conversely, only holoTC significantly correlated between semen and serum ($r = 0.538$, $r^2 = 0.290$, $P = 0.014$), while CBL did not.

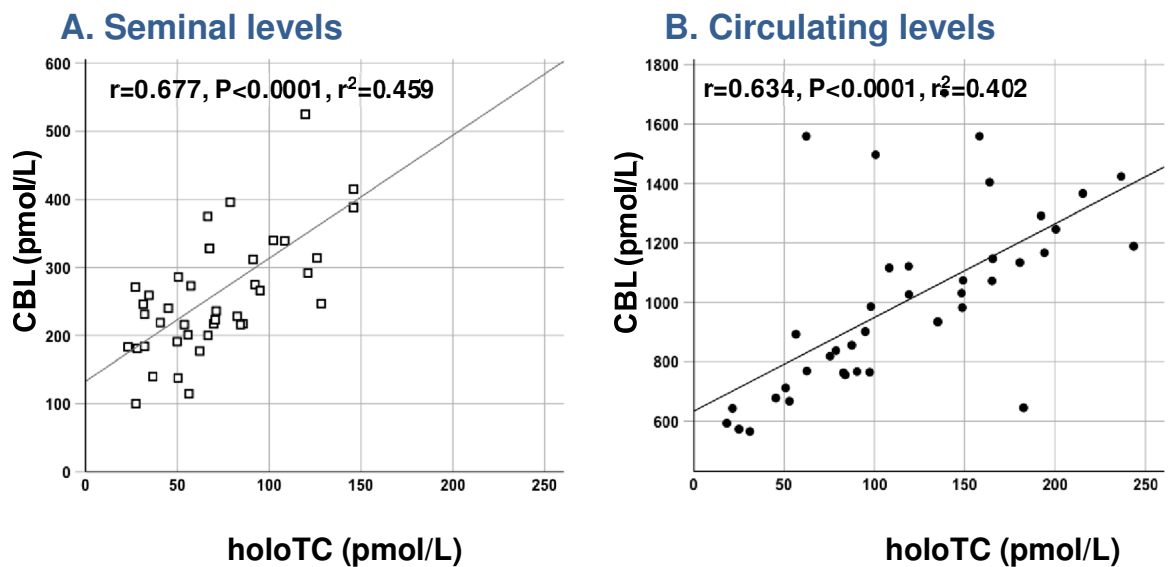


Figure 1. Positive correlations between CBL and holoTC concentrations in semen (A) and serum (B) in all subjects. Correlations were evaluated by Spearman’s test for not-normally distributed data in a univariate analysis: r , r^2 , and p from linear regression are indicated. CBL: cobalamin; holoTC: holotranscobalamin.

In both cohorts, CBL and holoTC concentrations were significantly higher in semen versus serum samples (CBL: $P < 0.001$, and holoTC: $P < 0.005$, Table 2, Figure 2). Morbidly obese subjects presented significantly lower concentrations of both CBL ($p = 0.006$) and holoTC ($p = 0.025$) in semen compared to lean subjects (Table 1, Figure 2), while the differences between the two cohorts reached a statistical significance in the serum for CBL only ($p = 0.012$, Table 1, Figure 2). Not-statistically significant correlations were found between holoTC/CBL and BMI, weight, waist circumference (WC), sex steroid hormones, and HbA1c.

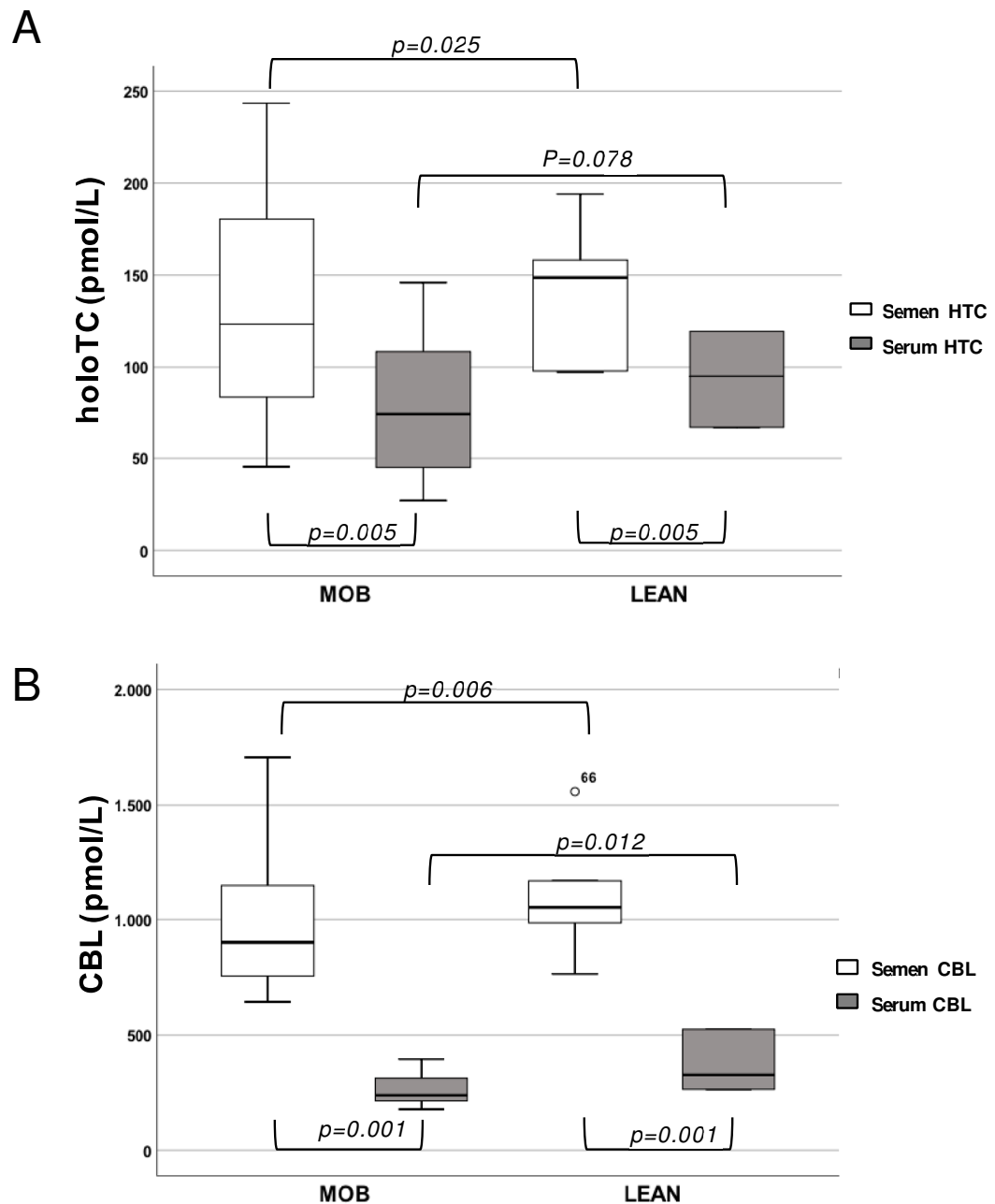


Figure 2. CBL and holoTC concentrations in serum and seminal samples from morbidly obese and lean subjects. Data is not-normally distributed and reported as box charts for holoTC (A) and CBL (B) serum and semen concentrations; p derived from the Mann-Whitney U test analysis is indicated. MOB: morbidly obese subjects; LEAN: lean subjects.

The quality of seminal parameters was significantly reduced in the morbidly obese cohort compared to the lean subjects, in particular when considering total sperm motility, vitality, concentration, and total number, as well as semen volume and pH (Table 3). In order to investigate the relationship between CBL and semen quality, seminal characteristics and sperm parameters were evaluated in the whole population. A statistically significant positive association was found between seminal holoTC, CBL, and total sperm motility, concentration and total number, as well as a negative correlation with semen pH (Table 4). Conversely, no association was found between any parameter and holoTC and CBL in both semen and serum.

Table 3. Seminal parameters: Seminal parameters of morbidly obese and lean subjects are reported as mean \pm SD for normally distributed and median (IQR) for not-normally distributed parameters and analysed with

the Mann–Whitney U test for comparison between obese and lean subjects. Significant *p* values are indicated in italics.

Seminal Parameter	Morbidly Obese <i>n</i> = 47	Lean <i>n</i> = 21	<i>p</i> Mann–Whitney U
Progressive Motility (%)	45.0 (31.0)	55.0 (19.5)	0.017
Total Motility (%)	52.0 (25.0)	65.0 (18.5)	0.004
Normal Morphology (%)	3.0 (5.0)	5.0 (6.0)	0.167
Vitality (%)	69.0 (29.0)	80.0 (6.0)	0.013
Total Sperm Number (millions)	34.5 (161.8)	220.0 (328.5)	0.001
Sperm Concentration(million/ml)	10.0 (52.6)	52.5 (75.0)	0.013
Volume (ml)	3.1 ± 1.7	4.2 ± 1.4	0.019
pH	7.6 ± 0.3	7.4 ± 0.2	0.001
Abstinence (days)	4.5 ± 3.8	3.8 ± 2.0	0.707

Table 4. Correlations between seminal holoTC or CBL and seminal parameters. *r* and *p* values of Spearman’s correlation are indicated. The analysis was performed on the whole population.

	Seminal HoloTC	Seminal CBL
Total Sperm Motility (%)	<i>r</i> = 0.394 <i>p</i> = 0.012	<i>r</i> = 0.345 <i>p</i> = 0.027
Total Sperm Number (millions)	<i>r</i> = 0.401 <i>p</i> = 0.009	<i>r</i> = 0.482 <i>p</i> = 0.001
Sperm Concentration (mil/ml)	<i>r</i> = 0.343 <i>p</i> = 0.028	<i>r</i> = 0.465 <i>p</i> = 0.002
pH	<i>r</i> = -0.535 <i>p</i> = 0.0001	<i>r</i> = -0.481 <i>p</i> = 0.001

When the whole cohort of morbidly obese and lean subjects were stratified in two classes of holoTC concentrations, according to the median value of holoTC (118 pmol/L) derived from the seminal distribution of the whole cohort, total sperm number and seminal volume were significantly higher and pH significantly lower in those subjects with high seminal holoTC (Figure 3A–C). Similar results were obtained for total sperm number and pH (Figure 3D,E) but not for semen volume (Figure 3F) when subjects were stratified according to the median seminal CBL levels (1,069 pmol/L).

A

D

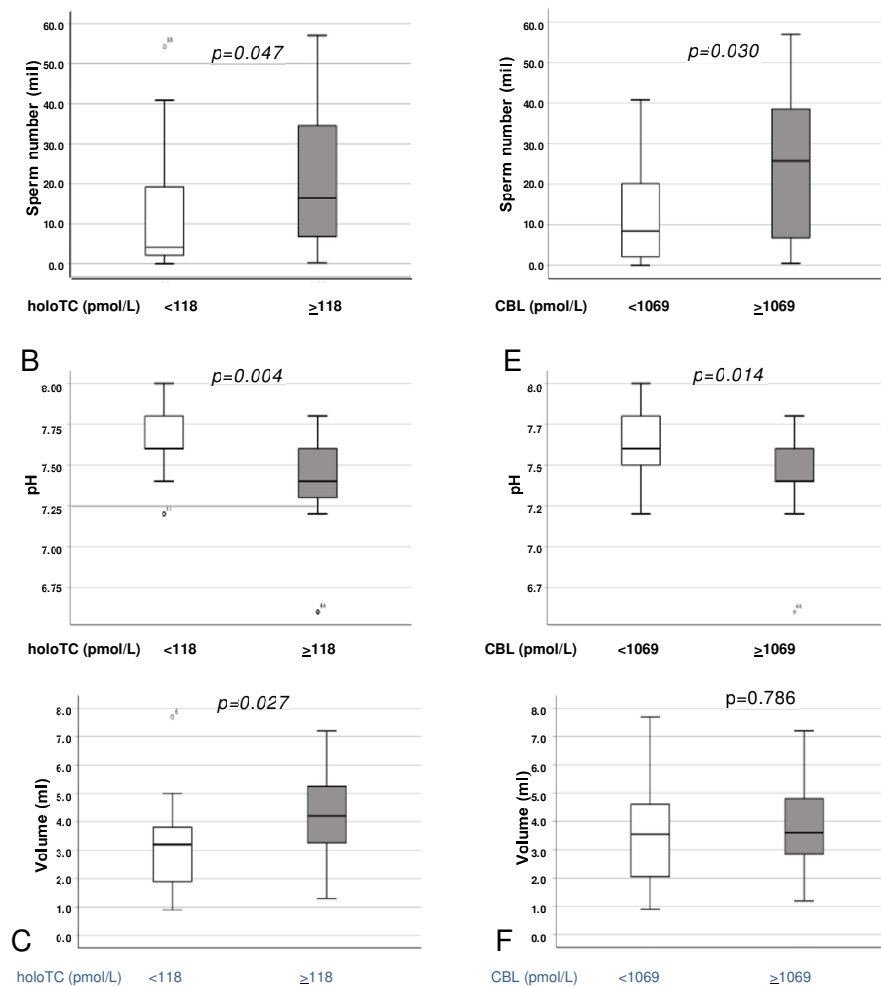


Figure 3. Seminal parameters stratified for seminal holoTC and CBL in the total population of obese and lean subjects. Data reported as box charts indicates sperm number (A,B), seminal pH (C,D), and volume (E,F) in all subjects stratified for low and high seminal holoTC (A,C,E) and CBL (B,D,F), respectively. Cut-off values were chosen as the median value of holoTC (118 pmol/L) and CBL (1,069 pmol/L) distribution in the whole population; p values were calculated with the Mann-Whitney U test.

Finally, in order to compare the sensitivity and specificity of seminal and serum holoTC and CBL in predicting semen quality, a ROC analysis was performed. A statistically significant accuracy in predicting sperm number and concentration was found for seminal holoTC and CBL (Figure 4A–D), but not for serum values. The cut-off values identifying the best combination of sensitivity and specificity of the methods are indicated. These cut-off values were similar to the median values of holoTC and CBL distribution in semen (see Figure 3).

A

B

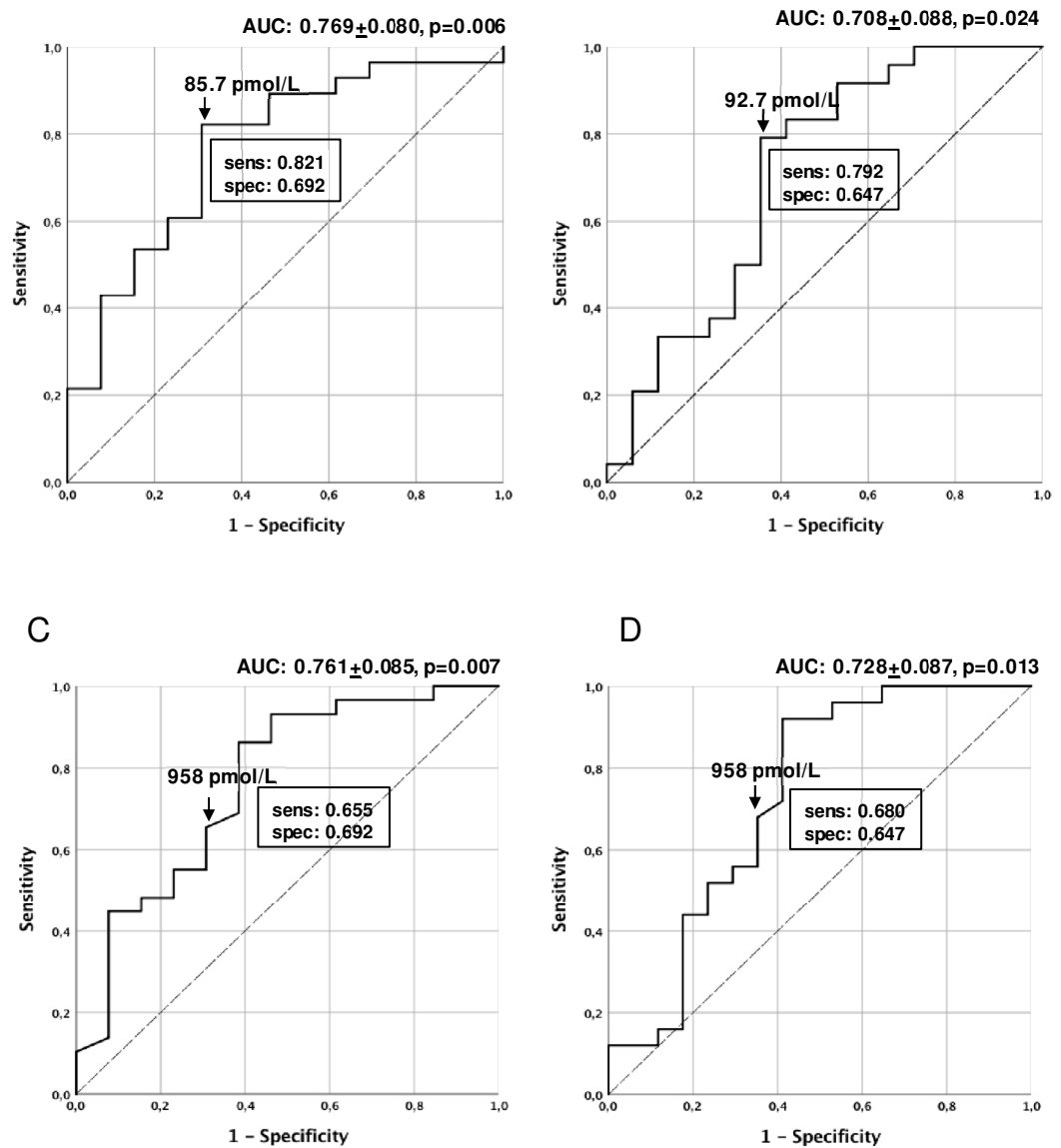


Figure 4. ROC analysis of seminal holoTC and CBL potency in predicting sperm number and concentration. ROC analysis of seminal holoTC predictive power for sperm number (A) and sperm concentration (B), and for seminal CBL predictive power for sperm number (C) and sperm concentration (D). Accuracy \pm SD (AUC) and p value are indicated for each analysis, along with the cut-off value of holoTC and CBL associated with the best value of sensitivity (sens) and specificity (spec) of the method.

Table 5 reports the mean values of the different parameters when all subjects were stratified by the best cut-off values of seminal holoTC and CBL, as identified in the ROC analysis. Parameters of statistically significant differences between the two groups are shown in Table 5.

Table 5. Stratification of the whole subject cohort according to seminal holoTC and CBL cut-off values identified by ROC analysis. Data is reported as median (IQR) for each parameter when all subjects were stratified in two groups according to the indicated cut-off values of seminal holoTC and CBL identified by ROC analysis. Significant p values after the Mann–Whitney U test are indicated.

	≥ 85.7 (pmol/L)	< 85.7 (pmol/L)					≥ 958 (pmol/L)	< 958 (pmol/L)	
BMI (Kg/m ²)	40.1 (39.0)	42.6 (15.8)				0.120	30.8 (27.1)	43.2 (36.1)	0.0
		142.0 (58.0)	0.032	120.0 (67.5)	144.0 (81.8)	0.018			
		52.0 (75.0)]	0.034	65 (61.0)	54.0 (75.0)	0.066			
		30.8 (281.0)	0.001	162.4 (567.6)	41.6 (408.0)	0.018			
ml)	44.0 (306.0)	7.8 (0.6)	0.0001	7.4 (1.2)	7.6 (0.8)	0.005	9. 0.001	44.0 (178.1)	10.0 (213.0)

4. Discussion

We observed some interesting and novel findings in this study. In line with the higher holoTC and CBL concentrations found in semen compared to serum in the whole population of subjects studied, it is likely that CBL may concentrate from the circulation to the seminal compartment. The higher concentrations found for holoTC and CBL in semen might support a role of CBL in controlling semen quality. Accordingly, a recent review showed that CBL and antioxidant supplementation can improve semen quality, in particular enhancing sperm count and motility [27].

Secondly, we found that seminal CBL and holoTC concentrations were significantly lower than serum levels in morbidly obese compared to lean subjects. This suggests that alterations associated with high grades of obesity may somehow affect the process of local concentration in the reproductive tract, at least in the ejaculate, thus contributing to the reduced semen quality observed in morbidly obese men [17–22]. Indeed, we found a significant positive correlation between seminal holoTC/CBL and some seminal parameters, which lost statistical significance when serum holoTC/CBL concentrations were considered. In particular, this was found for both holoTC and CBL and total sperm motility, total number, concentration, and seminal pH. Conversely, a not-statistically significant correlation with normal sperm morphology was evident. This finding indicates an active role of seminal B12 in controlling semen quality at the post-testicular level, such as in the epididymis, where spermatozoa are concentrated and acquire active motility [39], as well as in semen production, acting on the prostate and seminal vesicles. Interestingly, alterations at the post-testicular level in the control of sperm maturation have also been hypothesized to contribute to the reduced semen quality found in morbid obesity [17–22]. The extreme obesity may affect sperm quality at a post-testicular level, which was confirmed by the absence of any effect on the normal morphology parameter and conversely related to testicular maturation as previously shown [19].

The correlation between morbid obesity and hypogonadism has been extensively validated [17,18,40], and the high prevalence of hypogonadism in this type of obesity has been further confirmed. However, in our study no significant correlations were found between seminal holoTC or CBL and sex steroid hormones, and no influence of hypogonadism on CBL was evident, suggesting that hypogonadism does not contribute to regulate holoTC and CBL concentrations.

The role of seminal CBL in the post-testicular control of semen quality was further suggested by the significant difference observed in total sperm number and semen pH between subjects stratified according to low and high holoTC and CBL. In particular, seminal holoTC, but not CBL concentrations, significantly differentiated patients for semen volume, which was substantially lower in subjects with low seminal holoTC. Interestingly, semen volume was one of the parameters strictly associated with prostate and seminal vesicle functionality [41]. Finally, according to the higher level of accuracy and best sensitivity/specificity associated with seminal holoTC evaluation compared to CBL, seminal holoTC turned out to be the best predictor of semen quality (sperm number and concentration), suggesting that this parameter could be considered as a potential useful marker of patients' fertility.

One of the limitations recognized in our study was the limited number of subjects enrolled. Accordingly, our results should be considered as preliminary. However, the number of morbidly obese

subjects enrolled was in line with the other studies conducted on morbidly obese subjects recruited in bariatric surgery programs [17,18,21,42–44]. To exclude that the difference in the number of subjects in the two cohorts could somehow affect the difference observed in holoTC and CBL, we calculated Cohen's *d* values (Cd) for holoTC and CBL in semen and serum of the morbidly obese and lean groups. The values obtained (moderate level: semen holoTC Cd = 0.70, serum holoTC Cd = 0.77; high level: semen CBL Cd = 1.11, serum CBL Cd = 2.82) indicate that the small size of the groups was unlikely to affect the results. Our sample selection has two key strengths compared to previous papers [16,19,26]: a) absence of any recruitment bias – the morbidly obese subjects were not recruited in andrology centres, thus this does not affect fertility conditions, and b) parallel measurements of both holoTC and CBL concentrations in semen and serum of the same subjects.

5. Conclusions

In conclusion, our findings suggest that seminal rather than serum holoTC might represent a good marker of semen quality in morbidly obese subjects, whereby, despite the normal serum levels, low CBL and holoTC were found. Further studies investigating the local levels of holoTC on large populations including mildly obese/overweight subjects and elucidating the mechanisms which could affect semen quality are warranted.

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List of manuscripts awaiting publication

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- **J Samavat**, A Adaikalakoteswari, J Boachie, P Saravanan. Vitamin B12 deficiency leads to adipocyte dysfunction by enhancing triglyceride biosynthesis and impairing fatty-acid oxidation: a new protagonist in metabolic disease?
- **J Samavat**, A Adaikalakoteswari, J Boachie, P Saravanan. Increased pro-inflammatory cytokine production in vitamin B12 deficient adipocytes
- **J. Samavat**, A. Adaikalakoteswari, J. Boachie, I. Goljan, P. Saravanan. Expressions of Transcobalamin receptor CD320 and transporter TCN2 in low vitamin B12 treated human adipocytes

List of conference presentations

List of conference poster presentations

- **Samavat J. et al.** “Vitamin B12 Deficiency Leads To Fatty Acid Metabolism Dysregulation and Increased pro-inflammatory cytokine production in Human Adipocytes and Maternal Subcutaneous and Omental Adipose Tissue”. 73^o annual conference of Society of Endocrinology SfE BES 11-13 November 2019, Brighton -United Kingdom
- **Samavat J. et al** “Vitamin B12 Deficiency Increases pro-inflammatory cytokine production in Human Adipocytes and Maternal Subcutaneous and Omental Adipose Tissue” ° World Congress of Developmental Origins of Health and Disease DOHaD 20-23 October 2019, Melbourne, Australia.
- **Samavat J. et al.** “Vitamin B12 Deficiency Leads To Fatty Acid Metabolism Dysregulation and Increased pro-inflammatory cytokine production in Human Adipocytes and Maternal Subcutaneous and Omental Adipose Tissue”. CPHBAT 2019 Brown and white adipose in metabolism -Plasticity and metabolic crosstalk, 3-4 June, Copenhagen, Denmark
- **Samavat J. et al.** "Vitamin B12 deficiency leads to adipocyte dysfunction by decreasing fatty-acid oxidation: a new protagonist in metabolic disease?18th International Congress of Endocrinology and 53rd SEMSDA Congress 1-4 December, Cape Town, South Africa
- **Samavat J. et al.** 72^o annual conference of Society of Endocrinology SfE BES 19-21 November 2018, Glasgow, United Kingdom. “Increased pro-inflammatory cytokine production in vitamin B12 deficient adipocytes”; “Vitamin B12 deficiency leads to adipocyte dysfunction by enhancing triglyceride biosynthesis and impairing fatty-acid oxidation: a new protagonist in metabolic disease?”
- **Samavat J. et al.** Warwick Medical School’s annual Postgraduate Research Student Symposium 22nd -23rd May 2018, Coventry, United Kingdom “VitaminB12 deficiency triggers adipocyte dysfunction by enhancing triglyceride biosynthesis and pro-inflammatory cytokine production: a new agonist in metabolic disease?” Poster presentation winner
- **Samavat J. et al.** 71^o annual conference of Society of Endocrinology SfE BES 6-8 November 2017, Harrogate, United Kingdom “VitaminB12 deficiency triggers adipocyte dysfunction enhancing fatty acid biosynthesis and pro-inflammatory cytokine production: a new protagonist in Metabolic Disease?”

- **Samavat J. et al.** “Expression of Transcobalamin receptor CD320 and transporter TCN2 result increased in low vitaminB12 treated human adipocytes”; 10° World Congress of Developmental Origins of Health and Disease DOHaD 15-18 October 2017 Rotterdam, The Netherlands
- **Samavat J. et al.** “VitaminB12 deficiency triggers adipocyte dysfunction by enhancing triglyceride biosynthesis and pro-inflammatory cytokine production: a new agonist in metabolic disease?” 10° World Congress of Developmental Origins of Health and Disease DOHaD 15-18 October 2017 Rotterdam, The Netherlands
- **Samavat J. et al.** Warwick Medical School’s annual Postgraduate Research Student Symposium 6-7 June 2017, Coventry, United Kingdom.

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List of conference Oral presentations

- **Samavat J. et al.** “Vitamin B12 Deficiency Leads To Dysregulation of Fatty Acid Metabolism in Human Adipocytes and Maternal Subcutaneous and Omental Adipose Tissue” 11° World Congress of Developmental Origins of Health and Disease DOHaD 20-23 October 2019, Melbourne, Australia.
- **Samavat J. et al.** “The Role of Vitamin B12 Deficiency on Lipid Handling by Adipocytes: a new protagonist in metabolic disease?”. 3rd Midlands Academy of Medical Sciences Research Festival 27th March 2019, Birmingham, United Kingdom.
- **Samavat J. et al.** Effect of vitamin B12 on obesity and metabolic disorders 21st May 2019 PGR symposium Warwick Medical School
- **Samavat J. et al.** 3rd Midlands Academy of Medical Sciences Research Festival 23rd March 2018, Loughborough University, United Kingdom “VitaminB12 deficiency triggers adipocyte dysfunction by enhancing triglyceride biosynthesis and pro-inflammatory cytokine production: a new agonist in metabolic disease?”
- **Samavat J. et al** PRiDE annual conference, University of Warwick, UK, 2017 - Sep 2017 Effect of vitamin B12 deficiency on adipose metabolism of lipids.

Vitamin B12 Deficiency Leads To Fatty Acid Metabolism Dysregulation and Increased pro-inflammatory cytokine production in Human Adipocytes and Maternal Subcutaneous and Omental Adipose Tissue

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Vitamin B12(B12)is an essential micronutrient required for several metabolic reactions. Animal and clinical studies show that B12-deficiency is associated with the metabolic syndrome. Given the key metabolic role of adipose tissue we investigated whether B12 deficiency may affect triglyceride synthesis and lipid metabolism leading to adipose tissue inflammation. The AbdSc pre-adipocyte cell line(Chub-S7)and human AbdSc primary pre-adipocytes were differentiated under different B12 concentrations (25pM,100pM,1nM,500nM). Human Om, Sc-AT and blood samples were collected from 106 pregnant women at delivery. Serum B12 and relevant metabolic risk factors were measured. Gene expression was performed by q-RTPCR, *de novo* triglyceride synthesis was quantified by radioactive tracing, β -oxidation and palmitate-induced oxygen consumption rate was determined using seahorse-XF analyser. Adipocytes cultured in low-B12 conditions showed significantly increased expression($P<0.01$)of triglyceride biosynthesis genes(ELOVL6,SCD,GPAT,LPIN1 and DGAT2),a significantly decreased expression($P<0.01$)of β -oxidation genes(FAT/CD36,CPT1- β ,ACADL,ECHS1 andACAA2)and an increased expression($P<0.01$)of pro-inflammatory cytokines(IL-1, IL-6,IL-8,IL-18,TGF- β ,TNF- α and MCP-1).These data were also confirmed in the AT of B12-deficient pregnant women. Additionally,real-time fatty acid flux synthesis and fatty-acid-oxidation induced by palmitate were significantly altered($P<0.05$)in B12-deficient adipocytes. Our data highlights that B12-deficiency has profound effects on adipocyte dysfunction, opening new insights into the pathogenesis of maternal obesity and the relevance of micronutrient supplementation for pregnant mothers.

Vitamin B12 Deficiency Increases pro-inflammatory cytokine production in Human Adipocytes and Maternal Subcutaneous and Omental Adipose Tissue

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Background: Vitamin B12 (B12) is an essential micronutrient required for optimal hematopoietic, neurologic and other several metabolic reactions. Longitudinal studies and animal models showed that low maternal vitamin B12 deficiency is associated with the maternal obesity, development of insulin resistance and metabolic syndrome phenotype suggesting the crucial role of B12 in adipose tissue function. Although the mechanisms underpinning metabolic disorders remain poorly defined, the pathophysiology of obesity-induced metabolic diseases has been strongly related to white adipose tissue dysfunction through several mechanisms such as fibrosis, apoptosis and inflammation. Therefore, we investigated the role B12 deficiency in lipid metabolism arising from human abdominal subcutaneous adipocytes (AbdSc Ad), Subcutaneous (Sc) and Omental (Om) adipose tissue(AT). Therefore, the aim of this study is to investigate the role of B12 inflammation in human adipocytes.

Methods: AbdSc pre-adipocytes cell line (Chub-S7) and human AbdSc primary pre-adipocyte cells were differentiated under different B12 concentrations (25pM, 100pM, 1nM, 500nM) to assess B12 deficiency effects. Human Om, Sc AT and blood samples were also collected from 106 white pregnant women at delivery. Serum B12 as well as relevant metabolic risk factors were measured. Gene expression was performed by q-RTPCR.

Results: Adipocytes cultured in low vitamin B12 conditions showed significantly increased gene expression of pro-inflammatory cytokines ($P < 0.01$) such as interleukin-1 (IL-1) interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18), transforming growth factor beta (TGF- β), tumor necrosis factor alpha (TNF- α), monocyte chemoattractant protein-1 (MCP-1/CCL2). These data were also confirmed in both Omental and Subcutaneous AT of pregnant women with B12 deficiency. Our data highlights that low B12 induces higher gene expression and secretion of pro-inflammatory cytokines, which might lead to adipocyte dysfunction. This link between vitamin B12 deficiency and metabolic inflammation opens new insights into the pathogenesis of maternal obesity and the relevance of micronutrient supplementation for pregnant mothers.

Increased pro-inflammatory cytokine production in vitamin B12 deficient adipocytes.

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Vitamin B12 (B12) is an essential micronutrient required for optimal hematopoietic, neurologic and other several metabolic reactions. Longitudinal studies and animal models showed that low maternal vitamin B12 deficiency is associated with the maternal obesity, development of insulin resistance and metabolic syndrome phenotype suggesting the crucial role of B12 in adipose tissue function. Although the mechanisms underpinning metabolic disorders remain poorly defined, the pathophysiology of obesity-induced metabolic diseases has been strongly related to white adipose tissue dysfunction through several mechanisms such as fibrosis, apoptosis and inflammation. Therefore, the aim of this study is to investigate the role of B12 inflammation in human adipocytes.

Human pre-adipocytes cell line (Chub-S7) and primary adipocytes were obtained from lean, obese and morbid obese patients, grown to confluence, differentiated for one week, maintained in nutrition media for next 7 days (day 14) and then used for further experimental analysis. In order to analyse B12 deficiency effects, customized media with different concentrations of B12 (25pM, 100pM, 1nM, 500nM) were used. Gene expression was performed by q-RTPCR

Chub-S7 and primary adipocytes cultured in low vitamin B12 conditions showed significantly increased gene expression of pro-inflammatory cytokines such as interleukin-1 (IL-1) interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18), transforming growth factor beta (TGF- β), tumor necrosis factor alpha (TNF- α), monocyte chemoattractant protein-1 (MCP-1/CCL2). Our data highlights that low B12 in adipocytes induces higher gene expression and secretion of pro-inflammatory cytokines, which might lead to adipocyte dysfunction. This link between vitamin B12 deficiency and metabolic inflammation opens new insights into the pathogenesis of maternal obesity and the relevance of micronutrient supplementation for pregnant mothers.

VitaminB12 deficiency leads to adipocyte dysfunction by decreasing fatty-acid oxidation: a new protagonist in metabolic disease?

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Vitamin B12 (B12) is an essential micronutrient required several metabolic reactions. Longitudinal studies and animal models showed that pregnancy B12 deficiency is associated with the maternal obesity, development of insulin resistance and metabolic syndrome phenotype. Although the mechanisms underpinning metabolic disorders remain poorly defined, it has become increasingly clear that dysregulation of lipids is associated with obesity and its comorbidities. Therefore, the aim of this study is to investigate the role of B12 in lipid metabolism in human adipocytes. Human pre-adipocytes cell line (Chub-S7) were grown to confluence (day 0), differentiated for one week, maintained in nutrition media for next 7 days (day 14) and harvested for RNA analysis. In order to analyse B12 deficiency effects, customized media with different concentrations of B12 (25pM, 100pM, 1nM, 500nM) were used. Gene expression was performed by q-RTPCR and in order to Measure real-time fatty acid oxidation *Seahorse* XF Analyzer was conducted. Adipocytes cultured in low B12 conditions showed significantly decreased expression of genes involved in β -oxidation such as Fatty acid traslocase (FAT/CD36), Carnitine palmitoyltransferase I (CPT1- β), Acyl-CoA Dehydrogenase Long Chain (ACADL), Enoyl-CoA Hydratase Short Chain 1 (ECHS1) and Acetyl-CoA Acyltransferase 2 (ACAA2). In addition, we also observed that real-time fatty acid oxidation induced by palmitate was significantly decreased in B12 deficient cells. Our data highlights that low B12 cause a decrease of fatty acid metabolism, which might lead to adipocyte dysfunction and suggest a possible role of B12 deficiency into metabolic disorders.

Expressions of Transcobalamin receptor CD320 and transporter TCN2 in low vitamin B12 treated human adipocytes

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Background: Vitamin B12 (cobalamin) is an essential micronutrient in humans. Cellular uptake of B12 is facilitated by a complex process involving transcobalamin, a plasma protein and a cell surface receptor that specifically binds transcobalamin saturated with cobalamin. Earlier clinical studies and animal models showed that low maternal B12 deficiency is associated with maternal obesity, development of insulin resistance and metabolic syndrome, suggesting the crucial role of B12 in adipose tissue function. Although several studies explored the expression of cellular B12 receptor (CD320) and transporters (TCN2) that may regulate cellular uptake and storage of cobalamin in several tissues like placenta, liver and brain, no study has been conducted on adipocytes so far. Therefore the aim of this study is to investigate the expression of cobalamin receptor and transporter and intracellular B12 levels in human adipocyte

Methods: Human pre-adipocyte cell line (Chubs S7), and human primary pre-adipocytes were grown to confluence (day 0), differentiated in differentiation media for one week and maintained in nutrition media for a further 7 days (day 14). In order to analyse B12 deficiency effects, customized media with different concentrations of B12 (0pM, 25pM, 100pM, 1nM, 10nM, 100nM, 500nM) were used at all stages. On day 14, condition media were collected, cells were harvested for RNA analysis and stored at -80°C until use. Intracellular B12 concentration and their corresponding conditioned media were determined by electrochemiluminescent immunoassay using a Roche Cobas immunoassay analyzer (Roche Diagnostics UK, Burgess Hill, UK). Gene expression was performed by RT-PCR. **Results:** Intracellular levels of B12 in adipocytes cultured in low B12 (25pM and 100pM) showed 300-500% higher concentrations of B12 compared to the condition media. However, increasing concentrations of B12 in the condition media to 1nM, 10nM, 100nM and 500nM resulted in progressive reduction in cellular uptake of B12 to 9.1%, 3.9%, 1.6% and 1.3%, respectively. However, the intracellular levels are still higher than the normal physiological levels seen in humans. We also observed there was a significant increase in the gene expression of B12 transporter transcobalamin II (TCN2) and transcobalamin receptor (TCblR) (CD320) in adipocytes under each B12 condition.

Conclusion: Our study thus provides novel evidence that when extracellular B12 levels are low (0-100pM), the intracellular B12 levels and the gene expression of receptor and transporter are higher, whereas opposite effects are seen at higher extracellular B12 levels (1-500nM). This suggests active transport of B12 in adipocytes at low concentrations. In

addition, there might be a threshold for the B12 entry at the membrane level in higher concentrations of B12. Our findings support that optimal physiological levels of B12 is required. Further studies are required to assess whether the effects of non-physiological concentrations of B12 (low or high) result in adipocyte dysfunction.

Vitamin B12 Deficiency Leads To Dysregulation of Fatty Acid Metabolism in Human Adipocytes and Maternal Subcutaneous and Omental Adipose Tissue

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Background: Vitamin B12 (B12) is an essential micronutrient required for two key metabolic reactions. Longitudinal studies showed that B12 deficiency during pregnancy is associated with maternal obesity and metabolic syndrome phenotype. Although the mechanisms underpinning metabolic disorders remain poorly defined, it has become increasingly clear that dysregulation of lipids is associated with obesity and its comorbidities. Therefore, we investigated the role B12 deficiency in lipid metabolism in human adipocytes (and maternal Subcutaneous (Sc) and Omental (Om) adipose tissue (AT)).

Methods: AbdSc pre-adipocytes cell line (Chub-S7) and human AbdSc primary pre-adipocyte cells were differentiated under different B12 concentrations (25pM, 100pM, 1nM, 500nM) to assess B12 deficiency effects. Human Om, Sc AT and blood samples were also collected from 106 white pregnant women at delivery. Serum B12 as well as relevant metabolic risk factors were measured. Gene expression was performed by q-RT-PCR, de novo triglyceride synthesis was quantified using radioactive tracing technique by incorporation of ¹⁴C-oleate and β -oxidation and palmitate-induced oxygen consumption rate (OCR) was determined using Seahorse XF analyser.

Results: Adipocytes cultured in low B12 condition showed significantly increased expression of genes involved in triglyceride biosynthesis ($P < 0.01$) such as elongation of very long chain fatty acids protein 6 (ELOVL6), stearoyl-CoA desaturase (SCD), glycerol-3-phosphate acyltransferase (GPAT), phosphatidate phosphatase (LPIN1), diacylglycerol O-acyltransferase 2 (DGAT2) and a significantly decreased expression of genes involved in β -oxidation ($P < 0.01$) such as fatty acid translocase (FAT/CD36), carnitine palmitoyltransferase I (CPT1- β), acyl-CoA dehydrogenase long chain (ACADL), enoyl-CoA hydratase short chain 1 (ECHS1) and acetyl-CoA acyltransferase 2 (ACAA2). These data were also confirmed in the Sc and Om AT from pregnant women with B12 deficiency. We also observed the real-time fatty acid flux synthesis and fatty acid oxidation induced by palmitate was significantly altered in B12 deficient adipocytes.

Conclusion: Our data highlights that low B12 induces a dysregulation of fatty acid metabolism, which might lead to adipocyte dysfunction and suggest a possible role of B12 deficiency in metabolic disorders.

The Role of Vitamin B12 Deficiency on Lipid Handling by Adipocytes: a new protagonist in metabolic disease?

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Vitamin B12 (B12) is an essential micronutrient required for several metabolic reactions. Longitudinal studies and animal models showed that B12 deficiency during pregnancy is associated with maternal obesity, development of insulin resistance and metabolic syndrome phenotype. Although the mechanisms underpinning metabolic disorders remain poorly defined, it has become increasingly clear that dysregulation of lipids is associated with obesity and its comorbidities. Therefore, the aim of this study is to investigate the role of B12 deficiency in lipid metabolism arising from human abdominal subcutaneous adipocytes (AbdSc Ad) and Omental (Om) adipose tissue (AT). An AbdSc pre-adipocytes cell line (Chub-S7) and human AbdSc primary pre-adipocyte cells (lean, overweight, obese and morbid obese) were differentiated under low and high B12 concentrations (25pM, 100pM, 1nM, 500nM) to assess B12 deficiency effects. Human Abd Om AT and blood samples were also collected from 106 white pregnant women at delivery. Abd Sc was also assessed. Serum B12 as well as the relevant metabolic risk factors were measured. *In vitro* and *ex vivo* analysis of differentiated AbdSc Ad and OmAT respectively was performed for q-RTPCR, triglycerides synthesis quantified using radioactive tracing (¹⁴C-oleate), whilst β -oxidation and palmitate-induced oxygen consumption rate (OCR) was determined using seahorse XF analyser. Adipocytes cultured in low B12 condition showed significantly increased expression of genes ($P < 0.05$) involved in triglyceride biosynthesis such as elongation of very long chain fatty acids protein 6 (ELOVL6), stearoyl-CoA desaturase (SCD), glycerol-3-phosphate acyltransferases (GPAT), phosphatidate phosphatase (LPIN1), diacylglycerol O-acyltransferase 2 (DGAT2) and a significantly decreased expression of genes ($P < 0.05$) involved in β -oxidation such as fatty acid translocase (FAT/CD36), carnitine palmitoyltransferase I (CPT1- β), acyl-CoA dehydrogenase long chain (ACADL), enoyl-CoA hydratase short chain 1 (ECHS1) and acetyl-CoA acyltransferase 2 (ACAA2). These data were also confirmed in the AT tissue from pregnant women with B12 deficiency. In addition, we also observed the real-time fatty acid flux synthesis and fatty acid oxidation induced by palmitate was significantly altered in B12 deficient adipocytes.

Our data highlights that low B12 cause a dysregulation of fatty acid metabolism, which might lead to adipocyte dysfunction and suggest a possible role of B12 deficiency in metabolic disorders.

Vitamin B12 Deficiency Triggers Adipocyte Dysfunction by Enhancing Triglyceride Biosynthesis And Pro-inflammatory Cytokine Production: A New Agonist In Metabolic Disease?

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Background: Vitamin B12 (B12) is an essential micronutrient required for optimal hematopoietic, neurologic and other several metabolic reactions. Longitudinal studies and animal models showed that low maternal vitaminB12 deficiency is associated with the maternal obesity, development of insulin resistance and metabolic syndrome phenotype. Although the mechanisms underpinning metabolic disorders remain poorly defined, it has become increasingly clear that dysregulation of lipids and metabolic inflammation is associated with obesity and its comorbidities. Therefore, the aim of this study is to investigate the role of B12 in lipid regulation and inflammation in human adipocytes.

Methods: Human preadipocytes cell line (Chubs S7) and human primary preadipocytes were grown to confluence (day 0), differentiated in differentiation media for one week and maintained in nutrition media for next 7 days (day 14). In order to analyse B12 deficiency effects, customized media with different concentrations of B12 (25pM/L, 100pM/L, 1nM/L, 500nM/L) were used. On day 14, the condition media were collected and the cells were harvested for RNA and protein analysis and stored at -80°C until use. Cellular triglycerides (TG) synthesis was quantified using radioactive tracing technique by incorporation of ^{14}C -oleate. **Results:** Adipocytes cultured in low vitaminB12 conditions showed a significant increased expression of genes involved in triglyceride synthesis such as Elongation Of Very Long Chain Fatty Acids Protein 6 (ELOLV6), Stearoyl-CoA Desaturase (SCD), Glycerol-3-phosphate acyltransferases (GPAT), acylglycerol phosphate acyltransferase (AGPAT), phosphatidate phosphatase (LIPIN1) and Diacylglycerol O-Acyltransferase 2 (DGAT2). Also the expression of Fatty acid binding protein (FABP4), a key adipogenic gene that regulates lipid trafficking and is responsible for the formation of mature adipocytes resulted significantly increased in low B12 treated adipocytes. Cellular uptake of radio-labelled fatty acid (^{14}C -oleate) for *de novo* TG biosynthesis assessed by scintillation was significantly higher in low B12 condition. In addition, we also observed that the gene expression of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18), transforming growth factor beta (TGF- β), Monocyte chemoattractant protein-1 (MCP-1/CCL2) and were significantly increased. We analysed the levels of interleukin-1 beta (IL-1) in the condition media using the enzyme-linked immunoassorbent assay (ELISA) as well and an interesting significant increase expression of this cytokine was observed. **Conclusion:** Our data highlights that low B12 induces excess triglycerides biosynthesis and high gene expression of pro-inflammatory cytokines which might lead to adipocyte dysfunction. This link between vitamin B12 deficiency and metabolic inflammation opens new insights into the pathogenesis of maternal obesity and the relevance of micronutrient supplementation for pregnant mothers.