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Programa de Doctorado:
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Tesis Doctoral

EPIGENETICS OF ADIPOSE TISSUE AND ITS RELATED DISORDERS

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CERTIFICA: Que el trabajo expuesto en la memoria de la Tesis Doctoral desarrollada por Daniel Castellano Castillo con el título **"EPIGENETICS OF ADIPOSE TISSUE AND ITS RELATED DISORDERS"** corresponde fielmente a los resultados obtenidos.

La presente memoria ha sido realizada bajo mi dirección, considerando que tiene el contenido y rigor científico necesario para ser sometida a juicio por el tribunal nombrado por la Universidad de Málaga para optar al grado de Doctor.

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Málaga, Noviembre de 2018

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Directora de la tesis:

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Málaga, Noviembre de 2018

Yo, **Daniel Castellano Castillo**, declaro que soy el autor del presente trabajo de investigación titulado **"EPIGENETICS OF ADIPOSE TISSUE AND ITS RELATED DISORDERS",** el cual fue realizado bajo la dirección del Dr. Francisco José Tinahones Madueño, Dra. María Isabel Queipo Ortuño y la Dra. Isabel Moreno Indias.

Y para que así conste firmo el presente certificado.

Daniel Castellano Castillo

Málaga, Enero de 2017

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INDEX

ABBREVIATIONS

Body mass index: BMI

Tumor necrosis factor: TNF/ TNFα

Interleukin 6: IL6

Insulin resistance: IR

Cardiovascular disease: CVD

Metabolic Syndrome: MetS

Triglycerides: TG/Tg

High-density lipoprotein cholesterol: HDL-cho/HDL-C

Adipose tissue: AT

White adipose tissue: WAT

Brown adipose tissue: BAT

Uncoupling protein: UCP

Subcutaneous adipose tissue: SAT

Visceral adipose tissue: VAT

Weight/hip ration: WHR

Stromal vascular fraction: SVF

Adipose tissue-derived mesenchymal stem cells: ASCs

Scavenger receptor class A member 5: SCARA5

Bone morphogenetic protein 2: BMP2

Bone morphogenetic protein 4: BMP4

Transforming growth factor beta: TGFβ

Platelet-derived growth factor receptor A: PDGFRα

Platelet-derived growth factor receptor B: PDGFRβ

Platelet-derived growth factor subunit A: PDGFA

SMAD family member 1: SMAD1

SMAD family member 4: SMAD4

SMAD family member 5: SMAD5

SMAD family member 8: SMAD8

Zinc finger protein 423: Zfp423

Lysyl oxidase: Lox

Fibroblast growth factor 2: FGF2

Peroxisome proliferator activated receptor gamma: PPARγ

Peroxisome proliferator activated receptor gamma 2: PPARγ2

Peroxisome proliferator activated receptor alpha: PPAR α

CCAAT enhancer binding protein beta: C/EBPβ

CCAAT enhancer binding protein delta: C/EBPδ

CCAAT enhancer binding protein alpha: C/EBPα

Krüppel-like factor 5: KLF5

Krüppel-like factor 15: KLF15

Krüppel-like factor 2: KLF2

Sterol regulatory element binding transcription factor 1: SREBP1

Sterol regulatory element binding transcription factor 1 isoform c: SREBP1c

Sterol regulatory element binding transcription factor 1 isoform a: SREBP1a

Sterol regulatory element binding transcription factor 2: SREBP2

Lipopolysaccharides: LPS

Interferon-**γ:** IFN-γ

Interleukin 12: IL12

Interleukin 4: IL4

Interleukin 14: IL14

Interleukin 10: IL10

Chitinase-like 3: Ym1

Arginase 1: ARG1

Chylomicrons: CM

Apolipoprotein B48: ApoB48

Apolipoprotein CII: ApoCII

Apolipoprotein CIII: ApoCIII

Lipoprotein lipase: LPL

Apolipoprotein E: ApoE

Low-density lipoprotein cholesterol: LDL-cho/LDL-C

Low density lipoprotein receptor: LDLR

Low-density lipoprotein (LDL)-related protein 1: LRP1

Very low-density lipoprotein: VLDL

Intermediate density lipoproteins: IDL

Hepatic lipoprotein lipase: HLPL

Insulin receptor: INSR

Leptin: LEP

Leptin receptor Ob-Rb: Ob-Rb

Free fatty acids: FFA

Cluster of differentiation 36: CD36

Fatty acid binding protein 4: FABP4

Acyl-CoA synthase: ACS

Glycerol-3 phosphate: glycerol-3P

Acetyl-CoA carboxylase: ACC

Fatty acid synthase: FAS

Adipose TG lipase: ATGL

Hormone sensitive lipase: HSL

Perilipin 1: PLIN1

Caveolin 1: CAV-1

Janus kinase: JAK

Signal transducer and activator of transcription: STAT

Low-molecular-weight leptin trimer: LMW

Medium-molecular-weight leptin hexamer: MMW

High-molecular-weight leptin complex: HMW

Phosphoenolpyruvate carboxykinase: PEPCK

Glucose-6-phosphatase: G6P

AMP-activated protein kinase: AMPK

Beta cell: β-cell

Waist circumference: WC

Blood pressure: BP

Systolic blood pressure: SBP

Diastolic blood pressure: DBP

Non-alcoholic steatohepatitis: NASH

Neurological disorders: ND

Endoplasmic reticulum: ER

Lipase maturation factor 1: LMF1

Sel-1 suppressor of lin-12-like: Sel1L

Heparan sulfate proteoglycans: HSPG

Glycosylphosphatidylinositol (GPI)-anchored glycoprotein 1: GPIHBP1

Apolipoprotein A5: ApoA5

Angiopoietin-like protein 3: Angptl3

Angiopoietin-like protein 4: Angptl4

Prostaglandin E2: PGE2

Micro RNA 27: miR-27

Micro RNA 29: miR-29

Insulin-regulated aminopeptidase: IRAP

Cluster of differentiation 68: CD68

Macrophage chemoattractant protein 1: MCP1

C-C motif chemokine ligand 2: CCL2

High fat diet: HFD

Tricarboxylic acids cycle: TCA cycle

Thiazolidinediones: TZDs

Retinoid X receptor alpha: RXRα

Stearoyl-CoA-desaturase: SCD/SCD1

Monounsaturated fatty acids: MUFFAS

Polyunsaturated fatty acids: PUFFAS

Liver X receptor beta: LXRb

Liver X receptor alpha: LXRa

ATP binding cassette transporters A: ABCA

ATP binding cassette transporters G: ABCG

Neuropeptide Y: NPY

Agouti-related peptide: AgRP

Pro-opiomelanocortin: POMC

α -melanocyte stimulating hormone: α MSH

Phosphodiesterase-3B: PDE3B

Alpha-ketoglutarate dependent dioxygenase: FTO

transient receptor potential vanilloid chanel: TRPV

15-deoxy- Δ 512,14) -prostaglandin J2: 15d-PGJ2

Estradiol: E2

AMP-activated protein kinase kinase: AMPKK

Carnitine palmitoyltransferase 1: CPT1

Interleukin 1 beta: IL1B

Brain-derived neurotrophic factor: BDNF

Norepinephrine: NE

Complement C3: C3

Complement component C3a: C3a

Acylation stimulating protein: ASP

TNF receptor 1: TNFR1

TNF receptor 2: TNFR2

Nuclear factor kappa B subunit 1: Nf-κB1

Wingless-related integration site: Wnt

Insulin receptor substrate 1: IRS1

Colorectal cancer: CRC

Vitamin D: VD

Ultraviolet B rays: UVB

Cytochrome P450 family 27 subfamily A member 1: CYP27A1

Cytochrome P450 family 27 subfamily B member 1: CYP27B1

Cytochrome P450 family 24 subfamily A member 1: CYP24A1

25-hydroxyvitamin D: 25(OH)D

1,25-dihydroxyvitamin D: $1,25(OH)_2D$

Vitamin D receptor: VDR

Dinucleotide Citosin-guanine: CpG

5-methylcitosine: 5mC

Short interspersed nuclear elements: SINE

Long interspersed nuclear elements: LINE

DNA methyl-transferases: DNMTs

DNA methyl-transferase 1: DNMT1

DNA methyl-transferase 2: DNMT2

DNA methyl-transferase 3A: DNMT3A

DNA methyl-transferase 3B: DNMT3B

DNA methyl-transferase 3L: DNMT3L

S-Adenosylmethionine: SAM

S-adenosylhomocysteine: SAH

Ten-eleven translocation: TET

5-hydroximethylcytosine: 5hmC

5-formylcitosine: 5fC

5-carboxylcitosine: 5caC

DNA glycosylase: TDG

Base excision repair: VER

Cytosine: C

α -ketoglutarate: α -KG

Histone H2A: H2A

Histone H2B: H2B

Histone H3: H3

Histone H4: H4

Histone Acetyltransferases: HATs

Histone Mehtyltransferases: HMTs

Histone de-acetylases: HDACs

Histone de-methylases: HDMs

Lysine: K

Methyl-CpG binding domain: MBD

Methyl-CpG binding proteins: MeCPs

Methyl-CpG binding domain protein 1: Mbd1

Lysine 9 of Histone H3: H3K9

Suppressor of variegation 3-9 homolog 1: Suv39h1

Suppressor of variegation 3-9 homolog 2: Suv39h2

Di-methylation at Lysine 4 of Histone H3: H3K4me2

Tri-methylation at Lysine 4 of Histone H3: H3K4me3

Chromatin assembly factor 1: Caf1

Heterochromatin protein 1: HP1

SET domain bifurcated 1: Setdb1

M-phase phosphoprotein 8: MPP8

Lysine 36 of histone H3: H3K36

Zinc finger-CxxC domain: ZF-CxxC

CXXC finger protein 1: CFP1

Lysine demethylase 2a: Kdm2a

Lysine demethylase 2b: Kdm2b

Tri-methylation at Lysine 36 of Histone H3: H3K36me3

Polycomb repressive complex 2: PRC2

Enhancer of zeste 1 polycomb repressive complex 2 subunit: EZH1

Enhancer of zeste 2 polycomb repressive complex 2 subunit: EZH2

Embryonic ectoderm development: EED

SUZ12, polycomb repressive complex 2 subunit: SUZ12

Polycomblike: PCL

RB binding protein 7: RbAp46/RBBP7

RB binding protein 7: RbAp48/RBBP4

Jumonji and AT-rich interaction domain containing 2: JARID2

AE binding protein 2: AEBP2

Lysine 27 of histone H3: H3K27

Di-methylation at lysine 27 of histone H3: H3K27me2

Tri-methylation at lysine 27 of histone H3: H3K27me3

Adenosine triphosphate: ATP

Oxidized flavin adenine dinucleotide: FAD

Reduced flavin adenine dinucleotide: $FADH_2$

Lysine demethylase 5B: KDM5B

Jumonji and AT-rich interaction domain containing 1B: JARID1B

Jumonji domain-containing histone demethylases: Jmj-KDMs

Oxidized nicotinamide adenine dinucleotide: NAD+

Sirtuin 6: SIRT6

Pim-3 proto-oncogene, serine/threonine kinase: PIM3

ATP binding cassette subfamily G member 1: ABCG1

Thioredoxin interacting protein: TXNIP

6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3: PFKFB3

Methyltransferase like 8: METTL8

Insulin like growth factor 2: IGF2

Saturated fatty acids: SFA

Neuronal growth regulator 1: NEGR1

Fatty acid binding protein 1: FABP1

Fatty acid binding protein 2: FABP2

PPARG coactivator 1 alpha: PPARGC1 α

Melanocortin 2 receptor: MC2R

Melanocortin 3 receptor: MC3R

Transcription factor 7 like 2: TCF7L2

Potassium voltage-gated channel subfamily Q member 1: KCNQ1

Histone deacetylase 4: HDAC4

Nuclear receptor corepressor: NCOR

Plant homeodomain finger two: PHF2

n-3 polyunsaturated fatty acids: n-3 PUFFAs

Methyl-CpG-binding domain protein 2: MBD2

Acyl-CoA dehydrogenase medium chain: ACADM

Carnitine palmitoyltransferase 1B: CPT1B

Carnitine palmitoyltransferase 1C: CPT1C

Fatty acid desaturase 1: FADS1

Monoacylglycerol O-acyltransferase 1: MOGAT1

Monoacylglycerol O-acyltransferase 2: MOGAT2

Solute carrier family 44 member 2: CTL2/ SLC44A2

TAP binding protein: TAPBP

ATPase, H+ transporting, lysosomal V0 subunit D2: Atp6v0d2

Matrix metallopeptidase 12: Mmp12

Triggering receptor expressed on myeloid cells 2: Trem2

C-type lectin domain family 4, member d: Clec4d

Glycoprotein (transmembrane) nmb: Gpnmb

Chromatin inmunoprecipitation: ChIP

Homeostatic model assessment of insulin resistance: HOMA-IR

Postprandial triglycerides: Post TG

Glutamate-Oxaloacetate Transaminase: GOT

Glutamate-Pyruvate Transaminase: GPT

Gamma Glutamyl Transpeptidase: GGT

C-Reactive Protein: CRP

Proteinase K: PK

E2F transcription factor 1: E2F1

Transcription Start Site: TSS

Lean NG: Lean normoglycemic

MO NG: Morbid obese normoglycemic

MO PD: Morbid obese prediabetic

Parathyroid hormone: PT

INTRODUCTION

1. OBESITY

Nowadays, obesity has become one of the greatest causes for health disorders in developed countries. More than a third of the world population suffers of overweight or obesity, being expected to rise up to 38% of overweight and 20% of obesity by 2030, reaching the 85% of overweight/obese people in the USA (1).

Obesity is defined as an excess of body weight, which underlines with an accumulation of adiposity (adipose tissue). Obesity is usually interpreted by the Body mass index (BMI), measured as a ratio weight/height^2 (2). Though, subjects are mostly classified according to this index as lean or normal-weight subjects (when BMI<25), overweight subjects (when BMI≥25 and <30), obese subjects (BMI≥30 and <40) and morbid obese subjects (BMI≥40) (3,4).

The excess of adiposity is usually accompanied by a series of metabolic disturbances and a higher risk of suffering certain diseases (1). Obesity increases the risk of being diabetic between 4.9% and 9%, and inversely 60% to 90% of all diabetics are or have been obese (5). In fact, although bariatric surgery has as first consequence the weight loss, is also one of the most effective treatment to improve diabetes and metabolic disease, demonstrating the close relationship between BMI and diabetes (6). Although the connection between both, obesity and diabetes, is not totally clear, mechanisms in which are involved pro-inflammatory cytokines (tumor necrosis factor (TNF), interleukin 6 (IL6)), insulin resistance (IR), deranged fatty acid metabolism and cellular processes such as reticulum stress and mitochondrial dysfunction have been shown to be involved (7).

Obesity has also been widely associated to cardiovascular disease (CVD) (8). It has been shown that an increase in a unit of BMI is associated to a 4% increase in the risk of ischemic stroke and 6% increase in hemorrhagic stroke (9). Increase in BMI was also

associated to an increase in blood pressure, which was traduced to a 12% increase in coronary heart disease and 24% increase risk for stroke (9). Other study showed an increase in subclinical cardiovascular disease associated to a rise in BMI compared to lean subjects (10). Obesity was positively associated with a 1.17-1.28 relative risk increase in coronary artery calcium, 1.45 relative risk increase for common carotid artery intimal medial thickness, 1.32 relative risk increase for internal carotid artery intimal medial thickness and 2.69 relative risk increase in left ventricular mass (10). Atherosclerosis has also been associated to obesity in a process in which adipose tissue, low-grade inflammation, oxidative stress, impaired autophagy and gut microbiota among other factors could be related (11,12).

It is clear that cancer is one of the greatest health concerns in modern societies. Obesity has been strongly associated to several cancers, and what is more, has been proposed as the second biggest cause of cancer in the world only surpassed by smoking (13). Thus, the estimation is that over 20% of all cancers are caused for excess body weight, rising to 50% in postmenopausal women (14). Moreover, obesity also affects death rates in cancer, up to 20% of death in women and 14% of death in men that can be attributable to obesity (14).

Finally, obesity has traditionally been associated to what is known as metabolic syndrome (MetS). MetS is defined by a cluster of metabolic alterations which altogether increase the risk of suffering diabetes, cardiovascular disease and cancer (15,16).

2. ADIPOSE TISSUE

There are different adipose tissue (AT) depots with different metabolic roles (17). White AT (WAT) is composed by unilocular cells with a high capacity for lipid storage in the form of triglycerides (TG), since is the tissue in charge of accumulating the

surplus of energy in the organism. On the other hand, Brown AT (BAT) is made out of multilocular adipocytes with a high mitochondrial content that can consume high levels of energy when are stimulated by adrenergic signals or cold; the energy is used in heat production, a process that is carried out by the mitochondrial uncoupling protein (UCP) (17).

There are two types of WAT, which are mainly defined by their depot location: the subcutaneous AT (SAT) and the visceral AT (VAT) (18) (Figure 1).

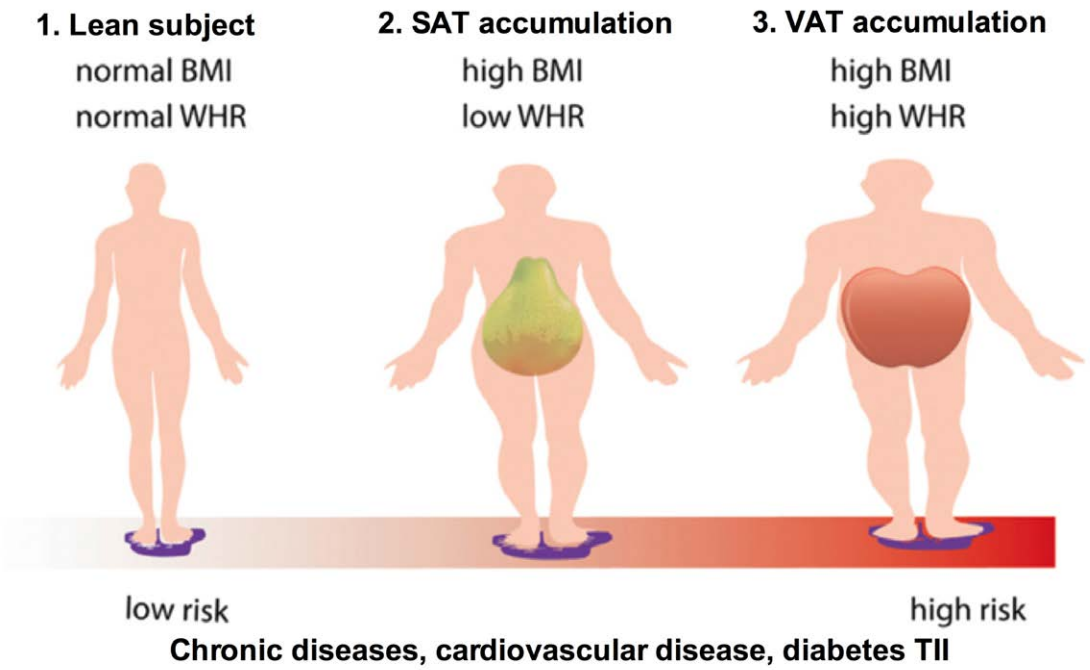

Figure 1. Patterns of fat distribution and its relationship with BMI, weight/hip circumference and risk of suffering disease. Pear shape (2) is associated to SAT and is usually refers as woman fat distribution. Apple shape (3) is associated to VAT and is most common to men fat distribution. Risk of associated chronic disease such as cardiovascular disease or diabetes is display by the red line intensity from the lowest risk for lean subjects (1) to the greatest risk characteristic of apple fat distribution (3). Abbreviations: Subcutaneous

adipose tissue (SAT); visceral adipose tissue (VAT); Body mass index (BMI); weight/hip ratio (WHR). **Adapted from** (19)**.**

Both SAT and VAT not only differ by their location, but also by their molecular profile, their metabolism and their implication in the etiology of the metabolic disease (18,20–22). Also the embryonic origin of both fat depots seems to be different. While VAT adipocytes are thought to have a mesodermal origin, SAT adipocytes come from different embryonic origins depending on the exact depots (mesoderm, neuroectoderm), and in some cases still undetermined what their origin are (17). Besides, while expansion of SAT has been long considered as a protective factor against metabolic disorders, fat accumulation in VAT (characteristic of central obesity or high wait/hip ratio) has been deemed to prompt metabolic disorders (19,23). However, there is an ongoing discussion about which tissue is first damaged and which are their roles in the generation of these diseases (20–22,24). What is undoubtedly is that AT has emerged as an important mediator of the whole body homeostasis, and its role as an endocrine organ has focused great interests.

2.1. Adipose Tissue cellularity: Adipocyte precursors and adipogenesis

AT is composed by a myriad of cell types in which adipocytes, the predominant cell, are in charge of lipid accumulation. Moreover, in its stromal vascular fraction (SVF) there are macrophages (which modulate adipose tissue function), lymphocytes, vascular cells, pericytes, adipose tissue-derived mesenchymal stem cells (ASCs) (with auto-renew properties and can differentiate to pre-adipocytes) all of them known as stromal vascular cells (SVC). The adipocyte cell turnover is carried out for ASCs, although the contribution of pericytes, endothelial cells and non-resident progenitors with

hematopoietic origin has been proposed to contribute at different degree depending on the specific fat depot and the metabolic condition of the subject (17). Thus, a proper balance among all these type of cells is needed for a correct function of AT.

Adipogenesis, the mechanism in which new adipose tissue is generated, assures the AT turnover and hyperplasia (AT expansion by cell number increase). ASCs have been shown to be able to differentiate to a wide range of cell types, including adipocytes, chondrocytes, osteocytes and miocytes. Although pre-adipocytes differentiation to adipocytes is a well-known process much less is known about ASCs commitment to pre-adipocytes. Basically, the differentiation from ASC to full differentiated adipocyte requires of four stages: 1) Commitment of ASCs to pre-adipocytes; 2) clonal expansion of pre-adipocytes; 3) growth arrest; 4) terminal differentiation to adipocytes (25). The commitment of ASC to pre-adipocyte lineage implies several factors as Scavenger receptor class A member 5 (SCARA5), Bone morphogenetic protein 2 (BMP2), Bone morphogenetic protein 4 (BMP4), Transforming growth factor beta (TGFβ) signaling, Platelet-derived growth factor receptor A (PDGFRα), Platelet-derived growth factor receptor B (PDGFRβ), Platelet-derived growth factor subunit A (PDGFA), SMAD family member 1 (SMAD1), SMAD family member 4 (SMAD4), SMAD family member 5 (SMAD5), SMAD family member 8 (SMAD8), Zinc finger protein 423 (Zfp423), Lysyl oxidase (Lox) or Fibroblast growth factor 2 (FGF2) among others (25–27). TGFβ signaling inhibits pre-adipocyte commitment, BMP (from the TGFβ protein family) exerts generally a positive effect over ASC to pre-adipocyte commitment, and the PDGF has been proposed to have both, adipogenic and anti-adipogenic effects. BMP2 and BMP4 have been demonstrated to stimulate the heterodimer Smad1/Smad4 (process that Zfp423 is thought to regulate) and the expression of Peroxisome proliferator activated receptor gamma 2 (PPARγ2) and Lox that are necessary to pre-

adipocyte commitment. Another pro-adipogenic effect different from the BMP signaling is the pro-adipogenic effect that FGFs provokes over ASCs. Thus, FGF2 has been shown to stimulate PPARγ2 overexpression and exogenous addition of FGF2 in SCV stimulates WAT formation (26,27).

Much more is known about pre-adipocyte differentiation to mature adipocyte. The adipogenesis pathway is controlled by several transcription factors, with PPARG as central factor in this process. Besides, several CCAAT enhancer binding protein (C/EBP) factors are involved at different stages of adipogenesis. First, an overexpression of C/EBPβ and C/EBPδ takes place, stimulating the expression of C/EBPα and PPARγ. In turn, both PPARγ and C/EBPα can stimulate each other in a regulatory loop. Eventually, PPARγ and C/EBPα activate the expression of genes related to adipocyte metabolism, producing final adipocyte maturation (Figure 2). Other factors have been also shown to be important in adipocyte differentiation. For instance, several genes from the Krüppel-like factors (KLFs) family are involved in the adipogenic pathway. KLF5 and KLF15 have been observed to stimulate PPARγ expression in early and terminal differentiation stages, respectively. Contrary, KFL2 has been shown to have an anti-adipogenic effect by inhibiting PPARγ expression (28,29). Other factor that has been proposed to play a role in adipocyte differentiation is the Sterol regulatory element binding transcription factor 1 (SREBP1), especially the Sterol regulatory element binding transcription factor 1 isoform c (SREBP1c), which is able to activate adipogenesis via PPARγ stimulation, although mice with overespression of nuclear SREBP1c has been shown to suffer for lipodystrophy (28).

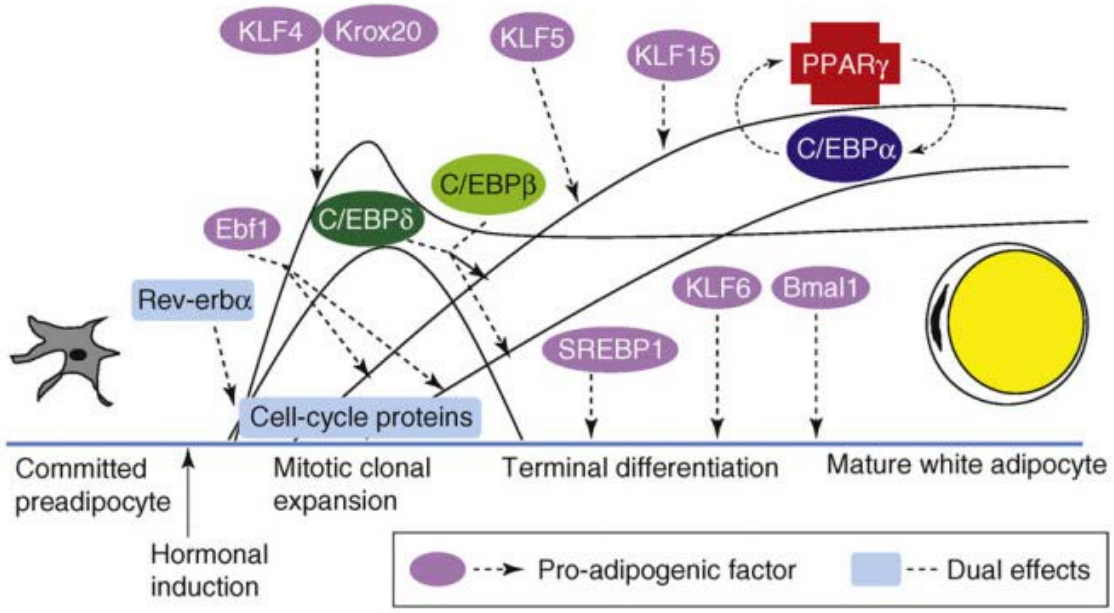

Figure 2. Stages and factors involved in the preadipocyte differentiation program. Committed preadipocytes start to express C/EBPβ and C/EBPδ, which can induce PPARγ and C/EBPα. PPARγ and C/EBPα in turn can stimulate each other in a regulatory positive loop, and can be stimulated by other factors such us KLF5 or KLF15. PPARγ and C/EBPα eventually stimulate the expression of adipose genes, which are typical of mature adipocyte. **Adapted from** (30)**.**

2.2. Adipose Tissue cellularity: Immune cells

Besides adipocyte cell lineage, AT contains leucocytes as dendritic cells, mast cells, neutrophils, macrophages, B cells and T cells, with the macrophage population as the most abundant population by far (31,32). Macrophages have been shown to modulate adipocyte and AT function as well as AT inflammation (31). Indeed, the primary and main function of AT macrophages is phagocytosis, eliminating debris and dead cells, thus contributing to the normal tissue turnover and homeostasis (33,34). In addition to its role in phagocytosis, AT macrophages seem to act as lipid buffer. It has been shown that these cells are capable of taking lipid released by adipocyte during lipolysis

(induced by fasting or adrenergic activation) in order to avoid an excess of lipids release (35,36). Lipids in macrophages are located into lipid droplets, which are taken for its degradation, at least in part, via lysosomes (36). This lipid accumulation in macrophages has been described to lead in an obesity context to an increase in macrophage lipid droplets, which are trying to buffer the excessive lipid content (36). AT macrophages also seem to modulate adipogenesis (31). Indeed, in lean AT has been observed that macrophages can recruit and stimulate adipocyte progenitors to differentiate to adipocyte. However, this phenomenon shifts in obese AT, where a pro-inflammatory nature of AT macrophages has been shown to inhibit adipogenesis. Therefore, the protective role of AT macrophages via lipid buffering and via stimulation of adipogenesis seems to failed in obesity, which might promote lipid delivery to the stream and promote AT growth via hypertrophy that has been related to metabolic disturbances (31). There are basically two phenotypes within the macrophage population: the M1 phenotype or pro-inflammatory phenotype, and the M2 phenotype or anti-inflammatory phenotype. AT homeostasis not only relies on the level of macrophage infiltration, but also in the prevalence and equilibrium between the M1-M2 phenotype (37). M1 macrophages are activated upon T-helper 1 type cytokines, microbial molecules such as lipopolysaccharides (LPS) and inflammatory mediators such interferon-**γ** (IFN-**γ**) (32). M1 macrophages have been shown to deliver pro-inflammatory cytokines and factors such as TNF**α**, interleukin 12 (IL-12) or interleukin 6 (IL-6) or reactive oxygen species (31,32,38). On the other hand, M2 activation is produced under T-helper 2 cytokines (as interleukin 4 (IL-4) and 13 (IL-13)), after what they have been observed to secrete anti-inflammatory factors such as chitinase-like 3 (Ym1), arginase 1 and interleukin 10 (IL-10) (31,32,38). Moreover, it has been demonstrated that the main fuel is different depending on this polarization. In this sense,

M1 macrophages has been shown to consume predominantly glucose while M2 phenotype has been observed to utilize fatty acids (38).

A shift from M2 phenotype to M1 phenotype has been described in AT from obese subjects, explaining the systemic chronic inflammation characteristic of obesity (39,40). Nevertheless, the predominance of M1 macrophage in AT is not only present in obese people, and can be enlarge to the presence of metabolic disturbance regardless of the BMI (37).

3. ADIPOSE TISSUE METABOLISM: LIPID AND GLUCOSE METABOLISM

3.1. Lipid metabolism

AT has an important role in storing energy surplus, which is performed via accumulation of TG in lipid droplets. During a meal, TG and cholesterol are packaged in chylomicrons (CM), within the intestine that then releases the particle to the lymph stream. CM are conformed by apolipoproteins B48, CII and CIII (ApoB48, ApoCII and ApoCIII, respectively), and by TG as the major lipid species. CM content is delivered to muscle tissue (in order to be used as fuel) and to AT (in order to be stored). This action needs from the lipoprotein lipase (LPL), which is stimulated by ApoCII present in CM. After LPL processing, CM loose their high TG content and ApoCII, and acquire ApoE until be converted to CM-remnants (with a higher proportion of cholesterol/TG). The lack of ApoCII provokes that LPL cannot further process this particles, while ApoE interacts with LDL receptors (LDLR) and Low-density lipoprotein (LDL)-related protein 1 (LRP1) in the liver where cholesterol is up-taken. The lipid content taken by the liver is re-packaged in Very-low density lipoproteins (VLDL) that are released again

to the blood stream being substrate for LPL. VLDL, as well as CM, can interact with LPL via ApoCII, delivering TG content and turning into intermediate density lipoproteins (IDL). IDL acquire ApoE and can either be removed from the circulation via interaction of ApoE with LDLR and LRP1 or be further hydrolyzed by hepatic lipoprotein lipase (HLPL) and converted to low-density lipoprotein (LDL) (41) (Figure 3).

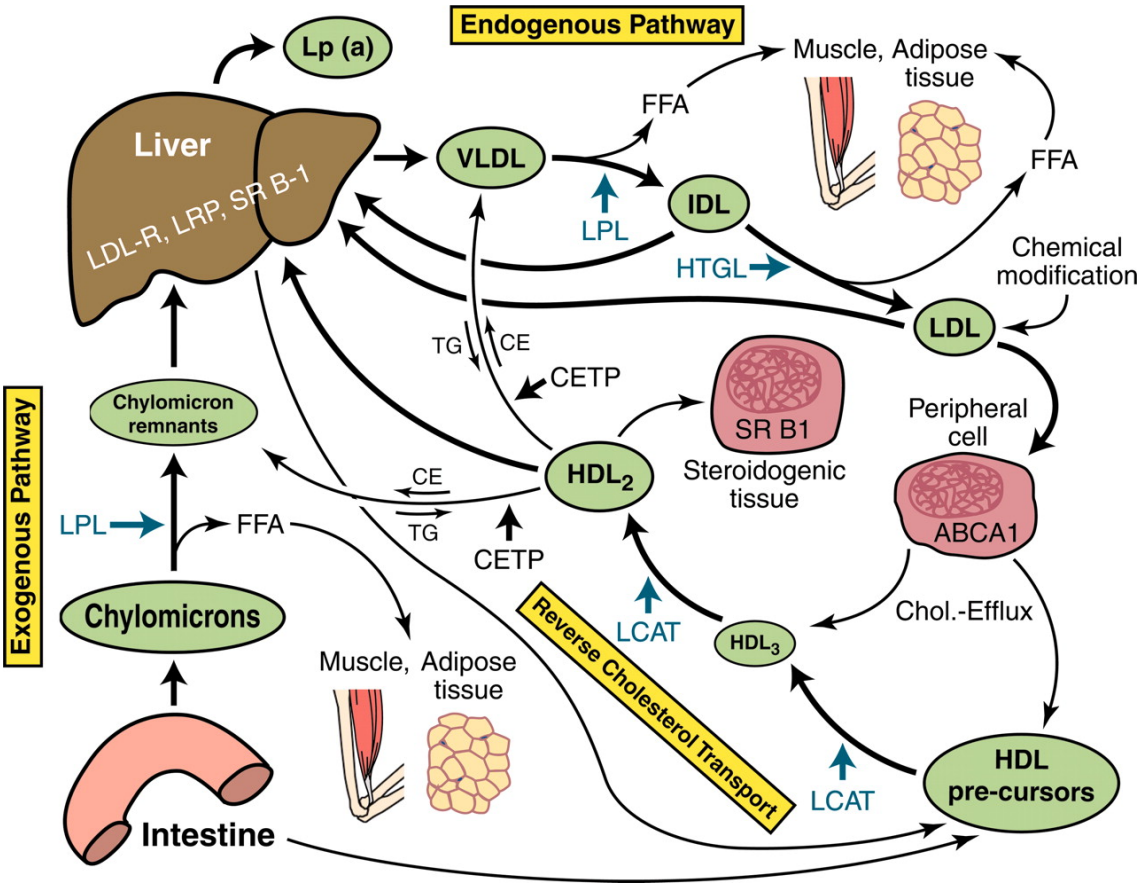

Figure 3. Lipoprotein metabolism representation in which exogenous pathway (coming from diet) and endogenous pathway (coming from hepatic synthesis) are shown. Briefly, TG are packaged in chylomicrons after a meal and delivered to muscles (fuel) and adipose tissue (storage) with the help of LPL action. Chylomicrons remnants are taken by the liver and the content is re-packaged in VLDL that as looses TG content by the action of LPL are converted to IDL that can be taken by the liver or further processed turning into LDL particles which deliver cholesterol to tissues or be withdrawn by the liver. **Adapted from** (42).

During this period of nutritional surplus, insulin stimulates through its receptor (Insulin receptor (INSR)) adipogenesis and lipogenesis as well as the uptake of glucose by enhancing the externalization of the glucose transporter 4 (GLUT4) (43,44). Together with insulin action, leptin (LEP), a hormone secreted by AT and that regulates energy balance in the body (45), acts through leptin receptor Ob-Rb sensitizing the action of insulin (44). As a result, FFA generated from LPL activity is taken with the help of CD36 transporters and fatty acid binding protein 4 (FABP4), while glucose is taken producing Acetyl-CoA and glycerol-3-phosphate (glycerol-3P) via glucolisis. FFA is transformed to Acyl-CoA by Acyl-CoA synthase (ACS) which in turn is re-esterified in TG that are stored in lipid droplets using the glycolytic intermediary glycerol-3P (43,44). Besides, Acetyl-CoA and glycerol-3P can be used to performed lipogenesis de novo via the lipogenic enzymes Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) producing TG as well (43,44) (Figure 4).

In fasting or when energy is demanded, lipolysis is stimulated and TG are breakdown in order to supply the required energy. Lipolysis can be activated by adrenergic stimulation and the lack of the inhibitory effect of insulin over lipolysis factors. Thus, adipose TG lipase (ATGL) and hormone sensitive lipase (HSL) emerge as key enzymes in lipolysis, since carry out the breakdown of TG to FFA (43,44,46). Other proteins can be involved in the regulation of this process as perilipin 1 (PLIN1) (which surround lipid droplets regulating lipase accessibility), caveolin 1 (CAV-1) (that is thought to facilitate perilipin phosphorylation after adrenergic stimulation, which stimulate lipase activity), or FABP4 which can help FFA transportation and is thought to stimulate HSL activity (46) (Figure 4).

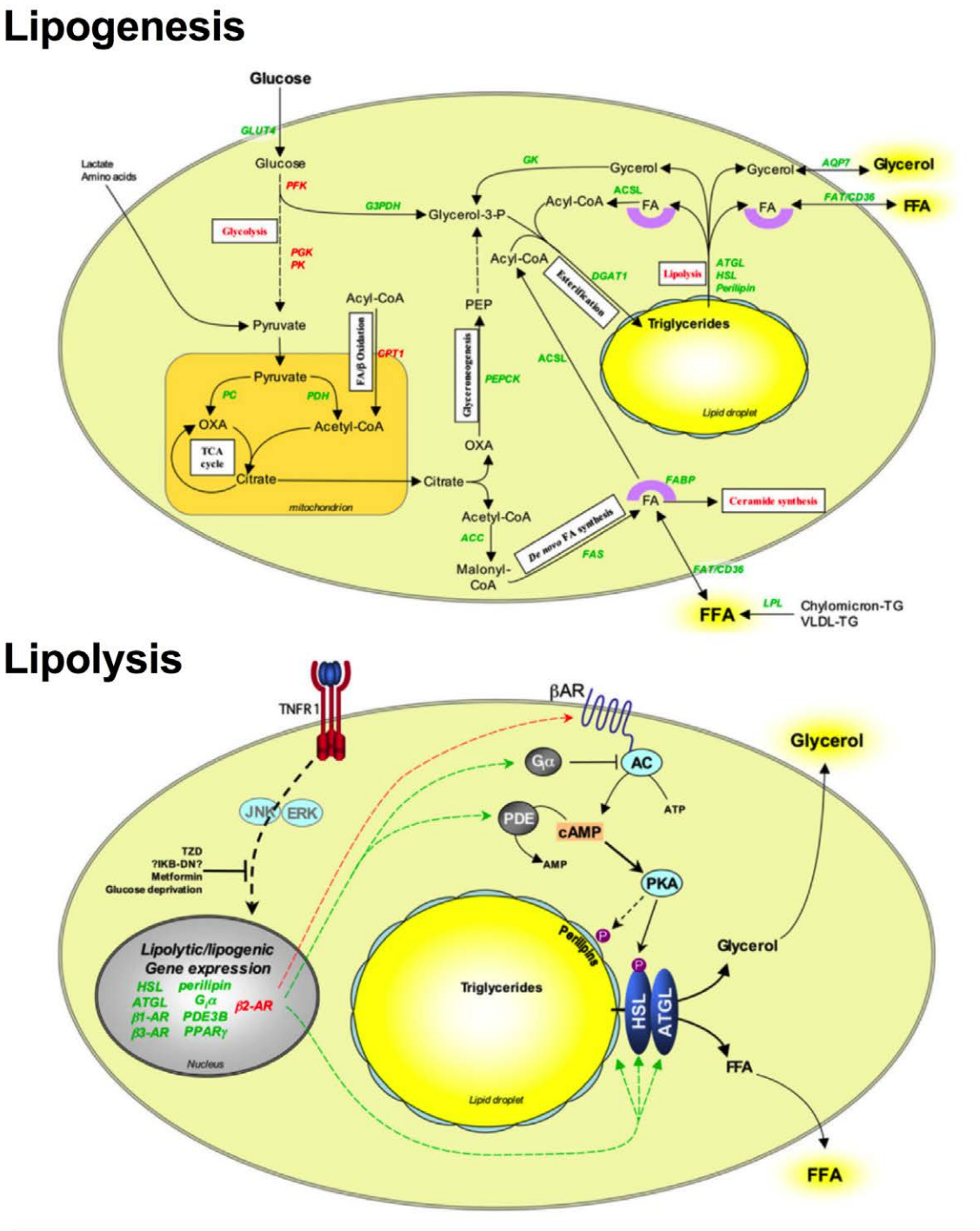

Figure 4. Schematic overview of lipogenesis and lipolysis in the adipocyte. During lipogenesis FFA are esterified with glycerol-3P from glycolysis or external sources giving TG to be stored in the lipid droplet. During lipolysis TG are broken down to FFA and glycerol that are delivered to the blood stream. **Adapted from (47).**

3.2. Glucose metabolism

AT not only plays a role in lipid metabolism but the closed interplay between both, lipid and glucose regulation, gives AT an important weight in glucose homeostasis. This is evident since AT-specific KO-mouse of GLUT4 has been shown to provoke a failure in systemic glucose homeostasis leading to insulin resistance. It has been shown that GLUT4 is down-regulated during fasting state and up-regulated during postprandial state. In AT, glucose and specifically the intermediary metabolite in glycolysis glycerol-3P, is necessary to the re-esterification of FFA to TG. When glucose is limited (as in fasting states) adipocytes rely on glyceroneogenesis in order to obtain the necessary source of glycerol-3P (48).

Moreover, some adipokines secreted by AT can exert a regulatory effect over glucose homeostasis (48). Thus, LEP has been shown to directly inhibit insulin gene (ISN) expression in β-cells via the activation of JAK/STAT signaling. LEP has also been demonstrated to inhibit insulin secretion and to regulate β-cell mass in pancreas (49) (Figure 5).

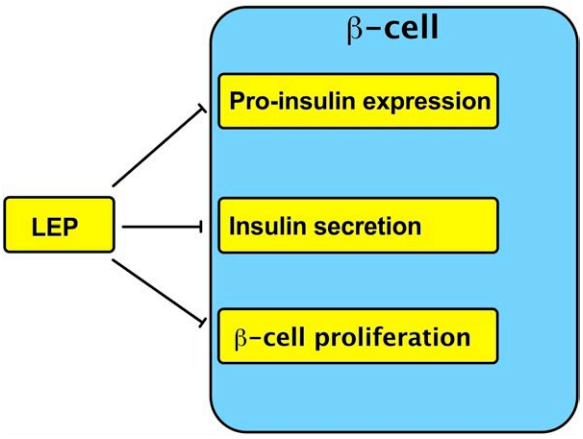

Figure 5. Schematic representation of the direct effects exerted by LEP over glucose metabolism. LEP inhibits insulin by down-regulation of pro-insulin expression, inhibition of insulin secretion in β-cell and by controlling β-cell mass.

Adiponectin is another molecule secreted by AT, with an active role in glucose homeostasis. This molecule is secreted in three isoforms: low-molecular-weight (LMW) trimers, medium-molecular-weight (MMW) hexamers and high-molecular-weight (HMW) complexes. Out of the three isoforms, the HMW has been described as the most active form. Adiponectin enhances hepatic insulin sensitivity and avoid gluconeogenesis by down-regulating both phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P). The increased hepatic sensitivity to adiponectin is thought to occur via either receptor-mediated activation of AMPK pathway or lowering hepatic ceramide levels (50,51). Apart from the effect on peripheral tissues, adiponectin affects β-cells. Thus, adiponectin can prevent β-cells from apoptosis, has a protective role against lipid cytotoxicity and it is thought to stimulate insulin secretion during challenged states (50).

4. ROLE OF ADIPOSE TISSUE IN METABOLIC DISORDERS

Since obesity is characterized by an increase of AT mass, the premise that adipose tissue may participate in the etiology and generation of metabolic disorders usually associated to obesity came out long time ago.

It is known that AT can deliver a wide range of molecules, as cytokines, prostaglandins, adipokines or free fatty acids, which can exert their activity in other tissues, which in cases can lead to disease (52–55). Therefore, AT has gained a central role in explaining metabolic disturbances and has been related to the appearance and development of diseases like dyslipidemia, hypertension, proinflammatory states, insulin resistance, MetS, diabetes, CVD, stroke, or cancer (37,56–59).

Although AT abnormalities has been usually associated to obesity, metabolic disturbances also account in normal-weight subjects (37), which agrees with the new

harmonization criteria to define MetS, in which central obesity is no longer a mandatory factor (15).

MetS is defined by a cluster of metabolic alterations, which altogether increases the risk of suffering diabetes, cardiovascular disease and cancer (15,16) (Figure 6). Genetic and lifestyle factors have been shown to be important for the development and etiology of MetS (60). MetS represents a serious problem in developed countries, with a high prevalence (30-40% by the age of 65 years) and both, its prevalence and incidence are increasing (61). The appearance of MetS has been mainly associated with lifestyle features like physical inactivity, smoking, alcohol intake and diet. However, genetic and epigenetic factors are now emerging as factors of paramount importance in its pathophysiology (62,63).

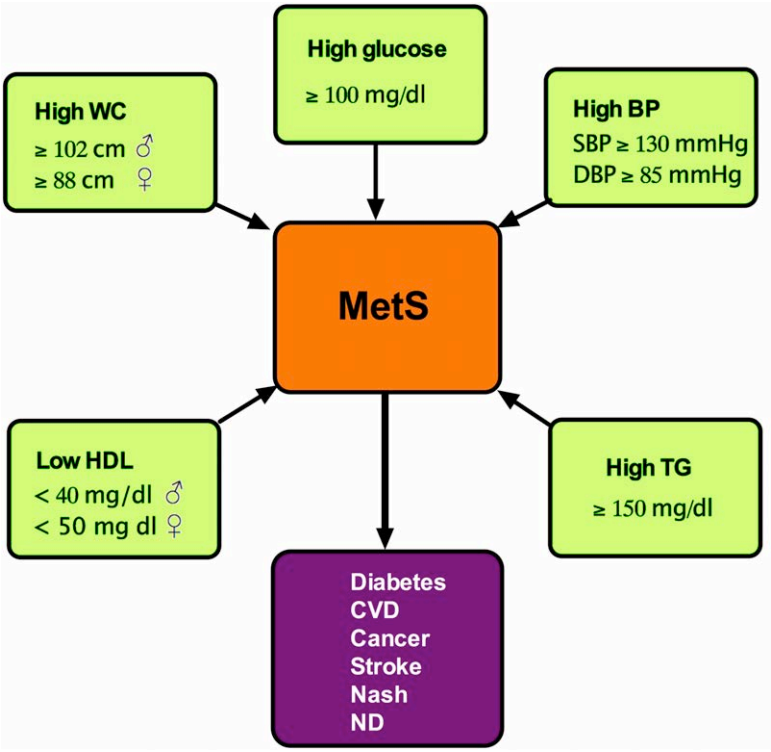

Figure 6. Metabolic syndrome variables and MetS associated disorders. MetS is composed by at least the alteration in 3 of the following parameters: HDL, TG, glucose, blood pressure and WC. Some of the parameters are sex dependent (as HDL or WC) and raze dependent (as WC, the showed values are for west countries). The MetS parameters are represented according

to the established in (15). Abbreviations: Metabolic syndrome (MetS); waist circumference (WC); blood pressure (BP); systolic blood pressure (SBP); diastolic blood pressure (DBP); high-density lipoprotein (HDL); triglycerides (TG); Cardiovascular diseases (CVD); Non-alcoholic steatohepatitis (NASH); Neurological disorders (ND).

4.1. Metabolic factors related to metabolic disorders

4.1.1. Lipoprotein Lipase (LPL)

LPL plays a key role in lipid metabolism (64) by hydrolyzing triglyceride-rich lipoprotein (CM or VLDL) to free fatty acids, that can be then incorporated into the AT for storage or energy utilization in other tissues (64,65) (Figure 7). LPL is synthetized and secreted to the endoplasmic reticulum (ER) where is folded with the help of Lipase maturation factor 1 (LMF1). LPL/LMF1 form a complex with Sel-1 suppressor of lin-12-like (Sel1L) and this LPL/LMF1/Sel1L complex helps to stabilize LPL homo-dimers, the active form of LPL, and allows LPL exits the ER (65).

After being secreted, LPL is bound to Heparan sulfate proteoglycans (HSPG) at the surface of the cell. HSPG not only serves as an anchorage molecule but also contribute to LPL translocation to the endothelial surface and acts as cofactor for LPL activity. Another factor, the GPIHBP1 (which is a Glycosylphosphatidylinositol (GPI)-anchored glycoprotein belonging to the lymphocyte antigen 6 family) has been shown to play an important role in LPL transportation from the interstitial space to the luminal space at the endothelial surface. Lack of this factor has been observed to impaired translocation of LPL to the endothelial surface provoking hypertriglyceridemia. Besides, it has been shown that GPIHBP1 is able to act over LPL activity by keeping the catalytic domain of the enzyme unfolded (65).

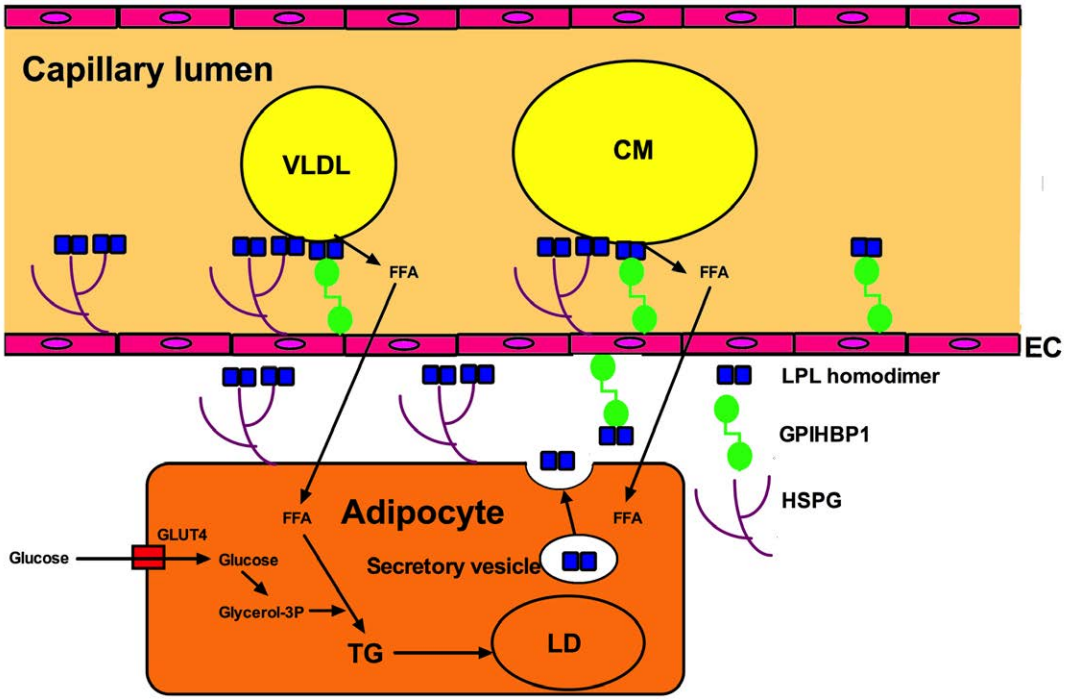

Figure 7. Schematic overview of LPL secretion and anchorage to EC surfaces. LPL forms a dimer in the endoplasmic reticulum and is then secreted. LPL is bound to HSPG in the surface of the adipocyte and then translocated to GPIHBP1. LPL hydrolyze TG from Triglyceride-rich lipoproteins as CM and VLDL producing FFA that are transported to the adipocyte where are re-esterified giving TG to be stored in LD. Abbreviations: Endothelial cells (EC); heparan sulfate proteoglycans (HSPG); triglycerides (TG); chylomicrons (CM); very-low density lipoproteins (VLDL); free fatty acid (FFA); lipid droplets (LD).

Given the importance of LPL in lipid homeostasis the enzyme undergoes a tight regulation at all levels. Thus, some factors have been postulated to control LPL activity like ApoCI/II/III, ApoA5, angiopoietins and hormones. ApoCII, which is carried in CM, has been shown to be a stimulator of LPL activity, while ApoCI/III are inhibitory factors. ApoA5 that is present in VLDL, HDL and CM to a less extend has been described to stimulate LPL activity, although the effect seems to be weaker than the positive action carries out by ApoCII. Angiopoietin-like protein 3 (Angptl3) has been described to inhibit LPL activity as well as by enhancing its cleavage by pro-protein

convertases. Angptl4 is produced in the liver and AT and has been shown to increase with fasting, causing an inhibitory effect over LPL. It is thought that inhibits LPL dimerization and reduces LPL affinity to GPIHBP1. Another member of angiopoietins, Angptl8 has also been described to inhibit LPL activity (65). It has been observed that insulin and glucose can regulate LPL. Thus, insulin leads to a raise of LPL mRNA levels, while glucose can activate LPL activity by glycosylation in its catalytic site (66). Other hormones or derivative hormones like Prostaglandin E2 (PGE2), 7-β-hydroxycholesterol and 25-hydroxycholesterol has been shown to exert an inhibitory effect over LPL production in macrophage cultures. Moreover, LPL gene expression has been shown to be regulated by miRNAs such miR-27 and miR-29 in AT (65). De-regulation of any of these factors can lead to de-regulation of LPL expression or its activity, which in turn might promote lipid metabolic failure. By contrast, the understanding of the regulation of LPL can also give clues to new treatment approaches based on the inhibition or stimulation of these factors. For instance, some molecules such as statins, fibrates, nicotinic acid, Ezetimibe or Orlistat have been used to treat hypertriglyceridemia by mean of their stimulatory effect over ApoCII, while other treatment as fibrates have been described to inhibit ApoCIII, therefore provoking LPL activity enhancement (65).

Indeed, AT is the main TG storage tissue. LPL mRNA increases during the adipocyte differentiation, being one of the earliest markers defined in the preadipocyte lineage (67). On the other hand, AT LPL mRNA expression has been negatively associated to the BMI (58). As we stated before, LPL activity in AT has been related with hypertriglyceridemia (68). Thus, lower activity of LPL can result in greater levels of plasma TG, which in turn can be accumulated in other tissues causing insulin-resistance (69).

4.1.2. Low-density lipoprotein receptor-related protein 1 (LRP1)

LRP1 is a member of the LDL receptor family that regulates lipid and glucose metabolism in the liver and AT (70). LRP1 is involved in CM-remnants, insulin receptor trafficking and regulation, and glucose metabolism, therefore being related to atherosclerosis (it is an atheroprotective factor) and diabetes (71). LRP1 translocation to the cell surface after glucose and insulin stimulation has been described in adipocytes. Moreover, this LRP1 induction by insulin and glucose is accompanied by an increase in the uptake of CM-remnants by adipocytes (71). In fact, it has been demonstrated that LRP1 are contained at GLUT4 rich vesicles and is delivered after insulin stimulation altogether. It has been shown that adipocyte-specific LRP1-knockout mice provokes a decrease of GLUT4, Insulin-regulated aminopeptidase (IRAP) and sortilin expressions, and that this three factors together with LRP1 form a complex that is involved in GLUT4 trafficking. Thus, LRP1-depleted 3T3-L1 adipocytes presents up to 50% decrease in glucose uptake, demonstrating the importance of LRP1 in the etiology of insulin resistance (71). Adipose-specific LRP1-knockout in mice has also been related to AT dysfunction (with the stimulation of CD68, MCP1/CCL2, IL6 and TNF) promoting atherosclerosis (72). VAT LRP1 mRNA has been described to be overexpressed both, in mice fed with high fat diet (HDF) compared to normal diet and in obese people compared to normal-weight subjects (73).

4.1.3. Glucose transporter type 4 (GLUT4)

GLUT4 in AT is the rate-controlling step in insulin-mediated glucose disposal, being its levels diminished in insulin resistance state and obesity. Glucose is necessary as a reliable source of glycerol-3P (a glycolytic intermediary) that is used to re-esterified

Free fatty acid (FFA) taken from the blood stream to form TG (48). A down-regulation of GLUT4 could impair this source of glycerol-3P for what glyceroneogenesis emerges as an important pathway for TG formation. In this pathway, lactate or pyruvate enters the Tricarboxylic acid cycle (TCA cycle) that produces oxaloacetate, which is then transformed to phosphoenolpyruvate by the cytosolic enzyme Phosphoenolpyruvate carboxykinase (PEPCK). Phosphoenolpyruvate is then converted to glycerol-3P by reverse glycolysis. A huge intake of FFA in subjects with insulin-resistance could overwhelmed this pathway, hindering FFA esterification and leading to FFA accumulation in peripheral tissues contributing to insulin resistance worsening. Thiazolidinediones (TZDs) is a potent insulin sensitizing used to treat diabetes. This enzyme can up-regulate glycerol kinase and PEPCK activity, triggering in an improvement in FFA removal by increasing FFA esterification in TG and resulting in an increase of insulin sensitivity (48).

4.1.4. Peroxisome proliferator-activated receptors (PPARs)

PPARγ is a key factor for adipocyte differentiation. Adipose-specific PPARγ knockout mice, has been carried out. In this model, there was an impaired AT expansion, presented adipocyte hypertrophy, and higher levels of plasmatic FFA. Besides, these mice were more sensitive to insulin resistance and liver steatosis induced by HFD. When these mice were treated with TZDs (anti-diabetic drug that has PPARγ as a target) liver insulin resistance remitted although no other effects were observed (74). Other member of PPAR receptor family, PPARα, is a target for the fibrates, molecules that are used for the treatment of hypertriglyceridemia. PPARα is a transcription factor that is involved in fatty acid oxidation in tissues with a high level of fat oxidation and peroxisomal metabolism (liver, heart muscle or brown adipose tissue). In AT, PPARα

has been shown to attenuate adiposity and to stimulate the secretion of adiponectin, which is an insulin-sensitizing hormone (75). Thus, 3T3L1 pre-adipocyte line and mice treated with PPARα agonists have been shown to induce adipogenesis, and to increase fat oxidation by direct induction of PPARα over adipogenic and fat oxidation genes (75). Both members of the PPAR family, PPARγ and PPARα carry out their function as heterodimer with the Retinoid X receptor alpha (RXRα) (76). Thus, it has been described that a adipocyte-specific RXRα-knockout mice can cause adipogenesis failure, and cannot increment AT even after HFD administration (77,78).

4.1.5. Sterol regulatory element binding factors (SREBFs)

SREBFs are transcription factors known to control cholesterol and fatty acid biosynthesis, as well as adipogenesis in AT. SREBF1 has two isoforms, the SREBF1c (the most expressed in AT with a key role in adipogenesis) and SREBF1a. These isoforms are determined by the transcription starting site (79). Although the different isoforms of SREBF present some functional overlap, SREBF1c has a more prevalent action in lipid biosynthesis while SREBF2 has a prevalent role in cholesterol biosynthesis. By contrast, SREBF1a has a role in both, fatty acid and cholesterol biosynthesis (80–82). SREBF1c and SREBF2 have lower gene expression levels in VAT of obese people respect to lean people. It has already been shown that this expression is reestablished to lean-like values after bariatric surgery in obese people (83). Furthermore, SREB1c expression in AT has been demonstrated to respond to food and caloric intake. Thus, fed can promote SREBF1c down-regulation while fasting and caloric restriction have been shown to stimulate AT SREBF1 expression (79). SREBF1 overexpression is responsible of the oxidative stress improvement observed in caloric restriction diets (79). In the other way around, oxidative stress is capable of inhibiting

healthy AT expansion through the suppression of the SREBF1c-mediated lipogenic pathway (84). In animal models, it has been demonstrated that SREBF2 protein in adipocyte hypertrophy is activated, which leads to an increase in the production of chemerin, and adipokine positively related to MetS (85).

4.1.6. Stearoyl-CoA-desaturase (SCD)

SCD is a key enzyme that catalyzes the rate-limiting step to monounsaturated fatty acids (MUFFAs) from saturated fatty acids, specially from stearoyl-CoA and palmitoyl-CoA, giving as a result oleate and palmitoleate (86,87). It is long known that polyunsaturated free fatty acids (PUFFAs) exerts an inhibitory effect over SCD gene expression (87). It has been proven in mice, that a lack of SCD lowers adiposity by the increase in the metabolic rate, the thermogenesis and the β-oxidation, and a decrease in the lipogenesis pathway (87).

4.1.7. Liver X receptor beta (LXRb)

LXRb is a transcription factor activated by ligand, which belongs to the nuclear receptor family and that is involved in gluconeogenesis, cholesterol and inflammation (88). LXRb is activated upon cholesterol breakdown products oxysterols and then dimerize with its partner RXRα to carry out its function (89,90). This biological function is mainly related to the lipogenesis pathway, being for instance Fatty acid synthase (FAS) and Acetyl-CoA carboxylase (ACC) some of the target genes implied. Other target genes for LXR involve the cholesterol transporters ATP binding cassette transporters A (ABCA) and G (ABCG), the SREBF1c transcription factor, and the key rate-limiting enzyme for the biosynthesis of monounsaturated fatty acids, the SCD (89). Thus, LXR activation has been shown to have beneficial effects in metabolism by: 1) increasing

lipogenesis, cholesterol clearance, bile acid synthesis and by lowering gluconeogenesis in the liver; 2) increasing cholesterol clearance in the intestine; 3) decreasing inflammation through macrophage modulation; 4) increasing GLUT4 expression in adipocytes; 5) stimulating insulin secretion and lipogenesis in pancreas (89) (Figure 8).

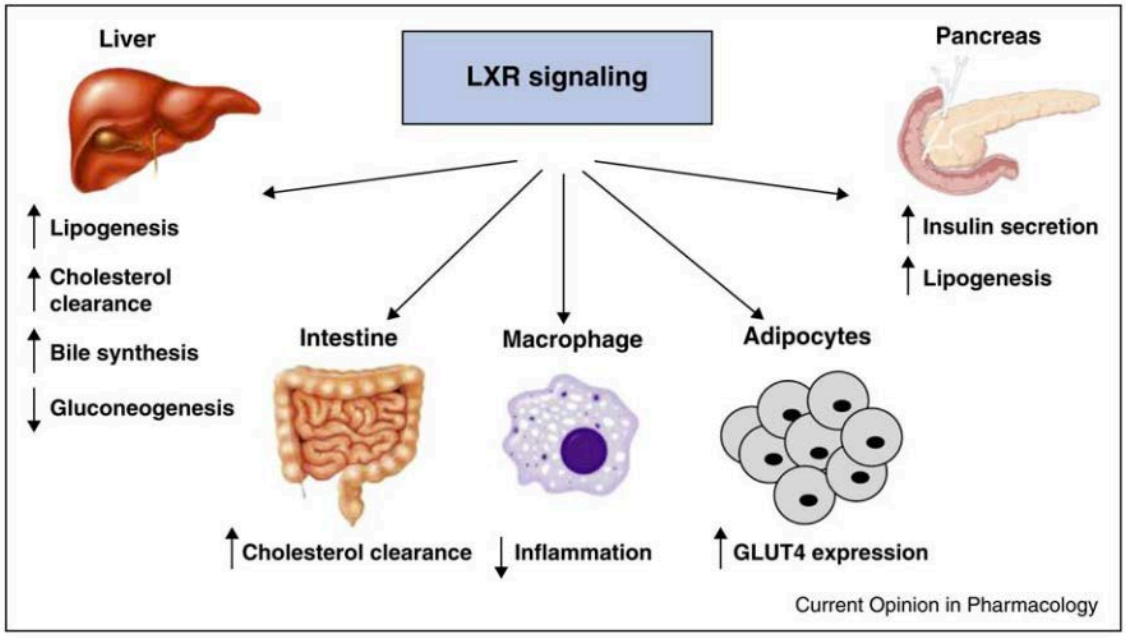

Figure 8. Organ-specific effects of LXR over general metabolism and inflammation. LXR stimulates cholesterol lowering by producing cholesterol clearance in liver and intestine and by increasing bile acid synthesis in the liver. Besides, it promotes lipogenesis in liver and pancreas and induces insulin secretion in pancreas. LXR also increases glucose uptake in AT by inducing GLUT4 expression, and lowers gluconeogenesis in the liver. **Adapted from** (89).

Therefore, it is not surprising the usage of LXR agonists (which activates the liver specific LXRa and the ubiquitously expressed LXRb) for the treatment of the metabolic disease, although a side-effect rises upon the unspecific activation of LXRa in the liver, which increases plasma TG and hepatic fat accumulation that can lead to liver disease eventually (88,90). Thus, the effort has been focused in the search of specific LXRb activators, that has been shown to ameliorate the MetS symptoms (88).

4.1.8. Leptin (LEP)

LEP is a hormone secreted by AT that regulates energy balance in the body by decreasing energy intake and increasing energy expenditure (45). Although LEP receptors are widely spread in multitude of cell types and tissues, LEP crosses the blood brain barrier and performs its principal role in the hypothalamus, where controls satiety and energy homeostasis. Thus, LEP has been described to inhibit food intake through the inhibition of Neuropeptide Y (NPY) and Agouti-related peptide (AgRP), and to stimulate factors like Pro-opiomelanocortin (POMC) that indirectly stimulates satiety through the activation of the α-Melanocyte stimulating hormone (αMSH) (45). Insulin and glucocorticoids has been observed to induce a long-term positive effect over LEP mRNA expression in AT from obese subjects (91). In line with this fact, LEP is known to be overexpressed in AT of obese subjects, both at mRNA and serum levels. Furthermore, a LEP resistance state has been described in obesity, process that can be due to the impairment in the transportation through the blood brain barrier or by the alteration in some molecular pathways downstream of LEP, for instance the Phosphodiesterase-3B (PDE3B)-cAMP- and Akt-pathways in the hypothalamus, the Fat mass and obesity-related (FTO) gene, the Transient receptor potential vanilloid type (TRPV)-1 channel, the 15-deoxy-Δ(12,14) -prostaglandin J2 (15d-PGJ2), the estradiol (E2) or the PPARγ pathways (45,92). Beside its typical role in energy balance, LEP has also been related to other processes like lipid and glucose homeostasis, immunity, and inflammation (92). In this sense, LEP has been shown to control β-cell function by inhibiting insulin mRNA expression and secretion and stimulating β-cell proliferation and survival (93), while LEP analogs administrations has been shown to improve insulin sensitivity (92).

It has also been demonstrated that LEP can stimulate fat oxidation in muscles, fact that could avoid the lipotoxic effect seen by the lipid accumulation in peripheral tissues, which in turn can lead to metabolic disorders such as insulin resistance and diabetes (94). This fatty acid activation observed after LEP administration has been proposed to be exerted through a direct activation of AMPK signaling via LEP receptor stimulation in the short-time response (15 minutes after administration) and through the hypothalamic-sympathetic axis and α-adrenergic receptor activation in the long-term response (6 hours after administration) that in turn also activates AMPK signaling in muscle (94) (Figure 9).

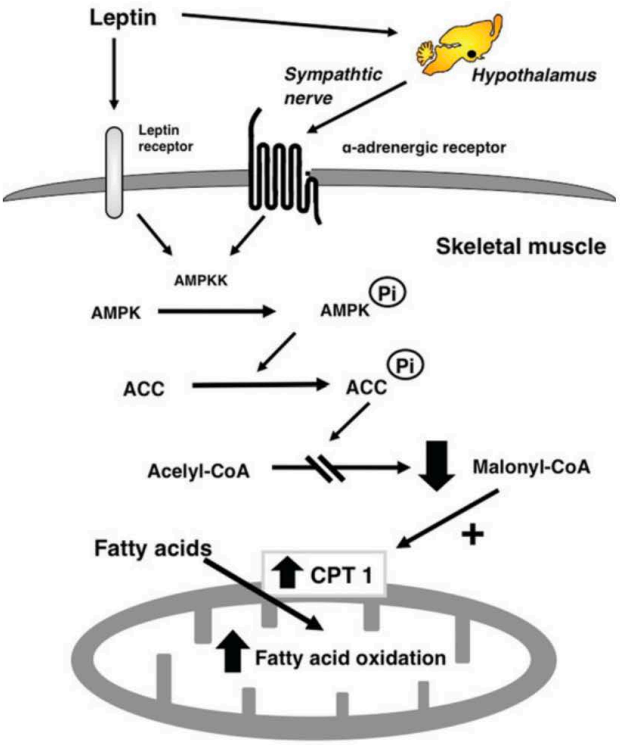

Figure 9. Mechanism of action of LEP promoting FFA oxidation in muscle. LEP can stimulate AMPK/AMPKK via direct action through its receptor (short term response) and through stimulation of the hypothalamus-sympathetic nerve and α-adrenergic receptor (long term response). This produces phosphorylation of ACC and its subsequent inactivation provoking a fall of malonyl-CoA, which stimulates CPT1. Increase of CPT1 at the

mitochondrial membrane leads to an increase of the transportation of FFA in order to be oxidated. **Adapted from** (94).

4.2. Inflammatory factors in metabolic disorders

Apart from the previous roles described above, LEP has been related to inflammation and immunity. Indeed, LEP is capable of stimulating the secretion of TNF, IL-6, and IL-12, and conversely TNF and IL-1B can induce LEP mRNA expression in AT, creating a vicious circle and contributing to the low-grade inflammatory state present in obesity (95,96). LEP has also ben related to cancer development by promoting inflammation, proliferation, angiogenesis, migration and inhibiting apoptosis (96). It has been shown that environmental enrichment stimulates Brain-derived neurotrophic factor (BDNF), which is capable of inhibiting LEP production in adipocytes via sympathoneural β-adrenergic signaling causing colorectal cancer growing inhibition in mice (97) (Figure 10).

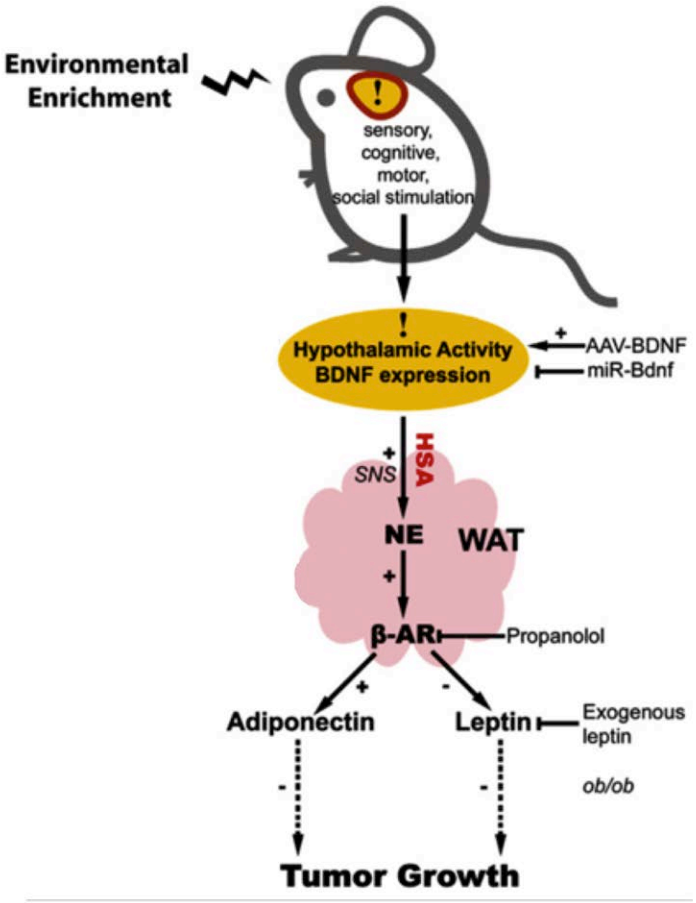

Figure 10. Involvement of white adipose tissue (WAT) to tumor growth in a mechanism in which LEP and adiponectin are involved. Environmental enrichment produces an increase of hypothalamic BDNF that stimulates outflow of sympathetic nervous system to WAT releasing norepinephrine (NE). NE then stimulates β-adrenergic receptors in WAT provoking an increase of adiponectin and a decrease of LEP that leads to an arrest of tumor growth. **Adapted from** (97)**.**

Human AT produces and secretes many factors of the complement pathway, being the complement component C3 a key molecule in this pathway. It is important to note that C3 secretion from AT is proportional to the quantity of adipose tissue, and therefore contributes to the systemic concentrations of this protein (98,99). C3a, a subproduct of C3, is converted to the Acylation stimulating protein (ASP), which has been described

to play an important role in lipid metabolism by stimulating lipid storage (100,101). Adipose tissue C3 production has been shown to be strongly stimulated in the postprandial state, and in parallel, a peak of ASP at the adipocyte microenvironment level has been reported (102 – 104). Also, the specific Adipocyte factor D (adipsin) has been shown to be necessary for ASP production. ASP could have a central role in the interplay between the complement system and the metabolism (100). In this line, obesity has been previously reported as an ASP-resistance situation and plasma levels of C3 have been associated with obesity (105,106). Also, serum C3 is associated with insulin resistance (107), is considered an independent predictor of type 2 diabetes mellitus (107,108), and is a risk marker for cardiovascular disease (98).

The tumor necrosis factor alpha (TNF) is a cytokine that can modulate a wide range of processes such as immune function, cell differentiation, proliferation, apoptosis and energy metabolism (47). TNF is produced both by the adipocyte and SVF in AT, acting as an endocrine and paracrine/autocrine factor through is interaction with type 1 and type 2 TNF receptors (TNFR1 and TNFR2) that are expressed in AT (47,109). In vitro experiments have suggested that TNF affects glucose homeostasis and lipolysis in adipocytes, and inhibits adipocyte differentiation and lipogenesis as well (47). TNF has been positively associated to insulin resistance, BMI and gene blockage in mice. In addition, TNF antibody neutralization in humans have been shown to increase insulin sensitivity (47,110). In line with this, TNF is capable of activating the inflammatory master nuclear factor kappa-light-chain-enhancer of activated B cells 1 (NfκB1). Moreover, TNF has been shown to inhibit adipogenesis by down-regulating PPARγ, CEBPα and CEBPδ, and by the up-regulation of Wnt/β-catenin and lipolysis-related signaling pathways, leading to FFA accumulation in peripheral tissues and therefore promoting inflammation and insulin resistance (109). TNF not only induces

inflammation indirectly via lipotoxicity, but a direct modulation of insulin pathway in the adipocyte has been described. This effect over insulin signaling implies the inhibition of the kinase activity by the insulin receptor (IR), via the inactivation of the Insulin receptor substrate 1 (IRS1) and by the destabilization of the interaction IR/caveolin 1 (CAV1) (109) (Figure 11).

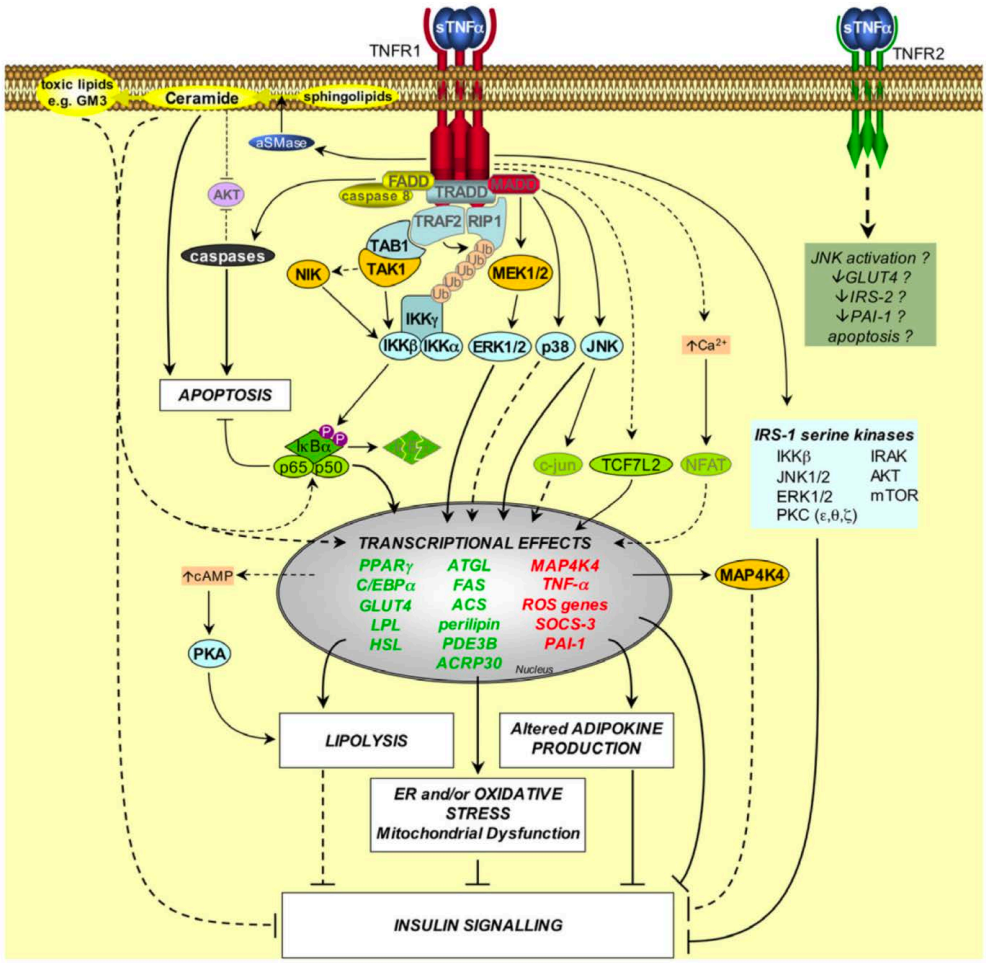

Figure 11. TNF signaling on AT. TNF interacts with its receptor TNFR1 which can induce apoptosis, ceramide production, lypolisis, ER oxidative stress, mitochondrial dysfunction and altered adipokine profile, factors that can eventually lead to inhibition of insulin signaling and therefore to insulin resistance. TNF might also act through TNFR2 which could also provoke an induction of apoptosis and a decrease to insulin sensitivity by down-regulation of GLUT4 and IRS-2 (47).

Since the low-grade inflammation has been associated to metabolic disease, treatments against TNF and/or its pathway could be suitable to treat disorders associated with this chronic inflammation, impaired glucose tolerance and dyslipidemia (47). It has also shown that TNF correlates positively with the size of the adipocytes, which in turn has been related to the generation of metabolic disorders (109,110). Given the direct and indirect action of TNF over the biology of AT and the lipid and glucose metabolism, it is not surprising the relevant role this factor could have in the etiology of the MetS and its complications (111).

5. ADIPOSE TISSUE AND COLORECTAL CANCER

AT can deliver molecules and promote metabolic states, which in turn can affect other tissues and promote disease. In this sense, the possible role of AT in the development of some kind of cancers has gained interest. As it is described previously, obesity and AT dysfunction has been related to a chronic low-grade inflammation (112). In connection with this, chronic inflammation is well known to be a risk factor of developing cancer including colorectal cancer (CRC) (113,114). In this manner, several studies have noted the relationship between CRC and low grade inflammation (115). CRC subjects present a dysfunctional AT, which might be a key contributor to the inflammatory state through the secretion of several proinflammatory factors such as TNF, IL-6, and NFκB pathway (116).

Inflammatory processes could not be the only relationship between AT and CRC. Metabolic deterioration has been also described to increase the cancer incidence, including CRC (117–120). Thus, the relationship between inflammation and metabolic deregulation has been described. For instance, NfκB pro-inflammatory pathway has been described to be a promoter of inflammation in AT, leading to metabolic disorders

(121). In this line, strategies based on the disruption of NfκB action has been proposed to ameliorate diabetes, hyperglycemia and insulin resistance (121).

Given this relationship between inflammation and CRC, and the capacity of AT to generate low-grade inflammation, it is of interest the study of the possible mechanisms that lead to this inflammation in AT. Besides, it would be of great interest the search of therapeutic approximations to avoid this pro-inflammatory state in AT. In this sense, metformin, one of the earliest drugs used to treat diabetes, has been described to inhibit inflammation in a process in which NfκB is involved. Metformin has been shown to ameliorate adipose tissue inflammation, and to promote macrophage polarization to the anti-inflammatory M2 phenotype (122). In addition, a lower risk of suffering cancer disease has been described in subjects who undergo metformin treatment (123,124).

A potent anti-inflammatory molecule is vitamin D (VD) (125–127). VD was first identified by its role in stimulating intestinal calcium intake and bone mineralization, being low levels of this vitamin related to decreased bone mineral density and osteoporosis (128). VD is synthetized from 7-dehydrocholesterol in the skin in a reaction carried out by UVB light, given as a result the pre-vitamin D, which is further converted by heat to VD. This form is not active yet, and VD is turned into 25-hydroxyvitamin D (25(OH)D) by CYP27A1, hydroxylation that occurs mainly in the liver. 25(OH)D is the major form of serum vitamin D and its levels have been observed to be a good indicator of VD status. This form is then further converter in the kidney to the active form, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) by CYP27B1. Kidney CYP27B1 expression is stimulated by parathyroid hormone (PTH) and down-regulated by FGF23 and its own product $1,25(OH)_2D$ (129). $1,25(OH)_2D$ stimulates its own degradation by the 24-hydroxylase CYP24A1, which catalyzes the conversion of

25(OH)D and 1,25$(OH)_2$D to calcitroic acid and other inactive metabolites (129,130) (Figure 12).

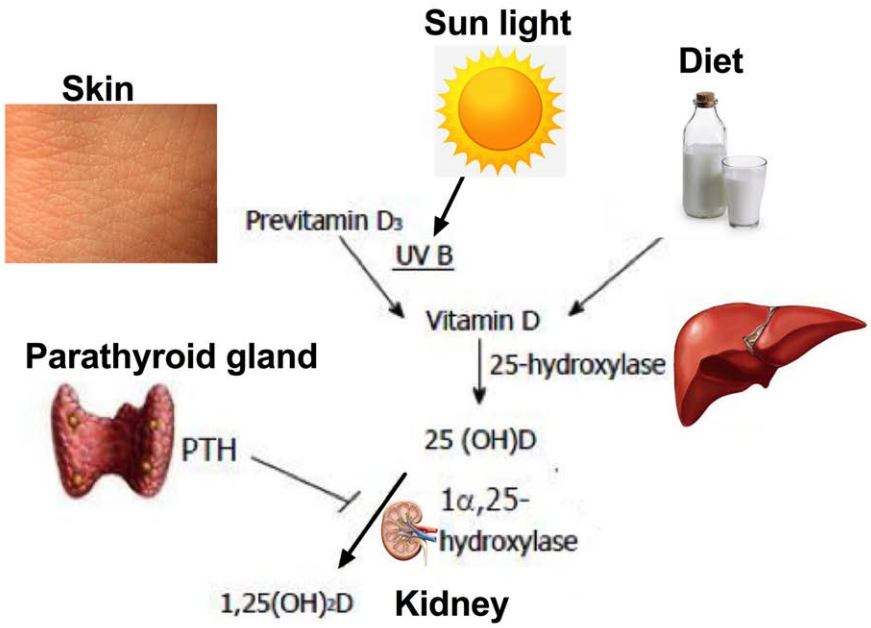

Figure 12. Vitamin D metabolism from pre-vitamin D to the active form 1,25-dihydroxy vitamin D.

VD receptor (VDR) has been described to be expressed in a wide range of tissues and cell types, and it is though that the non-classical actions of VD (such as inhibition of proliferation, macrophage modulation, terminal differentiation stimulation or insulin production stimulation) are exerted via its interaction with it (128). Lack of VD, specifically the major plasma form of VD, the 25(OH)D has been related to an increase in the development of CRC (131). AT can express proteins related to VD metabolism (132), and it has been proposed that it can act as VD storage tissue (133). It has been shown that the active form of VD, 1,25$(OH)_2D_3$, is able to modify adipocyte and AT physiology via VDR action (134,135), decreasing the expression of pro-inflammatory

cytokines in AT (136). Therefore, VD could be other factor that could be involved in the regulation of AT inflammation and could contribute to the biology of CRC.

6. EPIGENETICS

Epigenetics concerns the information conveyed through cell division and that not implies changes at the DNA sequence. Epigenetics shape differentiation processes, and it is the roof of the differences observed between cellular types or organs (137). There are basically two epigenetic regulation landscapes: DNA methylation, which occurs at cytosines adjacent to guanines (CpG); and histone modifications, which is more variable and diverse than DNA methylation at CpG (137).

6.1. DNA methylation

DNA methylation is a process in which a covalent methyl-group is added to the carbon 5 of a cytosine-pyrimidine ring (5mC) in a CpG nucleotide. Most of the DNA methylations are spread in transposons and mobile DNA sequences (SINE, LINE, etc), while DNA methylation at the promoter or first exon of genes represents a small percentage of whole DNA methylation in the genome (138). It is thought that DNA methylation was first selected evolutionary as a mechanism to stop the replication of mobile sequences in the genome (138). Un-methylated CpG can accumulate in the promoter of genes, elements that are called CpG islands. Un-methylated CpG island would assure that transcription factors and the transcription machinery are bound to the right place in the promoter (138). Around 75% of all genes have CpG island in their promoter, being susceptible of DNA methylation control (138). While DNA methylation at CpG islands in the promoter of genes are related to gene repression, DNA methylation in the body of the gene (which are usually hyper-methylated) has

been reported to activate gene expression, maybe by increasing the efficiency of the transcription (139–141).

DNA methylation is catalyzed by DNA methyl-transferases (DNMTs). These enzymes catalyze the conversion of un-methylated to methylated CpG in a process in which S-adenosylmethionine (SAM) acts as methyl-donor. In humans, there are several DNMTs: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L. From these, DNMT1, DNMT3A and DNMT3B have DNA-methyltransferase activity while DNMT3L and DNMT2 do not conserve this capacity even though can either act as cofactors for the other DNMTs (in the case of DNMT3L) or methylate tRNA (for DNMT2) (142). DNMT3A and DNMT3B have been described as methyltransferases "*de novo*", which are in charge of establishing new DNA methylated marks ubiquitously and in an un-specific way. Once the mark is established, DNMT1 has been shown to maintain the DNA pattern trough the replicative cell cycles by recognizing hemi-methylated DNA (142,143). Contrary to DNMT1 and DNMT3A/B, DNMT2 has been demonstrated to methylate tRNA instead of DNA, modification that has been proposed to avoid tRNA fragmentation and regulated protein transduction. Even though is a tRNA modifier, the enzyme evolved from DNMTs that with subtle changes finally acquired this new function (142) (Figure 13).

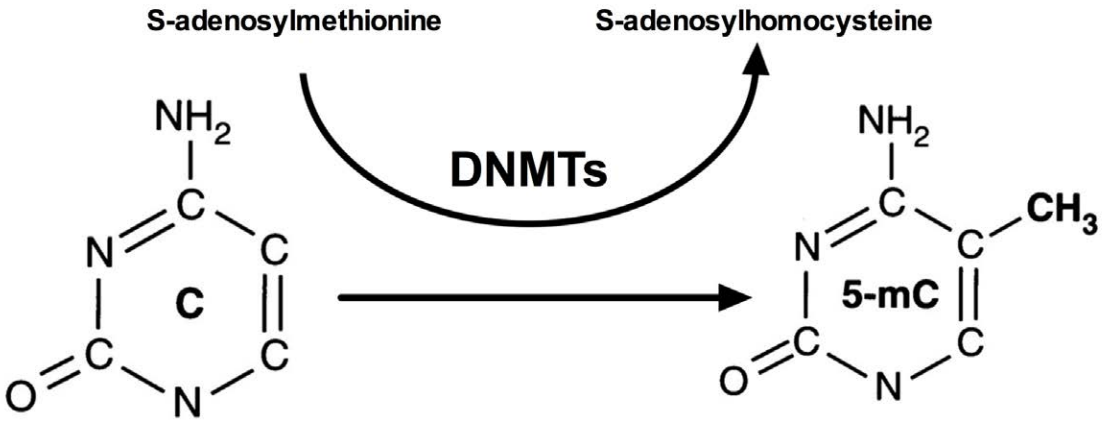

Figure 13. DNA methylation reaction. DNMTs use as substrates a cytosine near a guanine and S-adenosylmethionine (SAM). In the reaction, the methyl group is transferred to the carbon 5 of the cytosine ring, producing 5-methyl-cytosine. As a result S-adenosylhomocysteine (SAH) is also produced that can be recycle to form SAM. Abbreviations: Cytosine (C); DNA-methyltransferases (DNMTs); 5-methyl-cytosine (5mC).

DNA methylation is a dynamic process and can be modified according to environmental, genetics and stochastic factors. Therefore, a certain CpG can be methylated and un-methylated (144). DNA de-methylation can occurs both, by enzymatic action or by passive de-methylation. Passive de-methylation would take place in successive replications with a low activity of the methylation machinery, though producing a dilution of 5-methyl-cytosine (5mC) (formed by the transfers of the methyl group to the carbon 5 of the cytosine ring) giving as a result un-methylated DNA (145,146). The active de-methylation process implies the action of Ten-eleven translocation (TET) member family. These enzymes have been reported to catalyze the oxidation of 5mC to 5-hydroximethylcytosine (5hmC) in a process in which molecular oxygen and α-ketoglutarate (that is converted to succinate) are necessary. In turn, 5hmC can be further oxidized by TET to 5-formylcitosine (5fC), and 5fC to 5-carboxylcitosine (5caC), again

with the participation of oxygen and α-ketoglutarate (145,146). 5hmC has been related to active genes and is thought to contribute to the cell-specific set of expressed genes observed among cellular types, although the exact mechanism by which exerts this regulation is not fully understood (147–149). The restoration of these oxidized forms to cytosine can be carried out in a passive way as well, with dilution during rounds of cell replication, or in an active way. In the active way, Thymine DNA glycosylase (TDG) has been shown to excise 5caC and 5fC, after what the cytosine is restored by the base excision repair (BER) pathway (145,146) (Figure 14). Therefore, unlike the methylation process, the DNA de-methylation mechanism is a complex pathway where more studies would be necessary to expand the knowledge and weigh the importance of the different factors involved.

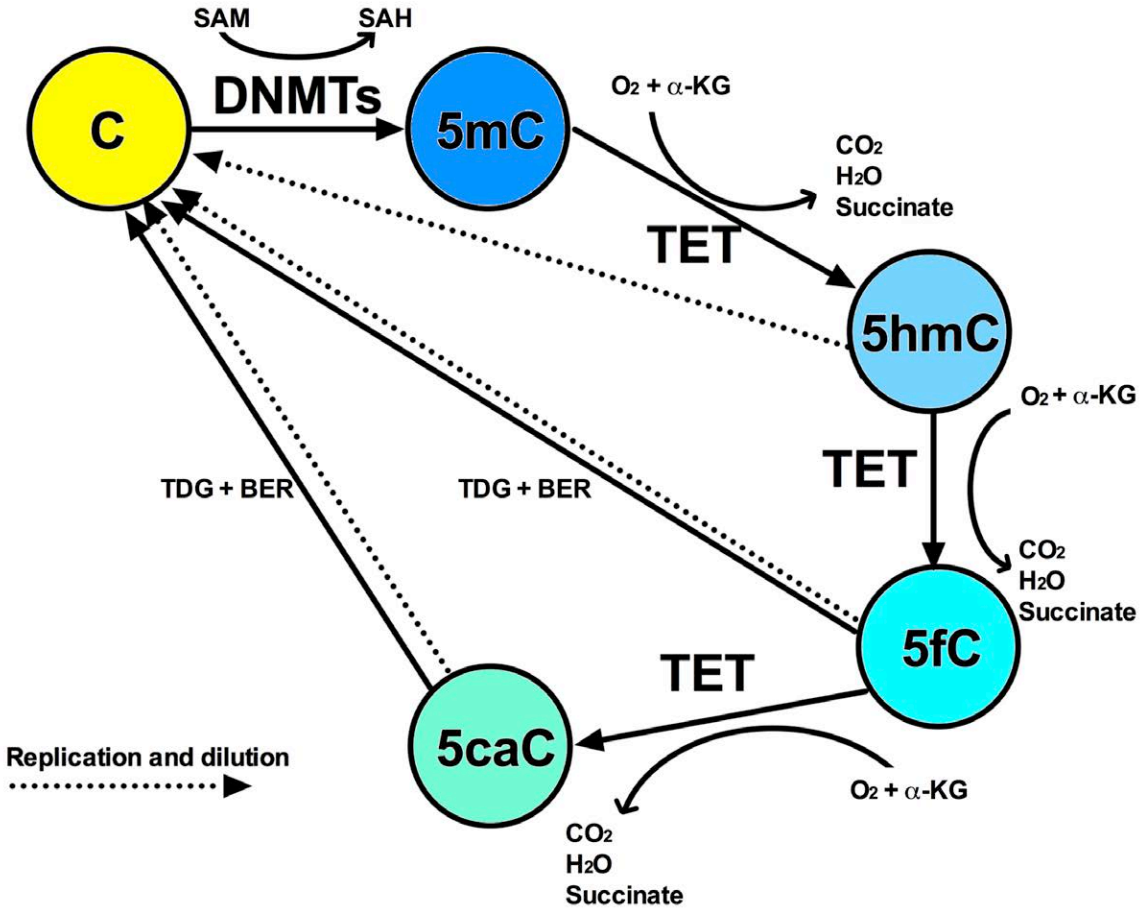

Figure 14. DNA de-methylation process. After methylation of cytosine by DNMTs, TET successively oxidizes 5mC to 5hmC, 5fC and 5caC. 5hC, 5fC and 5caC can be diluted to cytosine during the following rounds of replication (broken arrows) in what is known as passive de-methylation. Additionally, 5fC and 5caC can be excised by TDG after what the cytosine can be restored by the BER pathway. Abbreviations: Cytosine (C); 5-methylcytosine (5mC); 5-hydroxymethylcytosine (5hmC); 5-formylcytosine (5fC); 5-carboxylcytosine (5caC); DNA-methyl transferases (DNMTs); S-adenosylmethionine (SAM); S-adenosylhomocysteine (SAH); ten-eleven translocation enzymes (TET); α-ketoglutarate (α-KG); thymine DNA glycosylase (TDG); base excision repair pathway (BER).

6.2. Histone modifications

DNA in the nucleus is associated to histones in what is called the nucleosome. A pair of each H2A, H2B, H3 and H4 histone types forms the nucleosome (Figure 15). These histones are susceptible of post-translational modifications that can modified the strength of the DNA union with the complex, attract other regulatory factors and in turn regulate chromatin structure, gene activity and DNA repair. For instance, histone acetylation of lysine residues produces de blockage of positive charges, resulting in a weaker interaction with the DNA and making the DNA more accessible to other regulatory factors (polymerase, transcription factors, etc.) (150–152).

While DNA modifications are basically reduced to 5mC, histones can be modified at the same degree as any other proteins, modifications that mainly take place in the N-terminal tail. That means, there is a wide range of possible modifications such as methylation, phosphorylation, sumoylation, ubiquitination, glycosylation, acetylation, propionylation, butyrylation, crotonylation or citrullination. Lysines are the residue more often modified, although modifications in other amino acids like arginine, serine or threonine has been reported. These modifications have been described to be carried

out for a series of histone modifier such as histone acetyltransferases (HATs), histone mehtyltransferases (HMTs) or phosphatases among others. In turn, these marks have been shown to be removed by proteins called erasers, for instance histone deacetylases (HDACs) (that remove acetyl groups) or histone demethylases (HDMs) (which remove methyl groups). Different from DNA methylation that is usually linked to gene repression (although as it is discussed there are some exceptions), histone modifications can be involved in gene repression, gene activation, DNA repair, chromatin structure etc. Besides, the role of some modifications are difficult to understand since many of these modifications occurs at the same histone and myriads of possible combinations makes histone regulatory landscape a complex field of study (150,151).

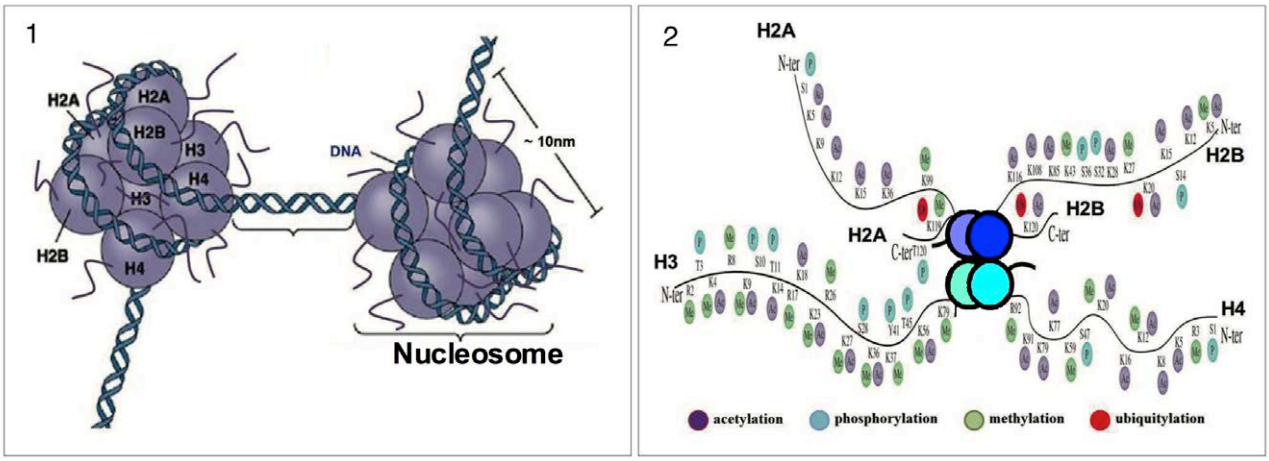

Figure 15. Nucleosome and histone modifications. Nucleosome is composed by a pair of each H2A, H2B, H3 and H4 histone types (1), where DNA is wrapped around the structure. Additionally, histones can suffer post-translational modifications in their aminoacidic chain. (2) Summarization of the most common N-terminal modifications at H2A, H2B, H3 and H4 histone types. **Adaptation from** (153)**.**

Even though its complexity, some histone modifications have been largely studied. Thus, di-methylation at lysine (K) 4 and tri-methylation at K4, K36 and K79 of H3 have been associated to transcription activation. On the contrary, Tri-methylation at K9 and

K27 from H3 and K20 from H4 have been related to gene repression. Acetylation of K9, K14, K18 and K56 of the H3; K5, K8, K12 and K16 of the H4; and K6, K7, K16 and K17 of the H2B have been described to promote gene transcription. Moreover, phosphorylation in serine 10 from H3 has been related to gene activation (154,155).

6.3. Interplay between DNA methylation and histone modifications

The epigenetics mechanisms, DNA methylation and histone modifications act coordinated. For instance, it has been shown that an increase at promoter DNA methylation lead to a decrease of histone acetylation, and conversely, histone hipo-acetylation sensitize to targeted DNA methylation. It has been reported that methylated DNA can bind to methyl-CpG binding domain (Mbd) containing proteins, which in turn has been shown to recruit HDAC leading to histone de-acetylation, reinforcing the transcriptional repression (156). It is thought that a certain environmental or physiological event could provoke the partial methylation of a promoter in an active gene. This fact could lead in turn to methyl-CpG binding proteins (MeCPs) target these sequences, for instance MeCP2 that has been demonstrated to recruit HDACs and DNMT1 provoking histone de-acetylation and DNA methylation. By contrast, the repressive signal can be triggered by histone de-acetylation. Thus, it could happen that and unbalanced HAT/HDACs ratio causes a hypo-acetylation state that could lead to HMT and DNMTs recruitment giving as a result a permanent gene blockage (repressive histone methylation marks seem to protect against DNA de-methylation). Both mechanisms, triggered by DNA modifications or histone modifications have been proposed, and it is thought this might rely on the specific loci, environmental or physiological conditions that cause these chromatin changes (156,157).

The study of the interplay between DNA methylation and histone modifications is somewhat difficult due to the redundant activity of some of the factors implied. It has been shown that the methyl-CpG domain proteins such as MeCP2 and Mbd1 can be implied in the cross-talk DNA methylation and di,tri-methylation at H3K9 (regarded as a repressive mark). It has been shown that MeCP2 can recruit the histone-lysine N-methyltransferase Suv39h1/2, which can methylate H3K9. Besides, Mbd1 lead to a raise in H3K4me2/3 levels by its interaction either with the histone methyltransferase Setdb1 and the chromatin assembling factor Caf1, or by forming a complex with Suv39h1/HP1 (heterochromatin protein 1) (157) (Figure 16).

Direct interaction between the DNMTs and the HMTs has also been reported. For example, it has been shown that DNMT3A/B can interact with Suv39h1 and Setdb1. Moreover, DNMT3A has been demonstrated to interact with the H3K9-HMT Ga9, in a complex in which M- phase phosphoprotein 8 (MPP8) is involved. This suggests that de-novo DNMTs might take some guidance by recognizing chromatin structure through direct interaction with histone modifiers (157).

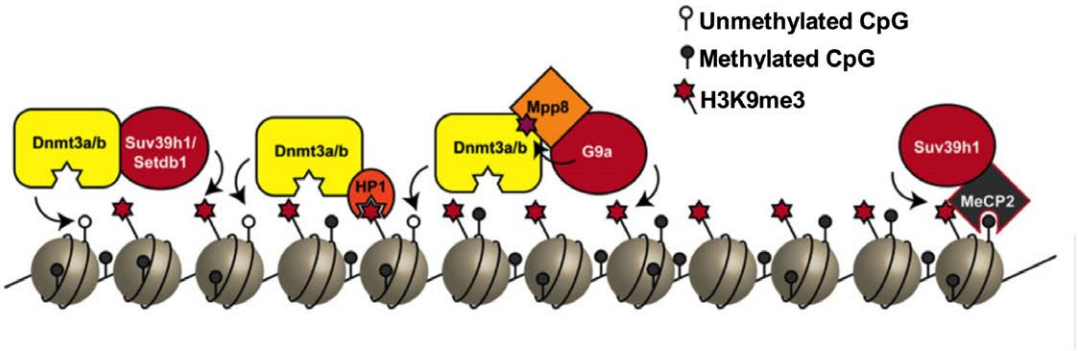

Figure 16. Interplay between H3K9 modifications and DNA methylation. Coordinate action of specific H3K9 HMTs and de-novo DNMTs by direct interaction with each other. In the other way around, DNA methylation can promote H3K9 methylation through the interaction between the HMT Suv39h1 with MeCP2. **Adapted from** (157)**.**

Gene body H3K36 methylation together with DNA methylation has been proposed to act in the splicing regulation, being H3K36 methylation enriched in exons compared to introns. A coordinate action between both, DNA methylation and H3K36 methylation has also been reported by the interaction of DNMTA/B and Setd2 (157).

By contrast, H3K4me3 has been associated to hinder DNA methylation in a process in which DNMT3L can be involved. DNMT3L has the capacity to bind to un-methylated H3K4, triggering to DNA methylation by modulating DNMT3A/B activity. However, DNMT3L cannot bind to H3K4me3 and therefore DNA methylation is impaired at these specific loci where this histone mark is up-regulated (157).

An important question is how epigenetic marks target the right places in the genome. In this sense, CpG island are usually hypo-methylated and with high levels of H3K4me3. It has been shown that some ZF-CxxC (zinc finger-CxxC) domain containing proteins (such as CFP1) can specifically target CpG island sequence. This protein has been shown to recruit Set1a/b, complex in charge of H3K4 tri-methylation. Other ZF-CxxC domain containing protein, the Kdm2a/b lysine demethylases, has been suggested to interact with CpG islands. Kdm2a/b act as histone demethylases and is thought that could be involved in the de-methylation of the repressive mark H3K36me2 in these CpG island (usually located at promoters) (157).

The Polycomb repressive complex 2 (PRC2) is a multiproteic complex (formed for a core complex and some accessory proteins such as Enhancer of zeste homolog 1 (EZH1) and 2 (EZH2), Embryonic ectoderm development (EED), SUZ12, Polycomb repressive complex 2 subunit (SUZ12), Polycomblike (PCL), RB binding proteins 46 and 48 (RbAp46/48), Jumonji and AT-rich interaction domain containing 2 (JARID2), AE binding protein 2 (AEBP2) that carries out gene repression through bi- and tri-methylation of the H3K27. PRC2 has been shown to be a key complex in

developmental processes and cell identity (158). The catalytic activity of PCR2 is performed by the subunits of Enhancer of zeste homologue 1 and 2 (EZH1/2) (158,159). Thus, PRC2/EZH2 complexes have been proved to have high methylation activity and PRC2/EZH1 have a weaker activity, suggesting that complexes that carry EZH2 are in charge of the H3K27me2/3 pattern establishment while; complexes with EZH1 are in charge of the pattern maintenance. Besides, the temporal and cellular pattern of expression for these two proteins are different, being EZH2 present in actively dividing cells and EZH1 present in both, dividing and differentiated cells (159). In AT, EZH2 has been shown to be necessary to adipogenesis by repressing genes from the WNT/β-catenin pathway, which has been described to inhibit the expression of PPARγ and CEBPα (160).

PRC2 recruitment has been shown to be induced by H3K27me3, process that help to maintain the pattern. Nevertheless, establishment of new H3K27me3 deposition has been demonstrated to rely on the DNA sequence. Thus, it has been shown that SUZ12 can target to un-methylated CpG island generating H3K27me2 at these positions (158,161). As it is described above, CpG islands are located in the promoter of genes, so deposition of H3K27me3 at these regions should account only to genes that are not active. In this sense, PRC2 recruitment also depends on the Transcription factor (TF) present. Thus, CpG islands with active genes (and TF stimulating expression) might avoid from PRC2 binding (161). Interestingly, it has been demonstrated that PRC2 can modulate DNMTs activity by direct interaction with EZH2 (162,163), again showing the regulatory interplay between the epigenetic phenomenon, DNA methylation and histone modifications. H3K4me3 and H3K27me3 marks are usually found in some promoters of genes that are thought to respond quickly to up- and down-regulation. These are known as bivalent genes and are usually genes that have to be switched on/off

during developmental processes or upon a given stimulus. Drug resistance against cancer treatment has been associated to the permanent repression of this bivalent loci due to DNA hyper-methylation (164).

In summary, histone modifications and DNA methylation are process closely connected. Cooperation between both mechanisms not only ensure correct gene regulation or chromatin structure but also help to target and set up a correct epigenetic pattern of modifications.

6.4. Interplay between metabolic status and epigenetics

Epigenetics unlike genetics is a plastic landscape of regulation. Indeed, epigenetics marks can be changed under developmental processes, nutritional conditions, exercise or metabolic status for instance (165–167). Given the plasticity observed in the epigenetics phenomenon, the study of DNA methylation and histone modifications has gained popularity to try to explain the etiology of complex disorders that have been traditionally associated to have a strong environmental component. Thus, obesity, MetS, diabetes or cancer (as CRC) could be modulated by epigenetics.

There is a closed relationship between epigenetics and metabolism, since epigenetics modifications need from molecules that are metabolic intermediaries for the main metabolic pathways. For instance, acetylation of histones is carried out using Acetyl-CoA as donor molecule. As it has been described above, DNA methylation and de-methylation processes are modulated by SAM, α-ketoglutarate or succinate levels. In turn, SAM levels depend on homocysteine, folic acid and methionine availability to be generated. Once SAM donates the methyl group is turned into S-adenosylhomocysteine (SAH), which is transformed to homocyteine that is used to restore methionine levels using L-methyltetrahydrofolate (which derives from folic acid) as a methyl donor.

Methionine is then transformed to SAM in a reaction in which adenosine triphosphate (ATP) is necessary (152,168). Nutrition and cell metabolism has been reported to altered the ratio SAM/SAH, which in turn can affect H3K4me3 levels (169–171). Besides, a positive correlation between SAM, BMI and adiposity mass has been demonstrated (172). DNA and histone de-methylation processes involve both, oxidized and reduced Flavin adenine dinucleotide (FAD/$FADH_2$ respectively); α-ketoglutarate; and succinate levels. All these molecules are part of the TCA cycle, therefore being biosensors that have an important role in the interplay between the energetic/metabolic status and the epigenetic modifications. Thus, reduction in fatty acid oxidation and glucose incorporation observed in insulin resistance states (173–175) could alter α-ketoglutarate availability affecting the epigenetic landscape by downregulation of DNA and histone methylation levels. Indeed, the de-methylation process in which α-ketoglutarate is oxidated to succinate relies not only in α-ketoglutarate and succinate levels but also in oxygen availability, and in fact hypoxia has been reported to increase H3K4me3 marks through reduction of de-methylation (169). It has been shown that SAM levels can directly inhibit the action of the histone de-methylase KDM5B/JARID1B, while fumarate and succinate (both byproduct down-stream α-ketoglutarate) can inhibit TET enzymes and Jumonji domain-containing histone demethylases (Jmj-KDMs) (152,176), demonstrating that these metabolic intermediaries cannot only exert a role in epigenetic regulation by substrate availability or affecting the substrate/product enzymatic rate but also by direct regulation of the enzymes implied in these epigenetic modifications.

It has been shown that histone acetylation levels directly rely on Acetyl-CoA levels and that Acetyl-CoA acts as a biosensor of the energetic state for histone modification. Thus, high levels of Acetyl-CoA can raise acetylation of histones resulting in a more

permissive chromatin, which gives to a raise of gene expression for genes related to growth or proliferation (152,176). By contrast, sirtuins (class III HDAC) has been shown to perform histone de-acetylation in a process in which oxidized nicotinamide adenine dinucleotide (NAD+) is required. NAD+ is a sensor of the metabolic status and it is implied in several oxidative pathways such us glycolysis, β-oxidation and the TCA cycle. NAD+ deficiency in diabetes, aging or in mice fed with HFD has been shown to impaired sirtuin action (152). It has been descried that sirtuin 6 (SIRT6) can be stimulated by FFA. Lack of SIRT6 has been shown to up-regulate the glycolytic pathway, leading to a severe hypoglycemic state. Thus, it is thought that SIRT6 stimulation through FFA (for example during β-oxidation) could produce the inactivation of glycolytic genes (152) (Figure 13).

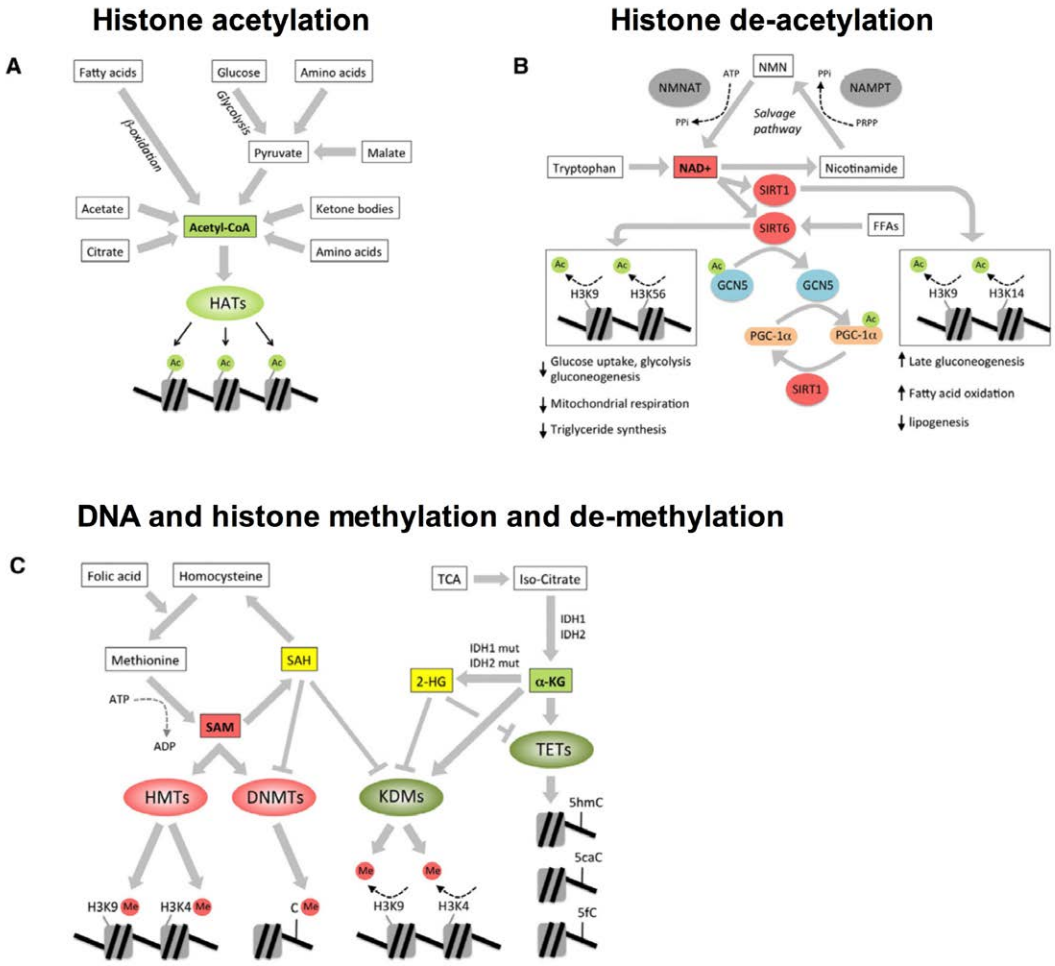

Figure 13. Interplay between metabolism and epigenetic modifications. Effects of metabolic intermediaries on histone acetylation (A), histone de-acetylation (B), and DNA and histone methylation and de-methylation (C). **Adaptation from** (152)**.**

6.4.1. Role of lifestyle and nutritional conditions in the epigenetics of obesity and metabolic disease

Given the interplay observed between epigenetics and metabolism, it is not surprising the efforts that researchers are putting in trying to understand what could be the role of epigenetics in the etiology of the metabolic diseases. In this sense, Dutch famine (famine that took place in Netherland during the 1944-1945 winter at the end of the War World II) has shed light on the nutritional effects during in-uterus condition over DNA methylation and adulthood metabolic disease. Thus, under-nutrition condition has been related to BMI and metabolic disease through specific DNA methylation marks at genes that regulated lipid or glucose homeostasis as well as adipogenesis (177). For instance, it has been shown in this Dutch famine population that DNA methylation at serine/threonine-protein kinase pim-3 (PIM3) (factor involved in glucose metabolism) could explain BMI in adulthood. DNA methylation at thioredoxin interacting protein (TXNIP), gene that regulates β-cell function, and ATP binding cassette subfamily G Member 1 (ABCG1), which is involved in lipid metabolism, was able to explain (together with other CpG positions) up to 80% of the association observed between the famine and the TG levels. During early stage gestational famine CpG marks near 6-phosphofructo-2-kinase/Fructose-2,6-biphosphatase 3 (PFKFB3) (involved in glycolysis) and Methyltransferase like 8 (METTL8) (adipogenesis) were shown to influence TG levels as well (177).

As it is described above, DNA methylation relies on SAM as the methyl-group donor. SAM levels depend on methyl-group donor nutrients such as choline, methionine, folate, etc. It has been described that maternal consumption levels of these methyl-donors just before pregnancy and during pregnancy can determined the DNA methylation levels in the newborn of genes related to metabolism and adipogenesis like RXRα, insulin like growth factor 2 (IGF2) and LEP, as well as of the DNA methyl-transferase DNMT1 (178).

DNA methylation pattern cannot only be established during the developmental period, but can also be modified in adulthood under several conditions. For instance, in a randomized control trial where subjects were exposed to Saturated or Polyunsaturated fatty acids (SFA and PUFA, respectively) overfeeding during 7 weeks, DNA methylation changes specific for SFA and PUFA emerged. There were changes at genes involved in metabolism and inflammation in AT like FTO, INSR, Neuronal growth regulator 1 (NEGR1), Fatty acid binding protein 1 (FABP1), Fatty acid binding protein 2 (FABP2), PPARG coactivator 1 alpha (PPARGC1α), Melanocortin 2 receptor (MC2R), melanocortin 3 receptor (MC3R), TNF or IL-6, among others. Moreover, DNA methylation at several loci at baseline were associated with the percentage of body weight increase after the trial (179). As well as food, exercise has also been related to DNA changes in adipose tissue. In a six-month interventional study, it has been described changes in DNA methylation in AT at several CpGs for genes which have been associated to obesity, diabetes and adipocyte metabolism such as Transcription factor 7 like 2 (TCF7L2), Potassium voltage-gated channel subfamily Q member 1 (KCNQ1), Histone deacetylase 4 (HDAC4) or Nuclear receptor corepressor (NCOR) (180). Lifestyle habits as smoking has been related to changes at DNA methylation in AT. These changes were at specific loci that were in turn associated to future weight

gain and metabolic disease risk after smoking cessation. Furthermore, it has been shown that after smoking cessation, the DNA methylation smoking-signature pattern had a longer lasting influence on DNA methylation than the mRNA pattern (181).

Histone deregulation has also been related to obesity, metabolic disease and related disorders. For example, Plant homeodomain finger two (PHF2) (which is an histone demethylase) has been shown to regulate CEBPα and PPARγ expression. Thus, it has been reported that specific Plant homeodomain finger two (PHF2) knockout mice display abnormal adipogenesis and a subsequent decrease in AT mass. Moreover, it is thought that PHF2 is important in the regulation of several metabolic tissues being involved in the metabolism of glucose and lipids (182). A total epigenetic remodeling at the AT LEP promoter has been described in Diet induced obese (DIO) mice feed with n-3 PUFAs. These changes comprised the promoter binding increase for Methyl-CpG-binding domain protein 2 (MBD2), DNMTs and several HDACs, with a subsequent increase of DNA methylation and a decrease in the acetylation of H3 and H4. Furthermore, a decrease of H3K4me3 was observed. All of these changes would be interpreted as a compensatory mechanism trying to deal with the extra-energetic consumption experimented (183). This study points out how the epigenetic state of a certain factor involved in the control of energy balance can deeply change in adulthood triggered for changes in the nutritional status.

As previously described, histone modifications are strongly associated to metabolism, and some of the erasers and writers involved have been associated to metabolic disorders (152). However, there are no studies about the histone marks profile in human AT, which could give us a better knowledge about the actual epigenetic status and about its possible role in the etiology of the AT related disturbances. Concerning AT, histone modifications studies have been mainly carried out in cultures (3T3L1 or primary pre-

adipocyte cultures) (184,185), and to a less extend in mice. Thus, it has been reported an increase of H3K4me2 enrichment in *db*/*db* mice compared with db/*m* at the promoter of ATPase, H+ transporting, Lysosomal V0 subunit D2 (Atp6v0d2), Matrix metallopeptidase 12 (Mmp12), Triggering receptor expressed on myeloid cells 2 (Trem2) and C-type lectin domain family 4, member d (Clec4d) genes, while this mark was lower in Glycoprotein (transmembrane) nmb (Gpnmb) (186).

6.4.2. Epigenetics alterations in colorectal cancer

Epigenetic aberrations in the context of CRC have been studied, which could lead to new subtypes classification based on pharmacological response, and therefore leading to new treatment strategies (187). Indeed, AT has been related to CRC appearance and progression (188), although the role of AT epigenetic regulation and its relationship to CRC has been poorly studied. Epigenetic modifications in peri-tumoral AT have been shown for breast and prostate cancers. Thus, an altered DNA methylation pattern has been described in the surrounding breast malignant cells, alterations that are related to chromosomal organization and with adverse clinical outcome (189). AT DNA methylation pattern has also been implicated not only in the appearance of metabolic diseases but also to the development of cancer by altering the metabolism and increasing the inflammatory environment (190). Differentiated DNA methylation has been reported for genes related to lipid metabolism and immune system (as Acyl-CoA dehydrogenase medium chain (ACADM), Carnitine palmitoyltransferase 1B (CPT1B), Carnitine palmitoyltransferase 1C CPT1C, Fatty acid desaturase 1 (FADS1), Monoacylglycerol O-acyltransferase 1 (MOGAT1), Monoacylglycerol O-acyltransferase 2 (MOGAT2), Solute carrier family 44 member 2 (CTL2) or TAP binding protein (TAPBP)) in peri-prostatic AT of obese and overweight versus lean

subjects with prostate cancer, which could be ultimately contributing to the worsening and different cancer progression observed in subjects with higher adiposity (190).

Since epigenetic can be the result of genetic, age, tissue specificity and a given environmental conditions, its study can point out functional factors or pathways that could be affecting to the AT functioning and contributing to metabolic disease and other disorders associated (as CRC), being a more accurate tool than genetic studies (which gives us a fixed scenario) to infer this relationships (137).

HYPOTHESIS

1- Adipose tissue DNA methylation at LPL promoter could be regulating LPL mRNA and be related to serum triglyceride levels, thus participating in the etiology of metabolic syndrome. Besides, these levels of LPL methylation could be related to the response in triglyceride clearance after a fat overload.

2- Overall DNA methylation state of the adipose tissue via LINE-1 as well as the DNA methylation promoter regions of genes related to adipogenesis, lipid metabolism and inflammation could be altered in metabolic syndrome playing a role in the etiology of the metabolic disease.

3- Adipose tissue DNA methylation at C3 promoter could be regulating C3 mRNA and be related to serum ASP levels in obesity, being evolved in the higher pro-inflammatory status and impaired lipid storage present in extreme obesity.

4- Adipose tissue H3K4me3 enrichment at the promoter of several genes related to adipogenesis, adipose tissue metabolism and inflammation could be modified in accordance with the obesity degree and metabolic status, which could explain in part the development of obesity and metabolic disease.

5- Low levels of vitamin D could be related to adipose tissue inflammation and adipose tissue DNA methylation in subjects with colorectal cancer, which in turn could be contributing to colorectal cancer development.

OBJECTIVES

1. To examine LPL DNA methylation status relating this to LPL gene and protein expression, as well as biochemical parameters associated with the etiology of MetS. Study how methylation at the LPL promoter could be related to triglyceride physiology by examining the postprandial triglyceride levels after a fat overload test.
2. To analyze the overall DNA methylation state of the adipose tissue via LINE-1 in both, MetS and Non-MetS subjects, as well as in the DNA methylation promoter regions of genes related to adipogenesis, lipid metabolism and inflammation in subjects with and without MetS.
3. To determine C3 DNA methylation level in adipose tissue from subjects with a different grade of obesity and to study whether C3 DNA methylation is associated to BMI or obesity-associated disorders.
4. To develop a series of improvements to the regular chromatin inmunoprecipitation (ChIP) protocol in order to make the technique applicable for the study of small frozen pieces of adipose tissue.
5. To analyze histone tri-methylation at lysine 4 in histone 3 (H3K4me3) in adipose tissue. Specifically we aimed to analyze promoter H3K4me3 levels and gene expressions of specific genes related to lipid metabolism, adipogenesis and inflammation in order to discern which are the associations between these marks with the obesity degree and metabolic status.
6. To explore the relationship between serum 25-hydroxyvitamin D (25(OH)D), adipose tissue gene expression of Vitamin D receptor (VDR), pro-inflammatory markers, and the epigenetic factor DNA methyltransferase 3a (DNMT3A) as well as VDR promoter methylation in colorectal cancer.

RESULTS

MANUSCRIPT 1. Adipose Tissue LPL Methylation is Associated with Triglyceride Concentrations in the Metabolic Syndrome

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In order to carry out the objective 1, the levels of DNA methylation of several LPL-promoter-CpG dinucleotides in a CpG island region were analyzed and related to the gene and protein expression levels visceral adipose tissue (VAT) in individuals with (MetS) and without (non-MetS) metabolic syndrome. To perform this, VAT samples were collected from laparoscopic surgical patients, and levels of LPL mRNA, LPL protein and LPL DNA methylation were measured by qPCR, western blot and pyrosequencing. Biochemical and anthropometric variables were analyzed. Moreover, a subset of individuals underwent a dietary fat challenge test and postprandial triglycerides were determined.

Anthropometric and biochemical characteristics of the patients

Table 1A shows the anthropometric and biochemical parameters of the non-MetS and MetS patients. As expected, the MetS patients had significantly increased glucose, triglycerides, waist circumference, systolic and diastolic blood pressures, BMI, insulin, HOMA-IR, total cholesterol, LDL cholesterol, ApoA1, ApoB and serum leptin levels in comparison to the non-MetS subjects, whereas HDL cholesterol, ApoA1 and adiponectin values were significantly lower compared with the non-MetS subjects.

Table 1. Biochemical and anthropometric parameters in non-metabolic syndrome subjects (Non-MetS) and metabolic syndrome subjects (MetS) in the descriptive study (A) and the subpopulation who underwent the fat overload test (B).

	(A) Study population		**(B) Fat overload subpopulation**	
	Non-MetS (N=70)	**MetS (N=64)**	**Non-MetS (n=11)**	**MetS (n=26)**
Age (years)	48.0±13.38	49.79±15.0	44.0±7.9	42.6±6.9
Male/Female (%)	51/49	40/60	27/73	48/52
BMI (Kg/m2)	32.2±10.8**	41.5±12.5	51.6±7.3	52.3±6.9
Waist circumference (cm)	101.5±21.2**	118.7±23.5	137.0±18.2	139.5±17.7
SBP (mm Hg)	124.7±17.6**	139.7±19.7	128.8±19.6	137.6±19.9

DBP (mm Hg)	76.2±11.6**	82.6±11.1	83.6±11.9	82.6±12.1
Glucose (mg/dL)	92.7±11.9**	116.4±30.7	93.0±7.9	104.4±18.0
Insulin (pmol/L)	12.0±13.6*	17.8±11.4	26.0±27.4	23.2±13.8
HOMA-IR	2.5±2.0**	5.1±3.4	4.3±3.2	6.1±4.1
Uric acid (mg/dL)	4.7±1.3**	5.7±1.3	5.4±1.0	6.1±1.4
TG (mg/dL)	102.7±43.0**	160.1±64.6	105.1±62.0	142.3±53.5
Post TG (mg/dL)	-	-	177.7±69.6	193.9±72.2
Cholesterol (mg/dL)	194.1±34.9**	212.3±42.1	174.7±48.5	201.5±38.6
HDL cholesterol (mg/dL)	54.5±12.8**	47.1±12.8	50.4±13.8	44.6±10.0
LDL cholesterol (Friedwald)	118.8±30.7*	133.2±32.5	95.6±37.0	124.9±33.6*
ApoA1 (mg/dL)	169.8±25.4*	155.6±26.6	158.3±30.9	148.0±21.6
ApoB (mg/dL)	93.8±22.9*	108.2±22.5	89.5±33.3	104.1±22.9
GOT (mg/dL)	21.0±12.3	20.0±11.5	22.3±8.8	24.8±13.7
GPT (mg/dL)	41.2±22.2	46.3±21.0	46.6±13.9	55.3±20.9
GGT (mg/dL)	56.6±186.5	40.4±27.7	28.6±10.2	34.8±22.9
CRP (mg/dL)	7.4±15.5	5.6±3.4	6.7±6.3	5.2±3.4
Leptin (ng/ml)**	22.5±26.3	47.1±31.3	58.3±22.0	65.8±26.1
Adiponectin (ug/ml)**	12.3±7.2	8.6±4.5	8.7±4.4	7.8±3.3

Definitions: Body Mass Index (BMI), Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), Baseline Triglycerides (TG), Postprandial Triglycerides (Post TG), High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), Apolipoprotein A1 (ApoA1), Apolipoprotein B (ApoB), Glutamate-Oxaloacetate Transaminase (GOT), Glutamate-Pyruvate Transaminase (GPT), Gamma Glutamyl Transpeptidase (GGT), Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), C-Reactive Protein (CRP). * P<0.05 and ** P<0.01 considered statistically significant between Non-MetS and MetS in each population (A) and (B).

On the other hand, **Table 1B** shows the anthropometric and biochemical parameters for the subset of people who underwent fat overload. Only mean LDL cholesterol was found significantly higher in MetS compared to non-MetS patients.

Measures of DNA methylation, LPL mRNA, and protein levels in adipose tissue

Figure 1 shows the LPL DNA methylation and mRNA levels. The MetS patients had significantly higher levels of DNA methylation (P<0.001) (**Figure 1A**) and lower levels of mRNA gene expression (P=0.012) (**Figure 1B**) compared to the non-MetS subjects.

Interestingly, this association was confirmed with a correlation analysis, which showed a negative correlation (r=-0.306, P=0.004) between the DNA methylation levels and mRNA levels of LPL (**Figure 1C**).

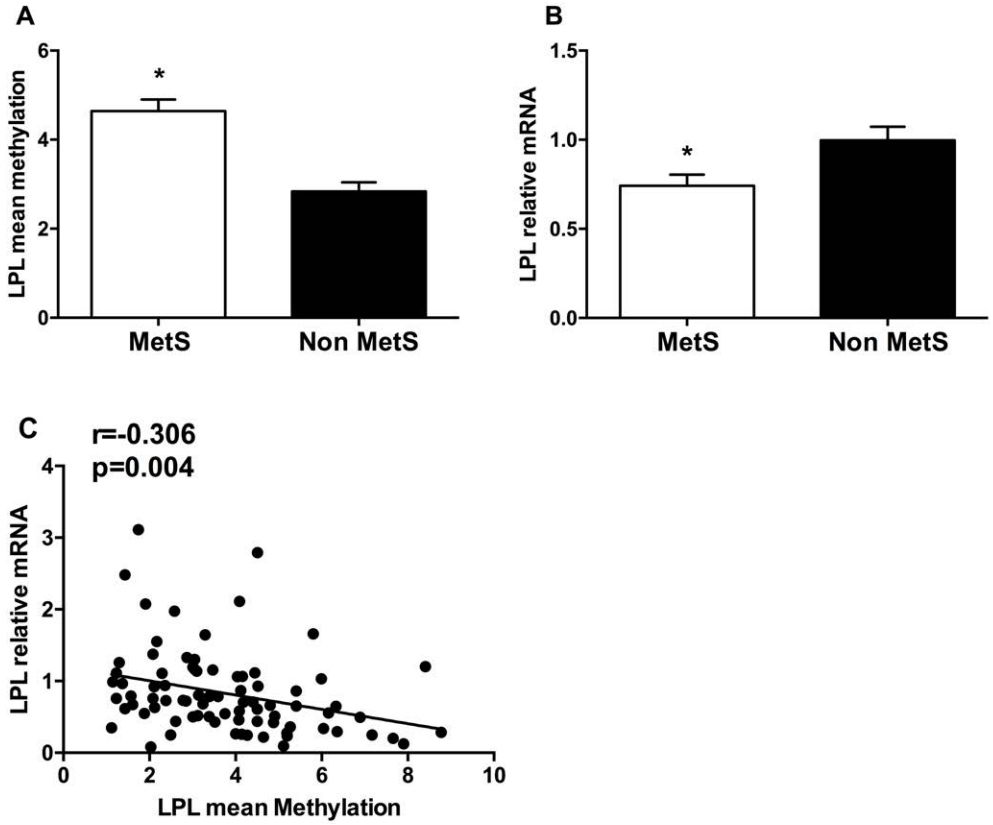

Figure 1.- Mean and SE of LPL DNA methylation levels (n=41 Non-MetS and n=46 MetS) **(A)** and LPL relative mRNA (n=70 Non-MetS and n=64 MetS) **(B)** in non-metabolic (Non-MetS) and metabolic syndrome (MetS) subjects. **Figure 1C** shows the correlation between both LPL methylation levels and LPL mRNA levels.

Finally, we used western blots to examine the protein level of LPL to assess whether the expression levels were translated into the final protein products, confirming a significantly lower LPL protein expression in the MetS patients in comparison with the non-MetS subjects (**Figure 2A** and **Figure 2B**).

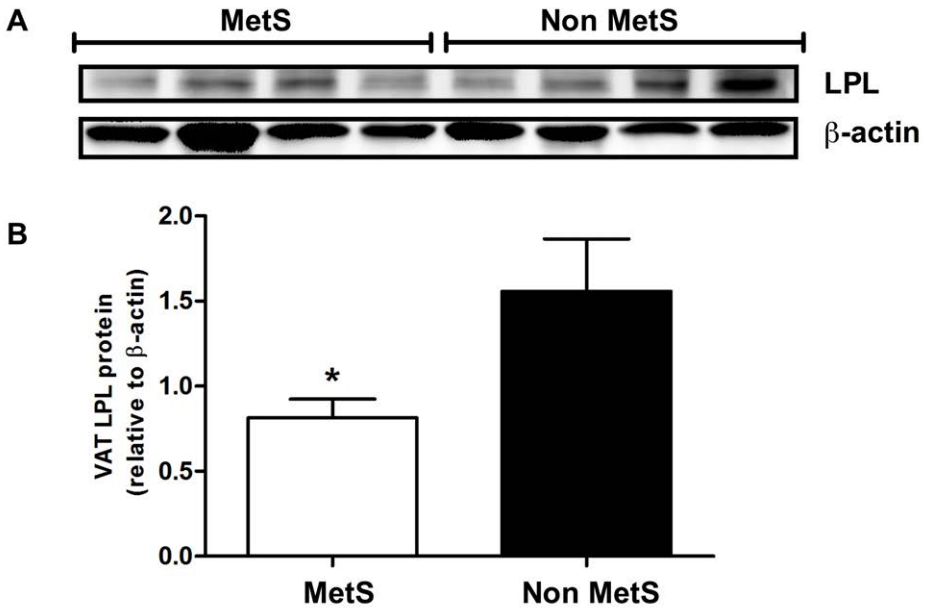

Figure 2.- Average and SE of LPL protein levels quantified by western blot in both non-metabolic syndrome (Non-MetS) and metabolic syndrome (MetS) subjects (n=10).

Associations between LPL levels and metabolic syndrome

We performed a correlation analysis to analyze the relationships between LPL DNA methylation and the LPL mRNA expression levels and the metabolic and anthropometric variables present in the study subjects (**Table 2**). We found that the number of MetS components, BMI, waist circunference, glucose, fasting triglyceride concentrations and serum leptin had significant and positive correlations with the LPL DNA methylation levels. Likewise, we found significant negative correlations between the LPL mRNA expression levels and the number of MetS components, BMI, waist circumference, HOMA-IR, glucose concentrations, baseline triglyceride concentrations, and ApoB (**Table 2**). Finally, we found significant positive correlations between LPL mRNA expression and the serum concentrations of HDL cholesterol and adiponectin levels (**Table 2**). These results are consistent with the role of the LPL gene in metabolism.

Table 2. Correlations between LPL DNA methylation (LPLmet) and LPL relative mRNA (LPL mRNA) and several anthropometric and biochemical parameters.

	LPL methylation	**LPL mRNA**
MetS variables (0-5)	0.421**	-0.244**
BMI (kg/m2)	0.344*	-0.239**
Waist circumference (cm)	0.297**	-0.222*
HOMA-IR	0.175	-0.183*
Insulin (pmol/L)	0.155	-0.165
Glucose (mg/dL)	0.269*	-0.220*
Cholesterol (mg/dL)	0.182	0.035
HDL cholesterol (mg/dL)	-0.068	0.210*
LDL cholesterol (mg/dL)	0.098	0.039
Log10(TG) (mg/dL)	0.246*	-0.259**
Log10(PostTG)	0.467**	-0.360*
SBP (mm Hg)	0.135	0.057
DBP (mm Hg)	0.111	0.026
ApoA1 (mg/dL)	-0.126	0.130
ApoB (mg/dL)	0.147	-0.301*
Leptin (ng/ml)	0.402**	-0.139
Adiponectin (ug/ml)	-0.039	0.378**

Definitions: Number of variables of MetS present (MetS variables), Body Mass Index (BMI), Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), Log10 of Fasting triglycerides (Log10(TG)), Log 10 of postprandial triglycerides (Log10(PostTG)), Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), Apolipoprotein A1 (ApoA1), Apolipoprotein B (ApoB). * $P<0.05$ and ** $P<0.01$ considered statistically significant.

Epigenetic factors associated with metabolic syndrome

In order to study the relationship between LPL DNA methylation and the parameters associated with MetS, and between the methylation and mRNA levels of LPL we performed regression analyses. Regression model 1 (**Table 3 model 1**) after adjustment for age, gender, BMI and HOMA-IR showed that the statistically significant variables in predicting the increase in LPL promoter methylation levels were the number of components of MetS and the BMI. When we considered LPL mRNA as dependent variable and adjusted by age, gender, BMI and HOMA-IR we found that LPL methylation was the only variable significantly associated with LPL mRNA variability (**Table 3 model 2**).

Table 3. Regression analysis with LPL DNA methylation (model 1) and LPL mRNA (model 2) levels as dependent variable.

	Model 1: LPL DNA Methylation ($R=0.472$; $R^2=0.223$)			**Model 2; LPL mRNA ($R=0.382$; $R^2=0.147$)**		
	β	***P***	**95% CI**	β	***P***	**95% CI**
Age	0.066	0.584	-0.023-0.041	-0.029	0.814	-0.012-0.010
BMI	0.298	0.037	0.003-0.080	-0.104	0.492	-0.018-0.009
Gender	-0.115	0.302	-1.121-0.383	0.113	0.331	-0.140-0.410
MetS variables (0-5)	0.349	0.009	0.126-0.837	-	-	-
HOMA-IR	-0.118	0.351	-0.216-0.078	-0.128	0.337	-0.077-0.027
LPL methylation	-	-	-	-0.266	0.025	-0.166-(-0.011)

Independent variables for model 1 are Age, BMI, Gender, MS variables and HOMA-IR; and age, BMI, gender, HOMA-IR and LPL methylation for model 2. Abbreviations: Body Mass Index (BMI), Number of variables of MetS present (MetS variables), Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), Lipoprotein Lipase (LPL).

Furthermore, we used logistic regression analysis to assess the risk of suffering MetS risk according to the methylation levels in LPL DNA promoter. LPL methylation level was found to be the only variable significantly predictive of the MetS state (dependent variable). In this regression, adjusted by age, gender and LPL mRNA levels, an increase in one unit in LPL methylation resulted in a more than two-fold higher likelihood of having MetS (**Table 4**).

Table 4. Logistic regression with membership or not membership to MetS group as dependent variable, and age, gender, LPL methylation and LPL mRNA as independent variables. Men are used as reference gender (0).

	NonMetS/MetS	
	OR (95% CI)	**P**
Age	1.003 (0.96-1.04)	0.865
Gender		
Male	1 (reference)	
Female	1.927 (0.73-6.41)	0.158
LPL methylation	2.092 (1.38-3.06)	0.000
LPL mRNA	0.464 (0.23-1.53)	0.282

Abbreviations: Lipoprotein Lipase (LPL).

Dietary Fat challenge

We designed a dietary fat challenge test in humans to evaluate the relationship between postprandial triglyceride and LPL methylation levels. Positive correlations were found between the baseline and postprandial triglyceride concentrations and the LPL DNA methylation levels (**Table 2**, **Figure 3A** and **Figure 3B**). Accordingly, we found a

negative correlation between LPL mRNA and both, the baseline and postprandial triglyceride concentrations (Table 2, **Figure 3C** and **Figure 3D**).

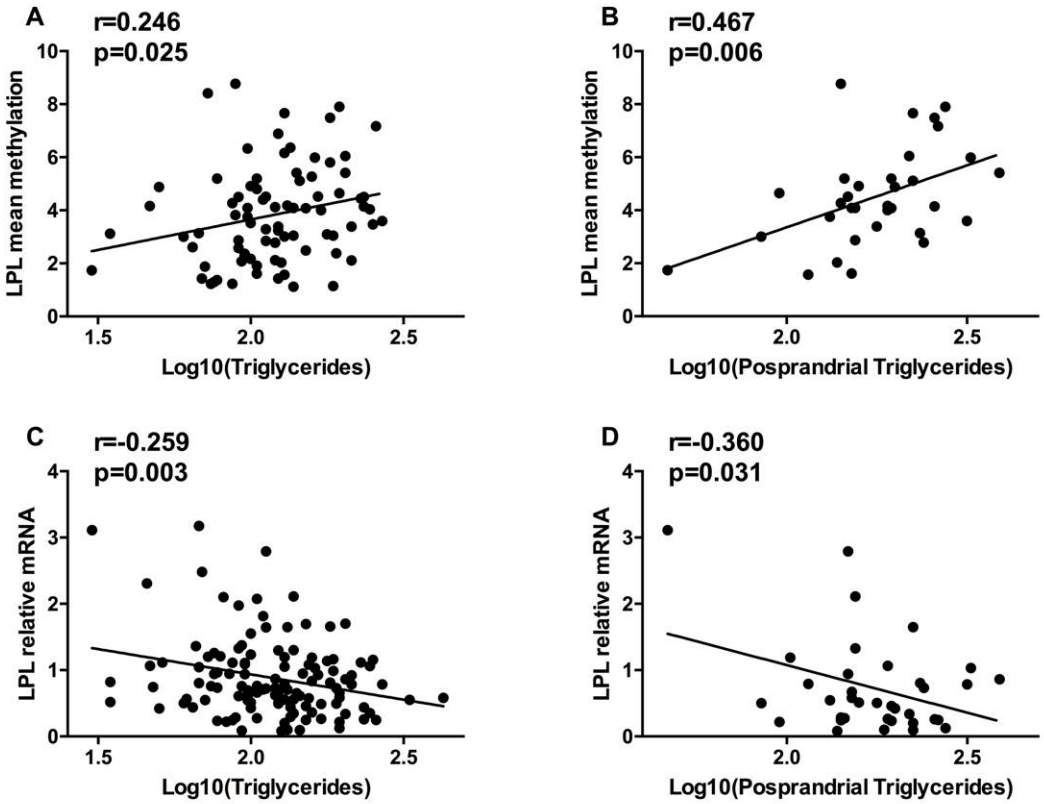

Figure 3. The figure shows the correlation of LPL DNA methylation with the Log of fasting triglycerides levels **(A)** and Log of postprandrial triglycerides levels **(B)**. **(C)** shows the correlation between LPL gene expression and Log of fasting triglycerides levels and **(D)** the association between LPL gene expression and Log of postprandrial triglycerides levels.

In a linear regression model adjusted for age, gender, BMI and HOMA-IR we found that LPL methylation was the only variable which explained the postprandial triglyceride concentrations (**Table 5**).

Table 5. Regression analysis with postprandial triglycerides as dependent variable, and LPL methylation, age, gender, BMI and HOMA-IR as independent variables.

	Log(TriglyceridesPost) ($R=0.628$; $R^2= 0.394$)		
	β	***P***	**95% CI**
Age	0.092	0.604	-0.006-0.009
Gender	0.016	0.925	-0.120-0.132
BMI	0.313	0.097	-0.002-0.017
HOMA-IR	-0.059	0.766	-0.023-0.017
LPL methylation	0.487	0.007	0.015-0.087

Abbreviations: Body Mass Index (BMI), Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), Lipoprotein Lipase (LPL).

MANUSCRIPT 2. Adipose Tissue DNA Methylation of Adipogenic, Lipid Metabolism and Inflammatory Genes in Metabolic Syndrome

Castellano-Castillo D. et al. Journal of Clinical Medicine (Under review)

The objective 3 is accomplished in this section. The aim of this study was to study the visceral adipose tissue (VAT) DNA methylation at several factors related to adipogenesis, lipid metabolism and inflammation as PPARG, PPARA, RXRA, SREBF2, SREBF1, SCD, LPL, LXRb, LRP1 C3, LEP and TNF in a population with or without MetS. To carry out this objective, VAT samples from Non-MetS and MetS subjects were obtained during laparoscopic surgery. DNA was then purified and treated with bisulfite. DNA methylation was performed using the PyroMark® Q96 ID Pyrosequencing System (Qiagen, South Korea).

Patients characterization and global methylation

MetS patients showed a clear metabolic deterioration as it is depicted in **Table 1**, with higher BMI (body mass index), waist circumference, glucose, insulin, HOMA-IR, TG, total cholesterol, LDL-cho, apolipoprotein B (ApoB), systolic blood pressure (SBP), diastolic blood pressure (DBP) and leptin than Non-MetS; and lower levels of HDL-chol and adiponectin compared to Non-MetS.

Table 1. Biochemical and anthropometric parameters in non-metabolic syndrome subjects (Non MetS) and metabolic syndrome subjects (MetS). * $p<0.05$ and ** $p<0.01$ considered statistically significant according to a Student's T-test and chi-squared test for gender.

	Non MetS (n=55)	**MetS (n=53)**
Age (years)	48.4±13.9	52.7±14.6
Male/female (%)	52/48	44/56
BMI (Kg/m²)**	29.8±7.9	36.4±10.9
Waist circumference (cm)**	97.6±14.8	112.6±22.4
Glucose (mg/dl)**	94.3±11.6	118.4±29.5
Insulin (pmol/L)**	9.8±7.4	16.2±11.4
HOMA-IR**	2.3±1.9	4.7±3.4
TG (mg/dl)**	101.6±38.1	164.2±65.1
Cholesterol (mg/dl)**	194.0±32.5	214.5±41.3
HDL-chol (mg/dl)*	55.0±11.0	48.5±14.2
LDL chol (Friedwald)*	119.0±31.8	135.1±30.2
ApoA1 (mg/dl)	171.6±21.8	160.5±29.5
ApoB (mg/dl)**	91.9±22.3	108.7±22.3
SBP (mm Hg)**	123.5±17.8	139.8±19.5
DBP (mm Hg)**	76.1±11.2	82.7±10.3
GOT (mg/dl)	20.0±13.1	19.3±8.7
GPT (mg/dl)	40.3±23.9	44.6±21.5
GGT (mg/dl)	57.4±203.4	42.1±27.9
Uric acid (mg/dl)**	4.6±1.2	5.6±1.2
Leptin (ng/ml)**	18.9±23.8	38.1±30.5
Adiponectin (µg/ml)*	11.2±5.3	8.2±4.1

Abbreviations: Body Mass Index (BMI), Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), Baseline Triglycerides (TG), High Density Lipoprotein cholesterol (HDL-chol), Low Density Lipoprotein cholesterol (LDL-chol), Apolipoprotein A1 (ApoA1), Apolipoprotein B (ApoB), Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), Glutamate-Oxaloacetate Transaminase (GOT), Glutamate-Pyruvate Transaminase (GPT), Gamma Glutamyl Transpeptidase (GGT).

In order to assess the global DNA methylation profile, DNA methylation at LINE-1 sequence were studied. Specifically, 6 CpG sites were included and pyrosequenced. The result showed no differences of global DNA methylation at any of the CpGs included between the groups (**Table 2**).

Table 2. DNA methylation level for each CpG position at LINE-1 pyrosequenced in both, Non MetS and MetS groups. Values are given as the mean±SE.

	Non MetS	**MetS**
LINE-1 P1 (%)	74.15±0.39	74.37±0.34
LINE-1 P2 (%)	65.84±0.20	65.75±0.33
LINE-1 P3 (%)	55.21±0,29	55.29±0.29
LINE-1 P4 (%)	61.37±0.33	61.48±0.24
LINE-1 P5 (%)	65.02±0.17	65.24±0,.21
LINE-1 P6 (%)	65.05±0.48	64.71±0.23

Abbreviations: Non metabolic syndrome group (Non MetS); Metabolic syndrome group (MetS); Long interspersed element 1 (LINE-1).

On the other hand, we performed an association analysis to observe the possible relationship between the global DNA methylation and the variables related to MetS (**Table 3**). We found a negative correlation between LINE-1 P2 and MetS index. Furthermore, there were negative correlations of glucose levels with LINE-1 P1, P2 and P5.

Table 3. Pearson's correlation between LINE-1 CpG positions (P1, P2, P3, P4, P5, P6) and the anthropometric and biochemical variables related to MetS. * p<0.05 and ** p<0.01 were considered statistically significant.

	MetS index	BMI	Waist	Glucose	Tg	HDL-cho	LDL-cho	SBP	DBP	HOMA-IR
LINE-1 P1	-0.167	0.057	-0.031	-0.246*	-0.088	0.113	0.082	0.162	0.02	-0.114
LINE-1 P2	-0.233*	0.025	-0.068	-0.334**	-0.208	0.074	0.028	0.171	0.010	-0.199
LINE-1 P3	-0.136	0.018	-0.011	-0.168	-0.072	-0.115	0.093	0.220	0.155	-0.101
LINE-1 P4	-0.068	0.042	0.012	-0.158	0.039	-0.112	0.077	0.168	0.010	-0.041
LINE-1 P5	-0.137	0.093	-0.037	-0.238*	0.016	-0.139	0.05	0.136	0.100	-0.088
LINE-1 P6	-0.19	-0.055	-0.05	-0.137	-0.166	0.028	0.052	0.066	0.016	-0.126

Abbreviations: Number of metabolic syndrome variables present in the subject of study (MetS index); Body mass index (BMI); Triglycerides (TG); High-density lipoprotein cholesterol (HDL-cho); Low-density lipoprotein cholesterol (LDL-cho); Systolic blood pressure (SBP); Diastolic blood pressure (DBP); Homeostatic model assessment of insulin resistance (HOMA-IR); Long interspersed element 1 DNA methylation at positions 1 to 6 (LINE-1 P1-P6). * and ** mean p<0.05 and p<0.01 respectively according to Pearson's correlation.

Gene specific DNA methylation in MetS versus Non MetS

Adipogenic and lipid metabolism factors

We studied genes related to adipose tissue development, as PPARA, PPARG and their heterodimer partner RXRA. There were no differences at any of the CpG sites included for PPARA, PPARG and RXRA (**Figure 1**). Nevertheless, a tendency to higher levels of DNA methylation in PPARA for MetS subjects than in Non MetS was observed.

Results for the association analyses showed a positive correlation between PPARA P2 with MetS index, TG levels and HOMA-IR. PPARG P1 correlated positively with BMI, while PPARG P1 and P3 were negatively associated to DBP. In the case of the PPAR's partner RXRA, we found a negative correlation between RXRA P1 with BMI and waist circumference.

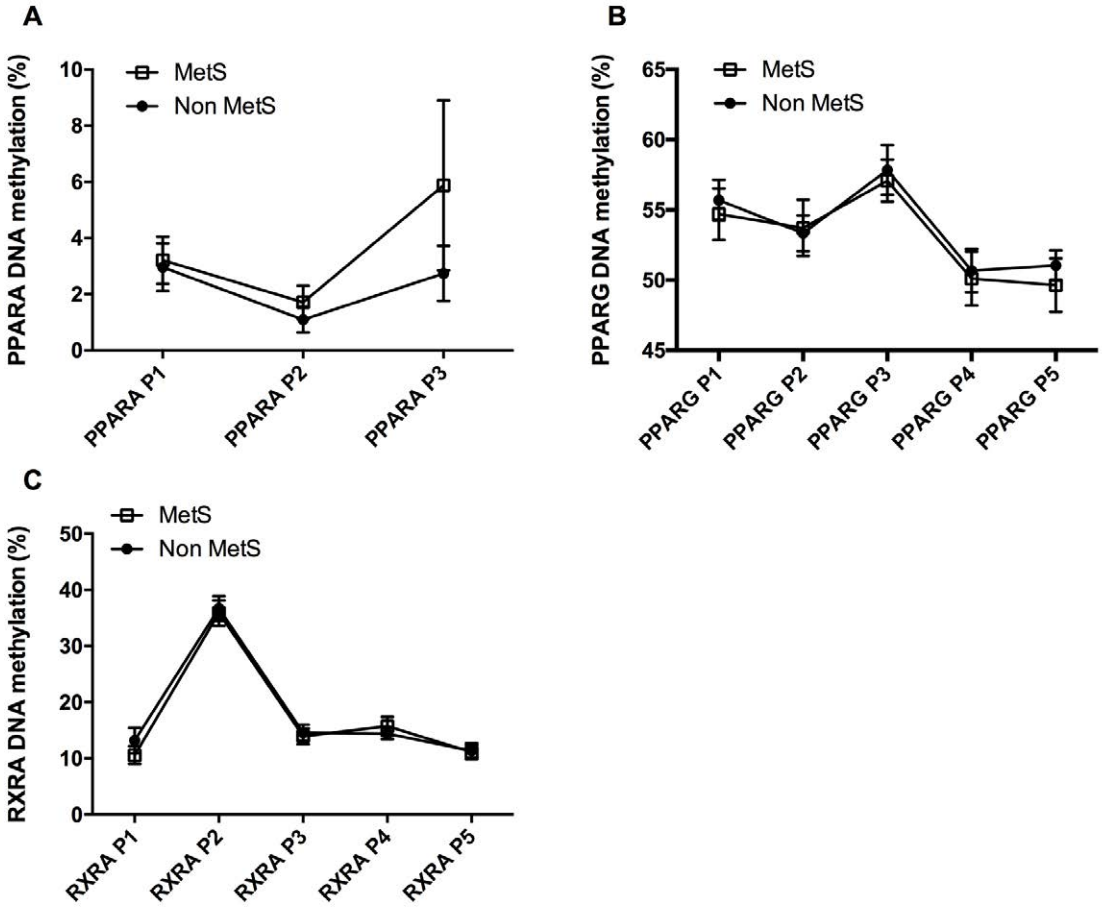

Figure 1. Adipogenic factors DNA methylation levels. DNA methylation profile across the CpG analyzed at the promoters of the adipogenic factors PPARA (A), PPARG (B) and the PPARs partner RXRA (C) in both, Non MetS and MetS groups. Values are given as the mean±SE. Peroxisome proliferator-activated receptor alpha (PPARA); Peroxisome proliferator-activated receptor gamma (PPARG); Retinoid X receptor alpha (RXRA).

Furthermore, a set of CpG sites inside genes related only to lipid metabolism was also pyrosequenced. No differences were found at any of the CpGs analyzed for SREBF1 and SREBF2 regulators (**Figure 2A** and **Figure 2B**, respectively). There were no significant DNA methylation differences at any of the LRP1 CpG sites studied either (**Figure 2C**). In the case of LPL, we found an increase of DNA methylation for the CpG situated at the position 2 (LPL P2) (**Figure 2D**). We did not find different levels of DNA methylation at any of the CpG studied for SCD and LXRB genes (**Figures 2E** and **2F**).

For these genes, we observed that MetS index correlated negatively with SCD P6, while SCD P3 was negatively associated to BMI. Positive associations existed between TG levels and LPL P3, and between HDL-cho and LRP1 P2. Furthermore, there was a negative association between the cholesterol regulator SREBF2 and DBP, specifically with SREBF2 P2.

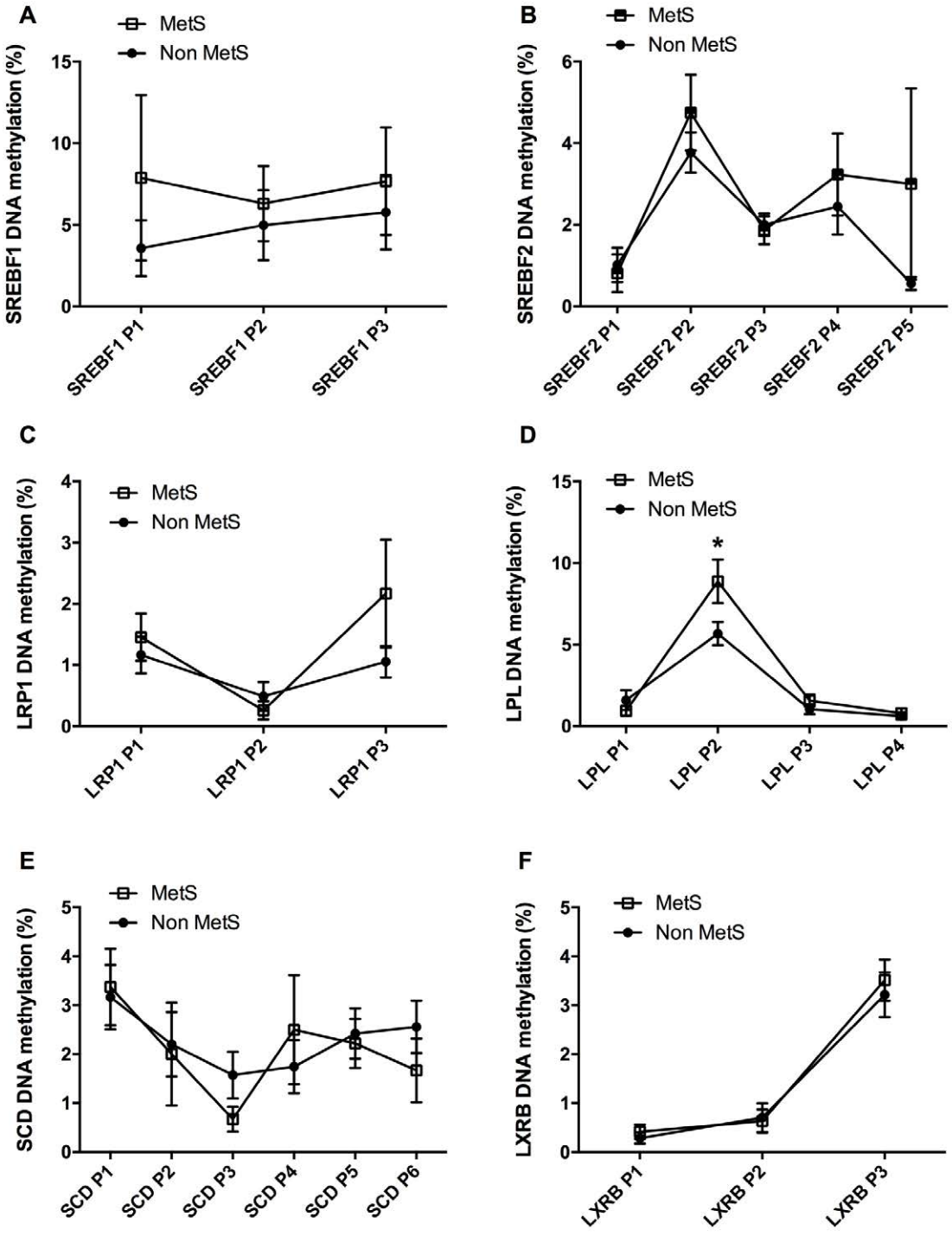

Figure 2. Lipid metabolism DNA methylation. The figure shows the DNA methylation in Non MetS and MetS groups at each CpG for several factors related to lipid metabolism as SREBF1 (A), SREBF2 (B), LRP1 (C), LPL (D), SCD (E) and LXRB (F). Values are given as the mean±SE. Sterol regulatory element binding transcription factor 1 (SREBF1); Sterol regulatory element binding transcription factor 2 (SREBF2); Low density lipoprotein receptor-related

protein 1 (LRP1); Lipoprotein lipase (LPL); Stearoyl-CoA desaturase (SCD); Liver X receptor beta (LXRB). * means $p<0.05$ according to a Student's T-test.

Inflammation factors

Due to the relationship between adipose tissue and inflammation, we analyzed some factors involved in this process. We analyzed 7 CpG sites inside the C3 gene promoter, and we did not find different DNA methylation levels between both, Non MetS and MetS subjects (**Figure 3A**). We studied 5 CpG sites for the tumor necrosis factor (TNF) as well. In this case, MetS subjects presented a lower DNA methylation levels at 3 out of the 5 CpG sites that were analyzed, concretely at position 1, 2 and 3 (TNF P1-P3) (**Figure 3B**). The third factor we studied was leptin (LEP), in which we analyzed 4 CpG sites at leptin sequence, which did not present significant differences between Non MetS and MetS subjects (**Figure 3C**).

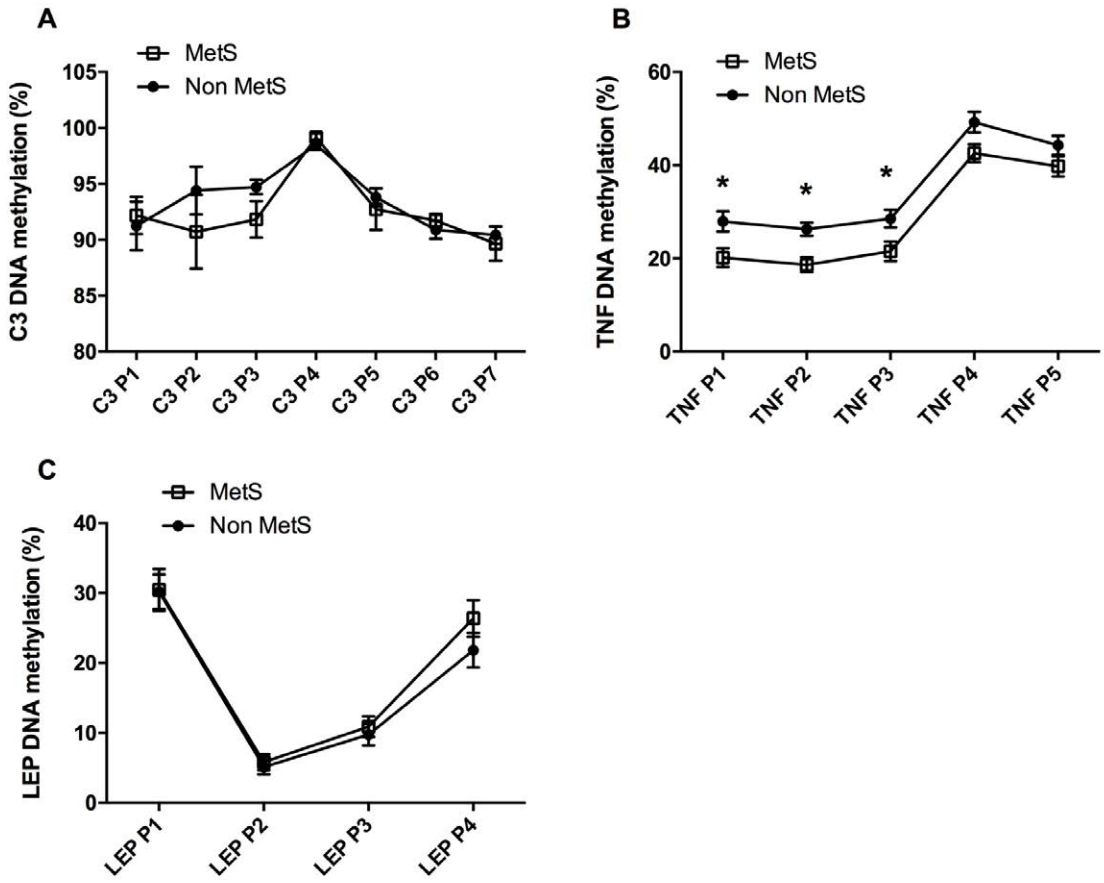

Figure 3. Inflammatory promoters DNA methylation. Comparisons between the Non MetS and the MetS group for DNA methylation at different CpG from genes implied in inflammatory processes as C3 (A), TNF (B) and LEP (C). Values are given as the mean±SE. Complement factor 3 (C3); Tumor necrosis factor (TNF); Leptin (LEP). * means $p<0.05$ according to a Student's T-test.

On the other hand, a negative relationship was found between MetS index and the DNA methylation levels of TNF P2, TNF P3, TNF P4 and TNF P5 (**Table 4**). Glucose correlated in a negative way with the DNA methylation of TNF P4 (**Table 4**). According to triglyceride levels, there were a negative correlations were found with TNF P2 and P5. Inversely to TG, HDL-cho correlated positively with TNF P1, P2, P5 (**Table 4**).

There was also a negative correlation between TNF P4 with LDL-cho and DBP. Furthermore, there were positive and significant correlations between LEP P1 with LDL-cho, SBP and DBP (**Table 4**).

Table 4. Correlation analyses between anthropometric and biochemical variables associated to MetS with some of the DNA methylation at the CpG analyzed. Only CpG that presented any significant association are represented.

	MetS V	BMI	Waist	Glucose	Tg	HDL-cho	LDL-cho	SBP	DBP	HOMA-IR
PPARA P2	0.276*	0.076	0.165	0.166	0.392**	0.061	0.08	0.066	-0.025	0.229*
PPARG P1	-0.072	0.306*	0.169	-0.224	-0.194	0.015	-0.197	-0.2	-0.293*	-0.058
PPARG P3	-0.078	0.138	0.174	0.03	-0.139	0.021	-0.218	0.037	-0.283*	0.112
RXRA P1	-0.102	-0.298**	-0.229*	-0.052	0.025	-0.095	0.127	-0.066	-0.225	-0.032
SREBF2 P2	0.056	0.006	0.144	0.112	0.136	-0.032	0.189	-0.224	-0.262*	0.121
LRP1 P2	0.09	-0.065	-0.048	0.114	-0.215	0.373*	-0.055	0.192	0.180	0.251
LPL P3	0.135	0.029	0.089	0.128	0.245*	-0.102	0.085	0.126	-0.111	0.149
SCD P3	-0.056	-0.340*	-0.283	-0.096	-0.018	0.108	0.22	0.087	-0.117	-0.03
SCD P6	-0.325*	-0.116	-0.17	-0.141	-0.134	0.121	0.102	-0.275	-0.232	-0.172
TNF P1	-0.212	0.132	0.046	-0.034	-0.188	0.283*	-0.02	-0.010	-0.115	0.029
TNF P2	-0.420**	0.054	-0.061	-0.192	-0.273*	0.304*	-0.195	-0.188	-0.217	-0.196
TNF P3	-0.320*	0.151	-0.021	-0.094	-0.155	0.222	-0.109	-0.237	-0.242	-0.03
TNF P4	-0.330*	-0.006	-0.096	-0.278*	-0.203	0.098	-0.295*	-0.245	-0.305*	-0.133
TNF P5	-0.281*	0.132	-0.100	-0.153	-0.281*	0.380**	-0.132	-0.097	-0.008	-0.074
LEP P1	0.088	0.081	-0.159	0.061	-0.071	0.015	0.229*	0.264*	0.230*	0.028

Abbreviations: Number of metabolic syndrome variables present in the subject of study (MetS V); Body mass index (BMI); Triglycerides (TG); High-density lipoprotein cholesterol (HDL-cho); Low-density lipoprotein cholesterol (LDL-cho); Systolic blood pressure (SBP); Diastolic blood pressure (DBP); Homeostatic model assessment of insulin resistance (HOMA-IR); Peroxisome proliferator-activated receptor alpha DNA methylation at position 2 (PPARA P2); Retinoid X receptor alpha methylation at position 1 (RXRA P1); Leptin DNA methylation at position 1 (LEP P1); Sterol regulatory element binding transcription factor DNA methylation at position 2 (SREBF2 P2); Stearoyl-CoA desaturase DNA methylation at positions 3 and 6 (SCD P3 and P6); Tumor necrosis factor DNA methylation at positions P1 to P5 (TNF P1-P5); Peroxisome proliferator-activated receptor gamma DNA methylation at positions 1and 2 (PPARG P1 and P2); Lipoprotein lipase DNA methylation at position 3 (LPL P3); Low density lipoprotein receptor-related protein 1 DNA methylation at position 2 (LRP1 P2). * and ** means $p<0.05$ and $p<0.01$ respectively

Regression analyses

To study the strength of the association observed in the correlation analyses we performed lineal regression analyses corrected by age, gender and BMI. We observed that the DNA methylation levels of PPARA P2 and LPL P3 could explain TG levels (**Table 5**).

Table 5. Lineal regression analysis with fasting triglycerides as dependent variable and PPARA P2, LPL P3 and TNF P2 as independent variables and corrected by age, gender and BMI.

	Fasting triglycerides (R=0.566; R^2=0.320)		
	β	**P**	**CI 95 %**
Age	0.111	0.425	-0.582-1.358
Gender	-0.268	0.047	-48.377-(-.312)

BMI	-0.101	0.446	-1.825-0.816
PPARA P2	0.332	0.012	1.32-10.012
LPL P3	0.264	0.046	0.099-10.72
TNF P2	-0.117	0.347	-1.867-0.669

Body mass index (BMI); Peroxisome proliferator-activated receptor alpha DNA methylation at position 2 (PPARA P2); Lipoprotein lipase DNA methylation at position 3 (LPL P3); Tumor necrosis factor DNA methylation at position 2 (TNF P2).

Furthermore, we performed a logistic regression analyses (harmonized by step method) to determine what factors could predict the risk of having MetS. We observed that TNF P2 remained as a protective variable; with a reduction of 23% of probability of being MetS per unit of DNA methylation increased (**Table 6**).

Table 6. Logistic regression analysis: risk of MetS. Variables that showed a significant association with MetS V at the correlation analyses such as age, gender, PPARA P2, SCD P6, TNF P2 and P5 were introduced as independent variables. A harmonized model in which gender, PPARA P2 and TNF P2 was maintained was generated.

	Non Mets/MetS (R^2=0.506-0.686)		
	β	**P**	**CI 95%**
Gender	5.813	0.094	0.739-45.699
PPARA P2	1.630	0.246	0.714-3.719
TNF P2	0.791	0.008	0.664-0.942

Non metabolic syndrome group (Non MetS); Metabolic syndrome group (MetS); Peroxisome proliferator-activated receptor alpha DNA methylation at position 2 (PPARA P2); Tumor necrosis factor DNA methylation at position 2 (TNF P2).

MANUSCRIPT 3. Complement Factor C3 Methylation and mRNA Expression Is Associated to BMI and Insulin Resistance in Obesity

Castellano-Castillo D. et al. Genes. 2018; 9(8).pii: E410.

The results detailed in this section answer the question proposed in the objective 2. In this study we analyzed C3 DNA methylation in adipose tissue from subjects with a different grade of obesity. Adipose tissue samples were collected from subjects with a different degree of obesity determined by their BMI as: overweight subjects (BMI⩾25 and <30), obese Class 1/2 subjects (BMI⩾30 and <40) and obese Class 3 subjects (BMI⩾40). C3 DNA methylation was measured for 7 CpGs by pyrosequencition using the Pyromark technology (Qiagen). C3 mRNA levels were analyzed by pre-designed Taqman assays (Applied biosystems) and ASP/C3a was measured using an ELISA kit.

Biochemical and anthropometric characteristics

Biochemical and anthropometric characteristics of each study group are summarized in **Table** 1. Briefly, there were statistics differences in BMI and waist among the three groups. Age was lower in Class 3 group respect to Overweight and Class 1/2 groups while HOMA-IR, cholesterol and leptin were higher.

Table 1. Biochemical and anthropometric variables for each study group.

	Overweight (n=23)	Class 1/2 (n=20)	Class 3 (n=17)
Age (years)	55.70±11.71 **a**	56.70±15.24 **a**	41.53±9.78 **b**
Gender (men/women)	10/13	7/13	6/11
BMI (kg/m^2)	27.41±1.29 **a**	33.23±2.76 **b**	49.78±6.49 **c**
Waist (cm)	93.96±5.48 **a**	106.75±9.23 **b**	133.69±17.77 **c**
Glucose (mmol/L)	6.12±1.20	5.99±1.47	5.79±1.15
HOMA-IR	2.318±0.98 **a**	3.124±1.59 **a**	5.30±4.91 **b**
Tg (mmol/L)	1.42±0.60	1.47±0.54	1.37±0.50
Cholesterol (mmol/L)	5.58±1.00**a**	5.48±1.28**a**	4.67±0.94**b**
HDL-cho (mmol/L)	1.37±0.34**a,b**	1.49±0.32**a**	1.26±0.32**b**
SBP (mmHg)	131.43±22.37	135.65±24.83	136.07±19.39
DBP (mmHg)	79.52±12.62	79.75±12.78	83.79±10.03
ApoA1 (mmol/L)	1.73±0.20	1.81±0.27	1.58±0.15
ApB (mmol/L)	1.10±0.27	1.01±0.21	0.89±0.28
GOT (µkat/L)	0.25±0.12**a**	0.34±0.18**a,b**	0.39±0.16**b**
GPT (µkat/L)	0.59±0.24	0.75±0.37	0.75±0.32
GGT (µkat/L)	0.63±0.50	0.61±0.29	0.55±0.38
Leptin (ng/ml)	14.31±7.13 **a**	21.83±11.12 **a**	68.21±30.09 **b**
Adiponectin (ng/ml)	9.21±3.92	10.49±4.64	6.90±3.83

Abbreviations: Body mass index (BMI); Homeostatic model assessment of insulin resistance (HOMA-IR); Triglycerides (Tg); High density lipoprotein cholesterol (HDL-cho); Systolic blood pressure (SBP); Diastolic blood pressure (DBP); Apolipoprotein A1 (ApoA1); Apolipoprotein B (ApoB); Glutamil oxaloacetate transaminase (GOT); glutamate pyruvic transaminase (GPT); Gamma-Glutamyl Transferase (GGT). Different letters mean significant differences between groups ($p<0.05$).

C3 methylation, C3 mRNA and serum ASP levels

C3 DNA methylation levels were lower in Class 3 patients compared to the other study groups (**Figure 1A**). However, this result was not translated into *C3* mRNA expression (**Figure 1B**). No differences were found for serum ASP values among the study groups either (**Figure 1C**).

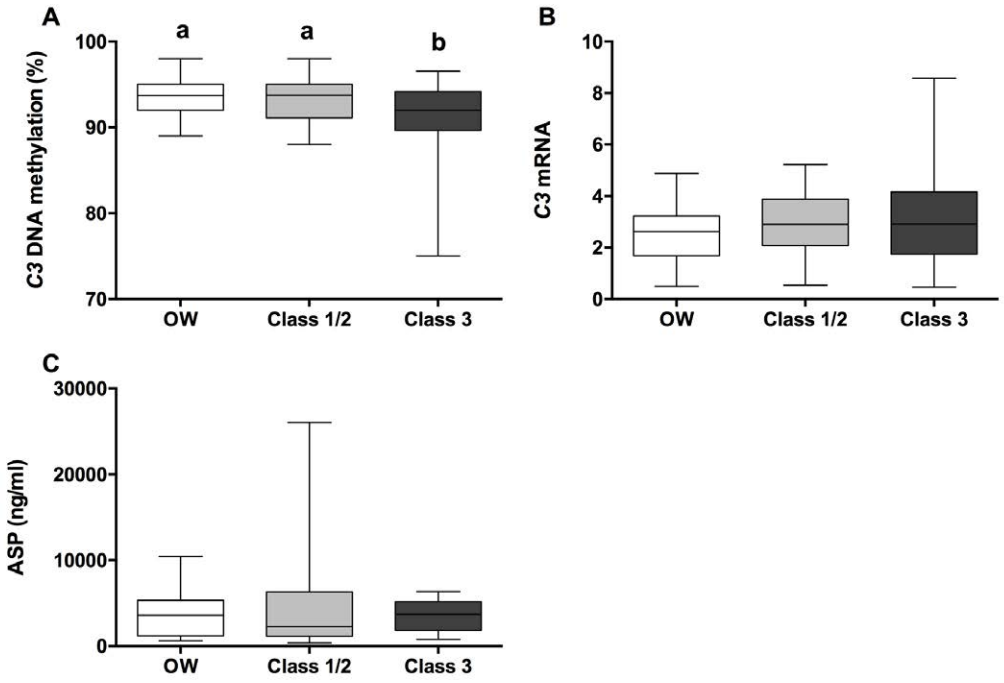

Figure 1. Figure shows the adipose tissue *C3* DNA methylation levels (A), adipose tissue *C3* mRNA levels (B) and serum levels of ASP (C) among the study groups: OW (overweight subjects; BMI=25-29.9 Kg/m^2), Class 1/2 (Class 1/2 obese subjects; BMI=30-39.9 Kg/m^2) and Class 3 (Class 3 obese subjects; BMI$\geqslant$40Kg/m^2). Values are presented as the means ± SD. ANOVA and post hoc analysis using Duncan and Tukey test was used to test differences among the groups. Different letters mean significant differences among the groups when $p<0.05$. Abbreviations: Acylation stimulating protein (ASP).

C3 methylation, C3 mRNA and ASP associations with biochemical and anthropometric variables

Biochemical and anthropometric characteristics were related with *C3* DNA methylation, *C3* mRNA expression and serum ASP (**Table 2**). Interestingly, while *C3* methylation correlated negatively with variables associated to adiposity, like BMI and serum leptin, *C3* mRNA expression showed a positive correlation with glucose, insulin and HOMA-IR and a negative association with serum adiponectin levels. When correction for age was made, the correlation observed between BMI and *C3* DNA methylation was still maintained (r=-0.353, p=0.006) while the association present between HOMA-IR and *C3* mRNA levels showed not statistical significance (r=0.252, p=0.052). In addition, we have observed a positive correlation between *C3* mRNA expression in VAT and the ASP levels (r=0.7, r=0.034) only in the Class 3 group. Finally, we observed a negative association between ASP serum levels and HDL cholesterol (**Table 2**).

Table 2. Correlations between *C3* DNA methylation, *C3* mRNA and serum ASP with anthropometric and biochemical variables.

	***C3* mRNA**	***C3* Methylation**	**ASP**
Age	0.076	0.226	0.035
BMI	0.177	-0.411**	-0.04
Waist	0.192	-0.26	0.062
Insulin	0.364**	-0.079	0.157
Glucose	0.324*	0.09	-0.102
HOMA-IR	0.417**	-0.079	0.124
HDL-cho	0.073	-0.176	-0.370**
Leptin	0.289	-0.528**	-0.077
Adiponectin	-0.316*	0.005	-0.071

Abbreviations: Acylation stimulating protein (ASP); Body mass index (BMI); Homeostatic model assessment of insulin resistance (HOMA-IR); High density lipoprotein cholesterol (HDL-cho). * and ** Indicates differences between the groups ($p<0.05$ and $p<0.01$ respectively).

These relationships, *C3* methylation with BMI and *C3* mRNA with insulin resistance, were reinforced by linear regression analyses. Thus, in a model with BMI as dependent variable and corrected with variables strongly related to obesity as age or HOMA-IR, *C3* DNA methylation reached a strong significance in the model, which was able to explain up to 48% of the BMI variability (**Table 3A**). Furthermore, when HOMA-IR was considered as the dependent variable in a multiple lineal regression analysis, *C3* mRNA levels and BMI could explain up to 35% of the variability present in HOMA-IR (**Table 3B**).

Table 3. Multiple regression analysis. Model A with BMI as dependent variable and model B with HOMA-IR as dependent variable. Model A was age-, gender-, HOMA-IR-, *C3* mRNA- and *C3* methylation-adjusted. Model B was age-, gender-, BMI-, *C3* mRNA- and *C3* methylation-adjusted.

A	**BMI (R=0.69, R^2=0.48)**		
	β	**p**	**CI (95%)**
Age	-0.28	0.00	-0.43-(-0.13)
Gender	0.48	0.81	-3.76-4.73
HOMA-IR	1.12	0.00	0.38-1.87
***C3* mRNA**	0.34	0.65	-1.17-1.86
***C3* methylation**	-0.79	0.00	-1.35-(-0.23)

B	HOMA-IR (R=0.59, R^2=0.35)		
	β	p	CI (95%)
Age	-0.00	0.96	-0.06-0.05
Gender	-0.71	0.32	-2.14-0.72
BMI	0.13	0.00	0.04-0.21
***C3* mRNA**	0.74	0.00	0.26-1.22
***C3* methylation**	0.07	0.44	-0.12-0.28

Abbreviations: HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; CI: Confidence Interval.

MANUSCRITP 4. Chromatin Immunoprecipitation Improvements for the Processing of Small Frozen Pieces of Adipose Tissue

Castellano-Castillo D. et al. PloS One. 2018;13(2):e0192314.

In this section we aimed to accomplish the objective 4. In this objective, we optimized the standard protocol of chromatin immunoprecipitation (ChIP) for small pieces of frozen human adipose tissue. In addition, we test ChIP for the histone mark H3K4m3, which is related to active promoters, and validate the performance of the ChIP by analyzing gene promoters for factors usually studied in adipose tissue using qPCR.

We have introduced crucial changes to the standard ChIP protocol, improving the homogenization, fixation and de-crosslinking steps, allowing enough immunoprecipited material to be obtained to perform further steps, as we demonstrated by testing H3K4me3 modifications. Thus, we have shown that the use of only 100 mg of frozen AT is enough for ChIP tests, which will help to advance knowledge about epigenetic marks of AT and their significance for metabolic homeostasis.

The high lipid content of the AT makes the fixation and subsequent steps difficult to work with. Adipocytes float in the upper layer due to their lipid content (**Figure 1.1**), which leads to a high loss of tissue in the processing. Thus, standard homogenization methods (mortar and pestle) were not able to extract a proper quantity of DNA, showing a very low performance. In this method, a high quantity of tissue remained stuck to the surfaces of the pestle and mortar that resulted in a high tissue loss, a very low nuclei recovery and no chromatin harvest. We therefore performed two other alternative methods where the fixation and washing of the tissue were prior to the homogenization step. This allowed better tissue manipulation, indeed avoiding loss of tissue. We compared the dounce homogenizer (**Figure 1.2**) with the ultraturrax homogenizer (**Figure 1.3**).

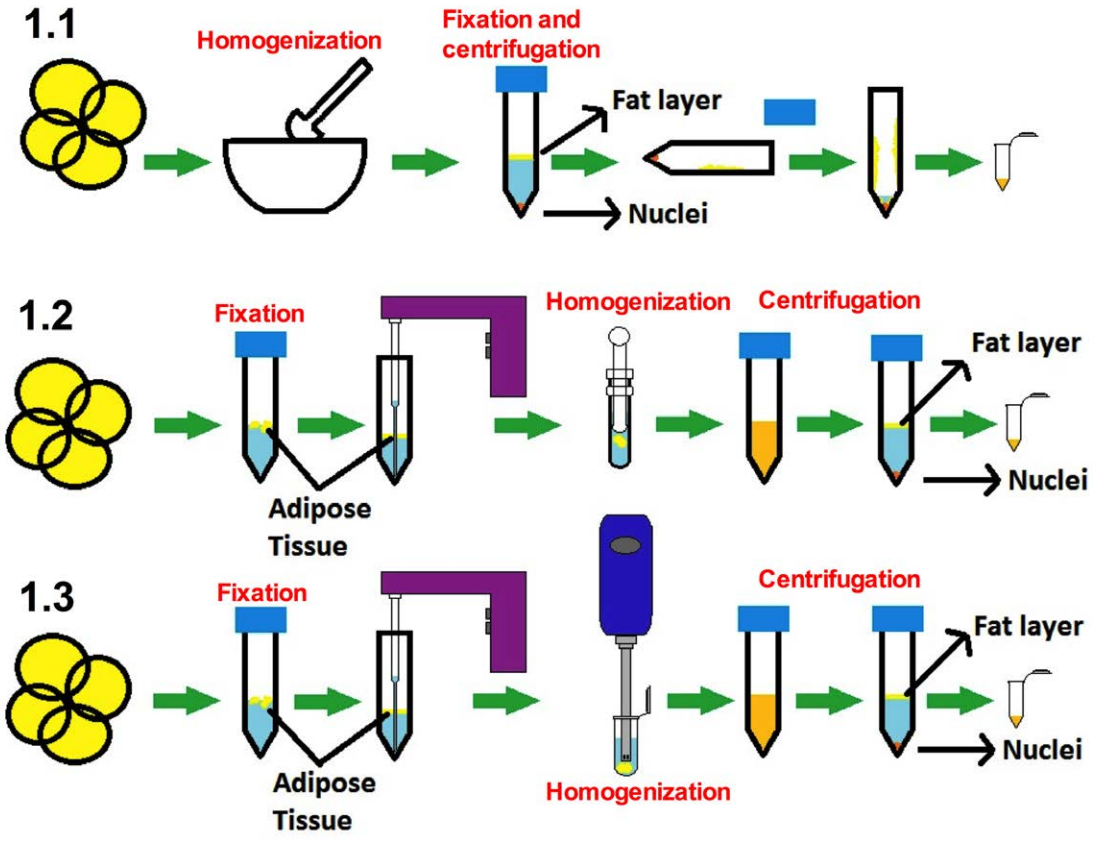

Figure 1. Figure shows the three different workflows performed, using the pestle and mortar **(1.1)**, the Dounce homogenizer **(1.2)** or the Ultraturrax homogenizer **(1.3)**. In **(1.1)**, the homogenization step was performed using liquid nitrogen, after which it was fixed. After this, the nuclei were pelleted and nucleus lysis buffer was added. Once incubated, the sample was sheared and the chromatin fragmentation and recovery were checked. In the other two alternative methods proposed (**1.2** and **1.3**), the tissue was cut in small pieces (3 mm) and the fixation step was performed prior to the homogenization. Once homogenized, the nuclei were recovered by centrifugation, nucleus lysis buffer was added and the sample was sheared and the chromatin fragmentation and recovery were checked.

The use of the ultraturrax homogenizer results in a higher total DNA recovery after the purification step (**Figure 2**).

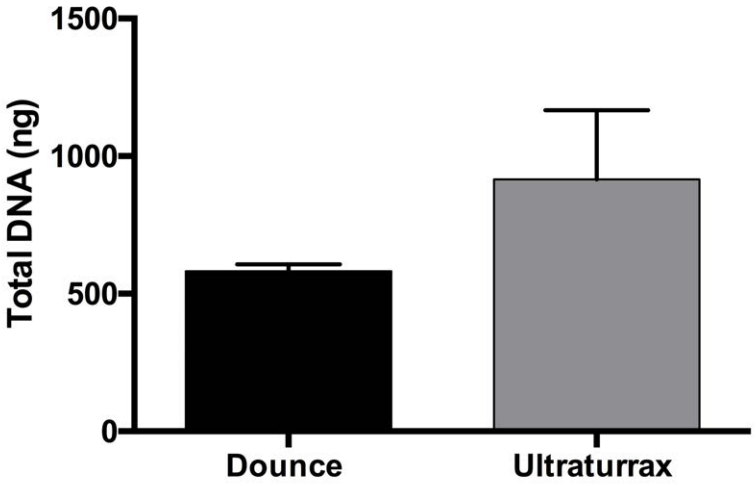

Figure 2. The figure shows the different efficiency in total DNA recovery between the Dounce and the Ultraturrax homogenizer. 100 mg of frozen adipose tissue was fixed in 1% paraformaldehyde, homogenized either, using the Dounce or Ultraturrax homogenizer and sheared for 40 cycles (30 seconds ON and 30 seconds OFF). A sample of 50 µl of the homogenized material was then taken, and the chromatin was de-crosslinked using the fast Chelex-100 method. Total DNA was extracted and quantified by nanodrop. Data are given as means with error bars. Abbreviations: Dounce, Dounce homogenization method; Ultraturrax, ultraturrax homogenization method. (n=6).

Once the optimal homogenization method was established, a proper fixation method for AT was found. Concentration of formaldehyde to crosslinking DNA/protein is an important step, which can affect the shearing of the DNA and, consequently, the performance of the immunoprecipitation (IP) and the recovery of DNA. For this reason, we tried two different formaldehyde concentrations in order to improve DNA recovery: the classical concentration of 1% formaldehyde was compared to a lower concentration of 0.5% formaldehyde. In addition, we also tried different incubation times (10, 8, 5 minutes) and temperatures (RT or 37ºC) to fix the sample, although no good results

were obtained for incubation times longer than 5 minutes and temperatures higher than RT (data not shown). 5 ml of each fixation solution were used to carry out the fixation step for 5 minutes at RT and shaking. Furthermore, the sonication step is highly variable depending on the sonicator and there is even moderate variation between different devices for the same technology. Indeed, it is recommendable to set up the proper shearing method not only for each kind of tissue but also for each device. At the same time, we also determined the sonication time to properly shear the DNA using a Bioruptor sonicator after tissue fixation. We tested 20, 30 and 40 cycles of 30 seconds ON / 30 seconds OFF at high power. We obtained better results using a low concentration of 0.5% formaldehyde together with a number of 40 cycles for DNA recovery (**Figure 3A** and **Figure 3B**). However, 1% formaldehyde hindered shearing of the chromatin, independently of the number of sonication cycles.

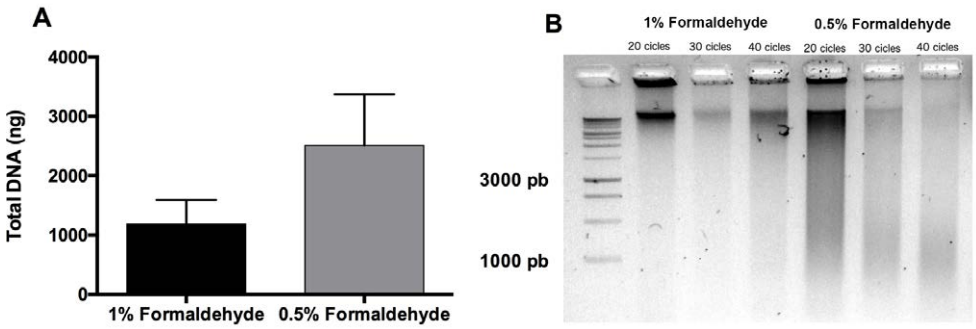

Figure 3. 100 mg of frozen adipose tissue was fixed in either 1% or 0.5% of paraformaldehyde and then homogenized using the Ultraturrax method. It was then sheared for 40 cycles (30 seconds ON and 30 seconds OFF), after which a sample of 50 µl of the homogenized material was taken, and the chromatin was de-crosslinked using the fast Chelex-100 method. Total DNA was extracted and quantified by nanodrop. Fixation at 0.5% presents higher levels of DNA recovery (A) after DNA purification and a better chromatin shear tested by electrophoresis in 2% agarose gel. (B) Comparison of the use of PBS+1% or PBS+0.5% formaldehyde in the

performance of DNA recovery after de-crosslinking and purifying the DNA. (n=6). Data are given as means with error bars.

Finally, due to the small pieces and nature of AT itself, added to the fact that it was frozen, the de-crosslinking and DNA recovery steps may be determinant for the success of the IP and downstream procedures. Two methods for chromatin de-crosslinking were tested: the standard method, which consists of incubating the chromatin at 65ºC for 5 hours followed by proteinase K (PK) treatment at 55º for 1 hour; and a faster method in which chromatin is heated to 100ºC during a shorter period of time of 10 minutes with 10% chelex-100 to protect the DNA. Once the DNA was purified, the data revealed a higher performance for the standard method, in which the chromatin is submitted to a moderate temperature for a long time (Fig 4).

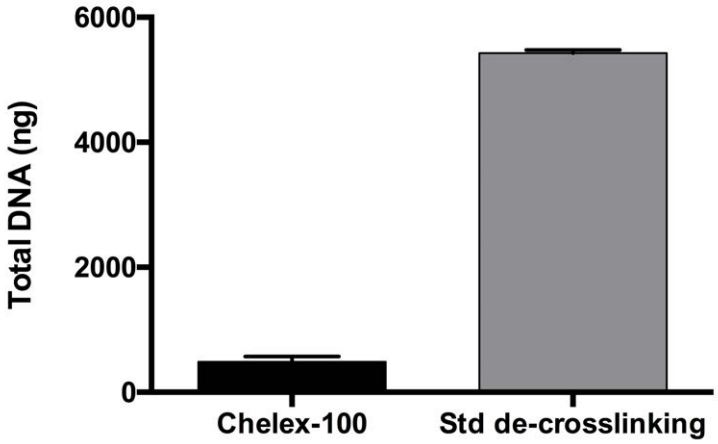

Figure 4. 100 mg of frozen adipose tissue was fixed in 0.5% paraformaldehyde and then homogenized using the Ultraturrax method. It was then sheared for 40 cycles (30 seconds ON and 30 seconds OFF), after which a sample of 50 µl of the homogenized material was taken, and the chromatin was de-crosslinked using either the fast Chelex-100 method or a moderate

temperature for 5 hours plus PK treatment. Figure shows the de-crosslinking step at a moderate temperature for 5 hours and then a PK step improves the quantity of DNA with respect to the method based on the use of Chelex-100. (n=6). Total DNA was extracted and quantified by nanodrop. Data are given as means with error bars.

Therefore, after testing different steps during the regular ChIP protocol, several changes have been introduced in order to match the method to small frozen AT samples. The high lipid content in AT hinders tissue manipulation, DNA extraction and even nucleus release and nucleus breakdown. This has led to the development of specialized extraction kits for AT, for example for RNA extraction. Thus, based on our data in AT manipulation (data not shown), we decided to increase the proportion of buffer with respect to the sample quantity compared to regular procedures for the following steps: fixation, washes after fixation, cell lysis and release of nuclei. This allowed us to deal with the high lipid content, avoiding a very thick cell lysate, which could hinder nucleus release. Furthermore, this allowed recovery of a cleaner nucleus pellet, improving the sonication and chromatin release. In these steps, we recommend the use of glass pipettes to remove the liquid discarded in each step since the high lipìd content of AT can become stuck to plastic surfaces, hindering manipulation and leading to tissue loss. Moreover, we determined use of the ultraturrax homogenizer as the best method for homogenization, a fixing solution of PBS+0.5% formaldehyde at RT and the standard de-crosslinking method as the most suitable procedures for small pieces of frozen AT. Up to now, the use of ChIP for AT has been limited to big amounts of tissue, and especially to mouse AT where the conditions are less limiting. Thus, the improvements shown in this work could help researchers study the proteome-DNA interaction in human AT, which is stored frozen in large tissue banks.

Once the best procedure was established, we applied the method to 100 mg of frozen samples of human AT. The yield of the method after the IP resulted in an average of almost 100 ng of DNA, enough to perform a posterior high throughput sequencing thanks to the high resolution of the latest next generation sequencing methods. On the other hand, in order to improve the performance and DNA recovery, we encourage others to perform ChIP experiments in small rounds of samples

Although we have provided an improved method to work with small frozen pieces of AT, we needed to confirm the correct assessment of the IP. We validated our ChIP protocol in mouse and human AT by testing H3K4me3 modifications, a mark of active promoter regions. By qPCR we identified H3K4me3 enrichment on several promoters of genes usually expressed in AT, such as PPARG, SCD, LPL, LEP, SREBF2, as well as a sequence 30 kb before PPARG TSS (Transcription Start Site) in mice (PPARG Out) and a sequence 35 kb before SCD TSS (SCD Out) for humans, both as control regions. We obtained a high percentage of enrichment in both mice and humans (**Figure 5A** and **Figure 5B** respectively) for genes usually expressed in AT, like SCD, PPARG, LPL or SREBF2, while control regions presented residual expressions. The presence of H3K4m3 at these promoters has already been demonstrated in several tissues and cell lines (ENCODE project), but to the best of our knowledge, no results are available in white AT. Nevertheless, these gene expressions are usually assessed in AT, which could agree with the high percentage of DNA immunoprecipitation observed in these promoter genes for H3K4m3, a histone associated with active genes.

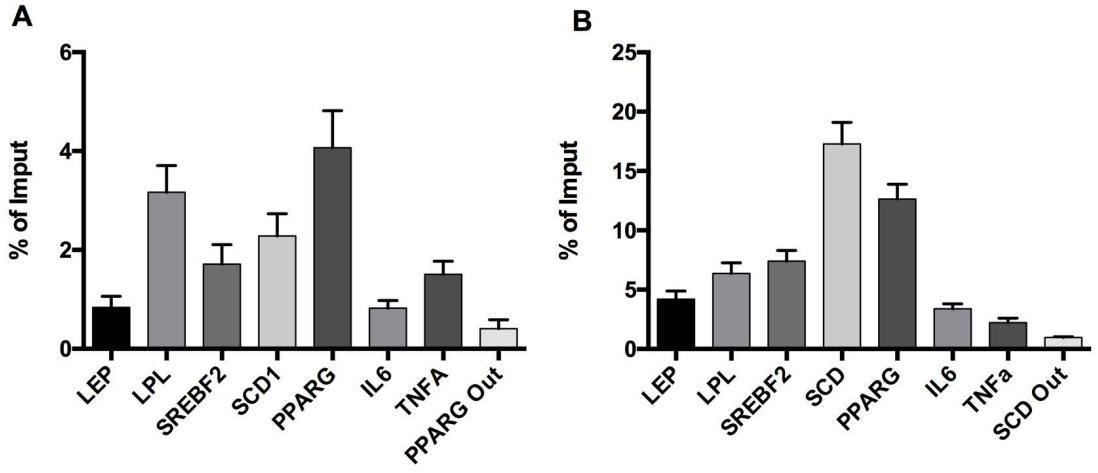

Figure 5. Figure shows the amplification of promoter zones for genes usually expressed in adipose tissue in mice (n=10) (A) and humans (n=39) (B), showing that DNA enrichment was successful since a higher quantification was found at the promoter of these active genes but not in the control sequence zones. Data are given as means with error bars. Abbreviations: LPL, Lipoprotein lipase; LEP, Leptin; SREBF2, Sterol regulatory element binding transcription factor 2; SCD, Stearoyl-CoA desaturase; SCD1, Stearoyl-CoA desaturase 1; PPARG, Peroxisome proliferator activated receptor gamma; IL6, Interleukin 6; TNF, tumor necrosis factor; PPARG Out, Sequence 30 kb before PPARG TSS; SCD Out, Sequence 35 kb before SCD TSS.

We have shown a novel, improved and reproducible ChIP method for small pieces of frozen AT with several critical steps. It is recommended to fix the whole tissue before mincing it in 5 ml of PBS + 0.5% formaldehyde, after which we recommend several washing and cell lysis steps with abundant cold PBS+PIC. Furthermore, the use of the ultraturrax homogenizer improves nucleus pellet recovery. Afterwards, optimization of the chromatin shear is a key step to success in the following IP. Finally, we recommend de-crosslinking the chromatin under a moderate temperature for 5 hours, adding PK in order to degrade the protein fraction and improve the DNA recovery.

MANUSCRIPT 5. Human Adipose Tissue H3K4me3 in Adipogenic, Lipid and Inflammatory genes are Positively Associated to BMI and HOMA-IR

Castellano-Castillo D. et al. PloS One. (Under review)

In this section we performed the objective 5. Thus, we studied, for the first time, H3K4 trimethylation (H3K4me3) (open chromatin) on the promoter of several adipogenic, lipid metabolism and inflammatory factors in visceral adipose tissue (VAT) from subjects with different degrees of BMI and metabolic disease. Frozen VAT samples (-80ºC) were fixed in formaldehyde, homogenized and the chromatin sheared. Chromatin inmunoprecipitation was performed with an antibody anti-H3K4me3 and promoter enrichment was analyzed by qPCR. mRNA extraction on the same samples was performed to quantify gene expression of these genes.

Antropometrical and biochemical variables

The clinical and anthropometric characteristics of the study groups are presented in **Table 1**. Statistical differences between groups were observed for BMI, waist, serum levels of glucose, insulin and HOMA-IR as expected, as well as in the HDL-C levels.

Table 1. Anthropometric and biochemical variables for each study groups. Different letters mean significant differences between groups ($p<0.05$).

	Lean NG (n=10)	**MO NG (n=10)**	**MO PD (n=9)**
Age (years)	54.40±13.93	40.50±8.34	47.11±8.28
Gender (male/female)	4/6	3/7	3/6
BMI (kg/m^2)	22.91±1.52**a**	50.70±8.78**b**	56.12±7.99**b**
Waist (cm)	86.70±8.74**a**	134.70±20.27**b**	142.74±14.41**b**
Glucose (mg/dl)	91.10±6.04**a**	90.90±4.95**a**	111.89±4.37**b**
Insulin (pmol/L)	6.02±3.13**a**	20.26±15.61**b**	22.29±9.10**b**
HOMA-IR	1.34±0.69**a**	4.61±3.53**b**	6.14±2.48**b**
Cholesterol (mg/dl)	226.30±56.60	183.10±48.66	190.44±25.48
HDL-C (mg/dl)	56.20±16.81**a**	41.20±9.37**a,b**	39.67±5.54**b,c**
LDL-C (mg/dl)	144.46±45.19	120.84±45.76	120.16±22.17
Tg (mg/dl)	128.40±69.38	93.99±31.09	159.53±60.84
SBP (mm Hg)	129.90±25.00	135.44±30.73	141.29±15.15
DBP (mm Hg)	80.80±9.36	80.78±13.04	86.86±9.22

Abbreviations: Body mass index (BMI); Homeostatic model assessment of insulin resistance (HOMA-IR); High-density lipoprotein cholesterol (HDL-C); Low-density lipoprotein cholesterol (LDL-C); Triglycerides (Tg); Postprandial triglycerides (Tg Post); Systolic blood pressure (SBP); Diastolic blood pressure (DBP).

H3K4me3 mark levels

Figure 1 shows the levels of the H3K4me3 mark. With respect to the Lean NG group, we observed an enrichment of the H3K4me3 mark at E2F1, LPL, SREBF2, SCD1, PPARG and IL6 promoters in the MO PD group.

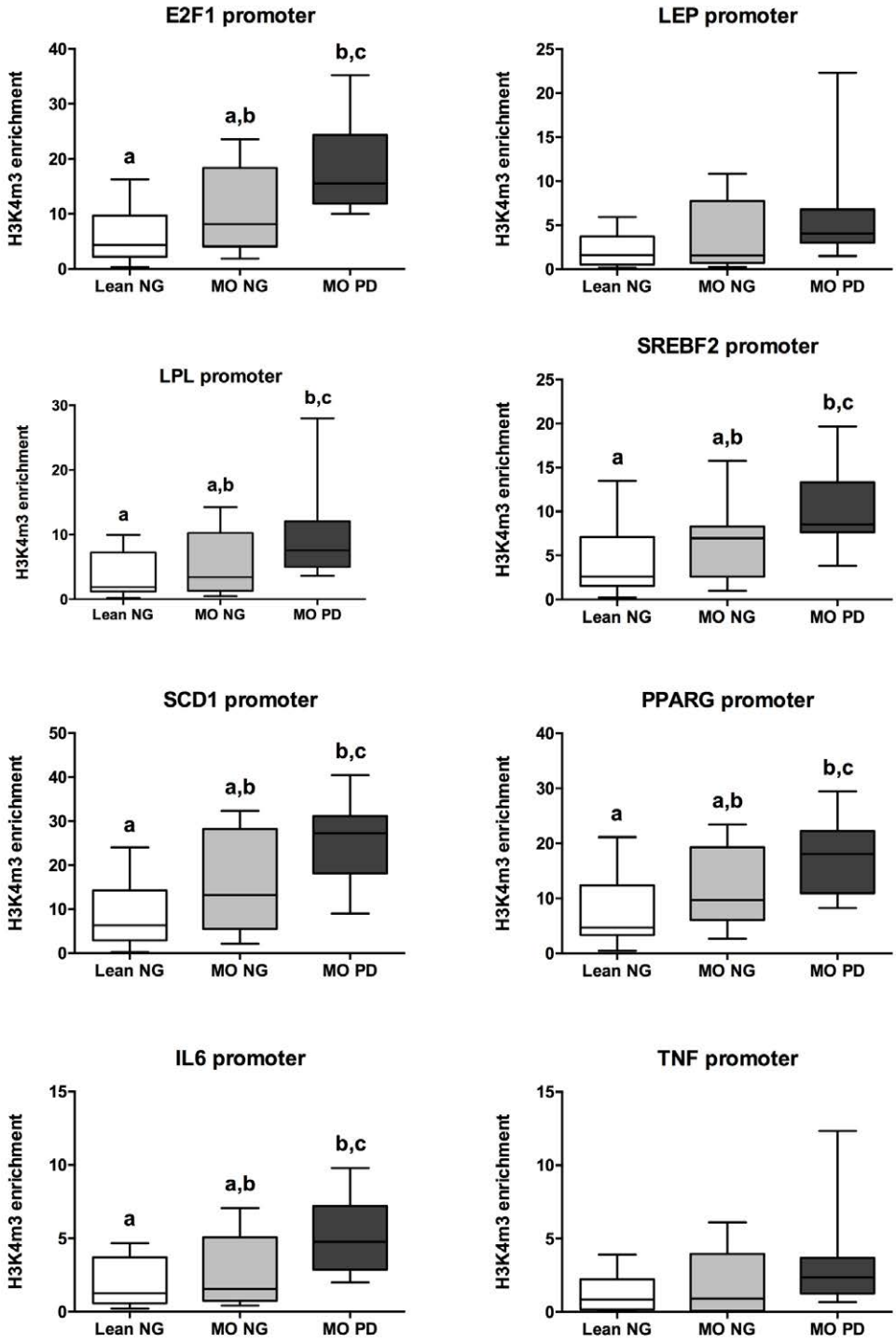

Figure 1. Group comparisons of H3K4me3 mark enrichment at the promoter of the study genes. Different letters mean significant differences at $p<0.05$ according to Kruskall-Wallis and Mann-Withney U-Test. Abbreviations: Lean Normoglycemic (Lean NG); Morbid obese normoglycemic (MO NG); Morbid obese prediabetic (MO PD); E2F transcription factor 1

(E2F1); Lipoprotein Lipase (LPL); Sterol regulatory element-binding factor 2 (SREBF2); Stearoyl-CoA desaturase 1 (SCD1); Peroxisome proliferator-activated receptor gamma (PPARG); Interleukin 6 (IL6); Tumor necrosis factor (TNF).

Accordingly, association analysis of the H3K4me3 mark in the studied genes with the measured clinical variables showed us a positive correlation for the H3K4me3 mark at E2F1, LEP, LPL, SREBF2, SCD1, PPARG, IL6 and TNF promoters with the BMI, HOMA-IR and insulin levels (**Table 2**). Moreover, there was a positive correlation between glucose and H3K4me3 mark enrichment at the promoter of SCD1, PPARG, E2F1 and IL6 (**Table 2**). We also observed a positive correlation between the H3K4me3 mark at E2F1, SREBF2 and SCD promoters with the number of metabolic syndrome (MetS) components (MetS Var) presents in the subject.

Table 2. Spearman correlation analysis between H3K4me3 mark enrichment at the study gene promoters and the anthropometric and biochemical variables. * and ** mean $p<0.05$ and $p<0.01$ respectively.

H3K4me3 enrichment	Age	BMI	Glucose	Insulin	HOMA-IR	Tg	Chol	HDL-C	LDL-C	SBP	DBP	MetS Var
E2F1	-0.214	0.530**	0.552**	0.573**	0.594**	-0.01	-0.256	-0.163	-0.257	0.123	0.072	0.448*
LEP	-0.148	0.364	0.361	0.348	0.367	0.044	-0.133	-0.12	-0.143	0.075	0.006	0.279
LPL	-0.168	0.430*	0.395*	0.437*	0.463*	-0.008	-0.208	-0.141	-0.198	0.154	0.064	0.333
SREBF2	-0.236	0.467*	0.442*	0.403*	0.441*	-0.048	-0.178	-0.232	-0.113	0.088	0.062	0.463*
SCD	-0.242	0.528**	0.513**	0.529**	0.548**	-0.034	-0.214	-0.186	-0.213	0.157	0.071	0.420*
PPARG	-0.243	0.488**	0.399*	0.460*	0.479**	-0.051	-0.263	-0.212	-0.246	0.073	0.038	0.369
IL6	-0.131	0.430*	0.497**	0.472*	0.501**	0.062	-0.094	-0.15	-0.065	0.191	0.047	0.327
TNF	0.009	0.23	0.379*	0.295	0.305	-0.051	-0.093	-0.008	-0.105	0.085	0.056	0.163

Abbreviations: E2F transcription factor 1 (E2F1); Leptin (LEP); Lipoprotein Lipase (LPL); Sterol regulatory element-binding factor 2 (SREBF2); Stearoyl-CoA desaturase 1 (SCD1); Peroxisome proliferator-activated receptor gamma (PPARG); Interleukin 6 (IL6); Tumor necrosis factor (TNF); Body mass index (BMI); Homeostatic model assessment of insulin resistance (HOMA-IR); Triglycerides (Tg); Total cholesterol (Chol); High-density lipoprotein cholesterol (HDL-C); Low-density lipoprotein cholesterol (LDL-C); Systolic blood pressure (SBP); Diastolic blood pressure (DBP); Number of MetS variables (MetS Var).

Gene expression levels

Levels of gene expression in the studied genes are despicted in **Figure 2**. With respect to the Lean NG group, LPL, SCD and PPARG mRNA levels were lower in the MO PD group, whilst higher mRNA levels were described for IL6 and TNF genes.

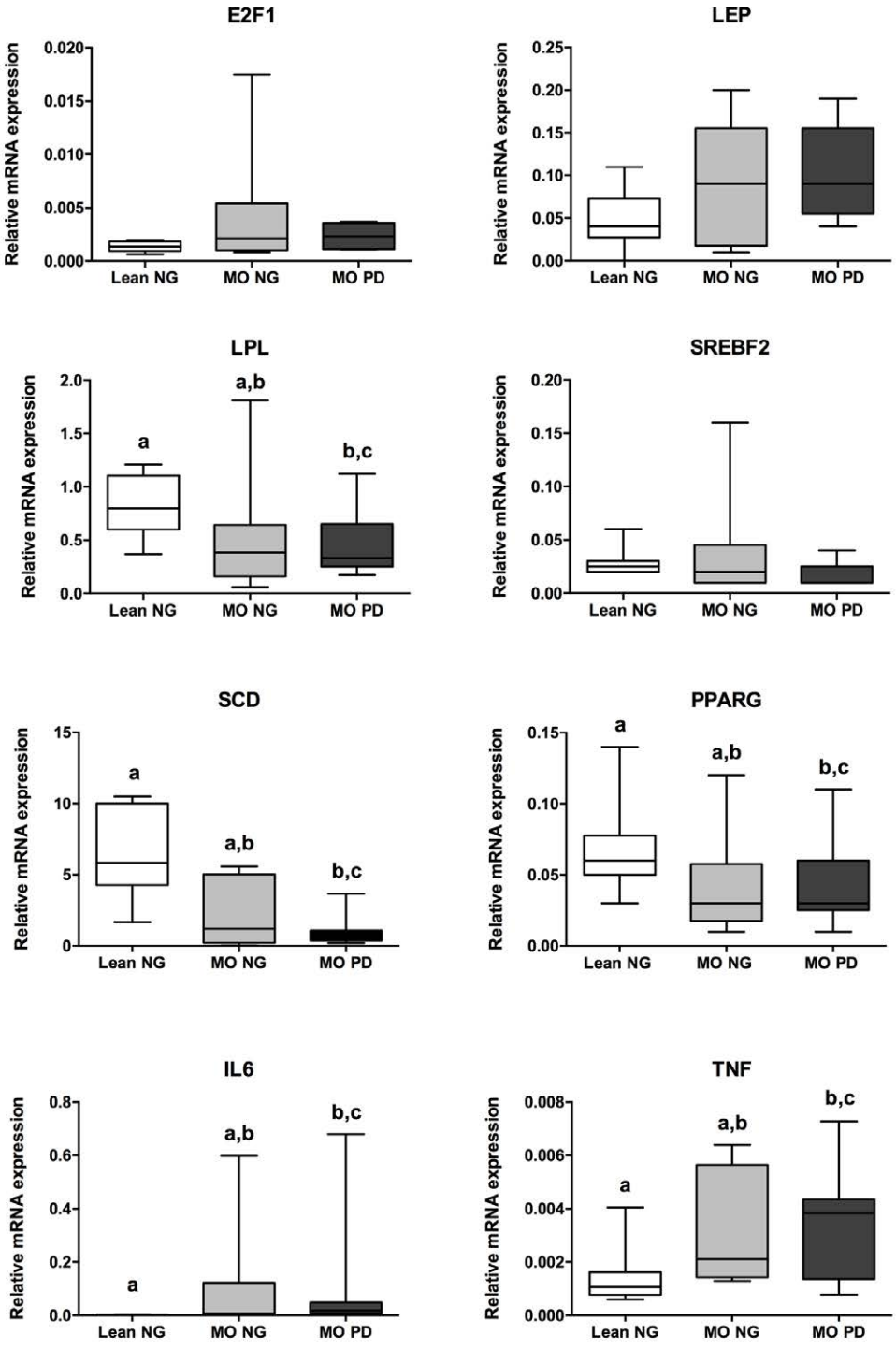

Figure 2. Group comparisons of the relative mRNA levels of the study genes. Different letters mean significant differences at $p<0.05$ according to Kruskall-Wallis and Mann-Withney U-Test. Abbreviations: Lean Normoglycemic (Lean NG); Morbid obese normoglycemic (MO NG); Morbid obese prediabetic (MO PD); E2F transcription factor 1 (E2F1); Lipoprotein Lipase (LPL); Sterol regulatory element-binding factor 2 (SREBF2); Stearoyl-CoA desaturase 1 (SCD1); Peroxisome proliferator-activated receptor gamma (PPARG); Interleukin 6 (IL6); Tumor necrosis factor (TNF).

Correlation analyses were in line with the expression results. Thus, LEP, IL6 and TNF mRNA levels were positively associated to BMI, while LPL, SCD and PPARG mRNA levels decreased in line with BMI (**Table 3**). On the other hand, HOMA-IR correlated with LEP, IL6 and TNF mRNA in a positive way, and negatively with SREBF2 and SCD (**Table 3**).

Regarding the rest of measured variables, positive associations were described between HDL-C and LPL, SCD and PPARG gene expressions. Interestingly, E2F1 mRNA levels were negatively associated with total cholesterol (Chol), HDL-C and LDL-C (**Table 3**).

Table 3. Spearman correlation analysis between the relative mRNA levels at the study genes and the anthropometric and biochemical variables. * and ** mean $p<0.05$ and $p<0.01$ respectively.

Relative mRNA	Age	BMI	Glucose	Insulin	HOMA-IR	Tg	Chol	HDL-C	LDL-C	SBP	DBP	MetS Var
E2F1	-0.256	0.359	0.116	0.194	0.19	-0.151	-0.559**	-0.407*	-0.534**	-0.144	0.097	0.284
LEP	-0.321	0.522**	0.334	0.673**	0.683**	0.179	-0.213	-0.308	-0.093	0.04	-0.095	0.533**
LPL	0.103	-0.500**	-0.295	-0.33	-0.327	-0.189	0.31	0.516**	0.203	0.008	-0.102	-0.454*
SREBF2	0.088	-0.258	-0.178	-0.391*	-0.409*	-0.238	-0.082	-0.024	-0.069	-0.209	-0.238	-0.373
SCD	0.375*	-0.681**	-0.319	-0.526**	-0.525**	-0.124	0.381*	0.555**	0.277	0.054	-0.165	-0.597**
PPARG	0.061	-0.408*	-0.214	-0.216	-0.204	-0.112	0.221	0.371*	0.182	0.097	0.161	-0.28
IL6	-0.291	0.729**	0.486*	0.571**	0.590**	0.036	-0.285	-0.223	-0.322	-0.025	0.144	0.533**
TNF	-0.298	0.679**	0.322	0.594**	0.612**	0.024	-0.263	-0.175	-0.335	0.048	-0.108	0.444*

E2F transcription factor 1 (E2F1); Leptin (LEP); Lipoprotein Lipase (LPL); Sterol regulatory element-binding factor 2 (SREBF2); Stearoyl-CoA desaturase 1 (SCD1); Peroxisome proliferator-activated receptor gamma (PPARG); Interleukin 6 (IL6); Tumor necrosis factor (TNF); Body mass index (BMI); Homeostatic model assessment of insulin resistance (HOMA-IR); Triglycerides (Tg); Total cholesterol (Chol); High-density lipoprotein cholesterol (HDL-C); Low-density lipoprotein cholesterol (LDL-C); Systolic blood pressure (SBP); Diastolic blood pressure (DBP); Number of MetS variables (MetS Var).

Relationship between H3K4me3 mark levels and gene expression levels

In order to analyze whether the promoter H3K4me3 levels could be related to gene expression we performed spearman's correlation analysis in the whole population between the mRNA levels and H3K4me3 enrichment at each gene. We did not observe any significant association between the promoter H3K4me3 levels and the mRNA levels for any gene except to E2F1, in which a positive correlation was observed (r=0.422, p=0.04).

Multivariate models

In addition, a harmonized lineal regression analyses showed that BMI was heavily explained by H3K4me3 enrichment levels at the promoter of E2F1 and LPL, and by the mRNA levels of LEP and SCD (**Table 4**). In this model, these four variables could explain up to 83% of the BMI variability present in our studied population.

Table 4. Harmonized lineal regression analysis with BMI as dependent variable, which was corrected by age and sex. H3K4me3 enrichment at gene promoters and gene expression of genes that showed significant association in the spearman correlation analysis were introduced in the model,.

	BMI (R=0.91, R^2=0.83)		
	Beta	**p**	**95% CI**
E2F1 H3K4me3	0.979	0.001	0.844 to 2.922
LPL H3K4me3	-0.813	0.003	-3.640 to -0.840
LEP mRNA	0.344	0.002	38.32 to 152.97
SCD mRNA	-0.516	0.000	-3.650 to -1.459

Abbreviations: Body mass index (BMI); E2F transcription factor 1 (E2F1); Lipoprotein Lipase (LPL); Leptin (LEP); Stearoyl-CoA desaturase 1 (SCD1).

On the other hand, when the studied variable is HOMA-IR, a harmonized lineal regression showed that the H3K4me3 enrichment at the promoter of SCD and IL6, together with the mRNA levels of LEP and SCD could explain a 79% of the variation observed in the HOMA-IR (**Table 5**).

Table 5. Harmonized lineal regression analysis with HOMA-IR as dependent variable, which was corrected by age and sex. H3K4me3 enrichment at gene promoters and gene expression of genes that showed significant association in the spearman correlation analysis were introduced in the model,.

	HOMA-IR (R=0.89, R^2=0.79)		
	Beta	**p**	**95% CI**
Gender	-0.259	0.047	-3.598 to -0.027
SCD H3K4me3	0.792	0.016	0.049 to 0.417
IL6 H3K4me3	-0.666	0.030	-1.769 to -0.105
LEP mRNA	0.564	0.000	16.60 to 44.82
SCD mRNA	-0.261	0.065	-0.541 to 0.018

Homeostatic model assessment of insulin resistance (HOMA-IR); Stearoyl-CoA desaturase 1 (SCD1); Interleukin 6 (IL6); Leptin (LEP).

MANUSCRIPT 6. Adipose Tissue Inflammation and VDR Expression and Methylation in Colorectal Cancer

Castellano-Castillo D. et al. Clinical Epigenetics. 2018;10:60.

The objective 6 was accomplished in this section. The aim of this study was to explore the relationship between serum 25-hydroxyvitamin D (25(OH)D), adipose tissue gene expression of VD receptor (VDR), pro-inflammatory markers and the epigenetic factor DNA methyltransferase 3a (DNMT3A) as well as VDR and NFκB1 promoters methylation in subjects with colorectal cancer (CRC) and witouth CRC (Control). Blood and visceral adipose tissue from 57 CRC and 50 healthy control subjects were collected. mRNA was measured by qPCR using Taqman technology while bisulfite treated DNA was pyrosequensed using the PyromarkQ96 technology in order to analyze DNA methylation. Protein levels were measured by Western-blot.

Anthropometric and biochemical variables

Table 1 shows the biochemical and anthropometric characteristics of the study groups. There were no differences in age, BMI or gender between the control and CRC groups. The CRC group had lower levels of insulin, total cholesterol, HDL-C and LDL-C than the control group. In contrast, the CRC group presented higher levels of plasma triglycerides when compared with the control group.

Table 1. Anthropometric and biochemical variables of the study groups

	Control (n=57)	**CRC (n=50)**
Age (years)	64.94±8.84	68.035±8.43
Male/Female (%)*	68/32	45/55
BMI (kg/m^2)	28.51±4.21	27.61±3.91
Waist (cm)	96.55±11.64	97±12.74
Glucose (mg/dl)	111.72±28.77	125.035±46.87

Insulin (µUI/ml)**	11.638±6.54	6.23±5.19
Triglycerides (mg/dl)*	142.3±70.69	172.821±87.54
Cho (mg/dl)**	220.68±39.84	169.625±43.57
HDL-C (mg/dl)**	53.28±14.4	40.053±15.12
LDL-C (mg/dl)**	136.61±29.80	101.58±35.67
Corrected calcium (mg/dl)**	8.99±0.44	9.67±0.65
Alkaline phosphatase (U/L)	72.67±21.63	64.66±22.81

Results are presented as means ± S.D *p<0.05 CRC vs. Control; **p<0.01 CRC vs. control according to t student's test and Chi squared test for variables expressed as percentage. Colorectal Cancer Group (CRC), Body Mass Index (BMI), Diabetes Mellitus (DM), Total Cholesterol (Cho), High Density Lipoprotein Cholesterol (HDL-C), Low density Lipoprotein Cholesterol (LDL-C).

Serum 25(OH)D levels and adipose tissue VDR gene and protein expression

Our results showed that 12% in the control group and 26% in the CRC group were vitamin D deficient (25(OH)D lower than 20 nmol/L) according to the Endocrine Society Clinical Practice Guideline, although no significant differences were found according to a Fisher's test. Serum 25(OH)D levels were significantly lower in the CRC group than in the control group (**Figure 1A**), while parathyroid hormone levels showed an inverse result (**Figure 1B**). Contrary to serum 25(OH)D levels, adipose tissue *VDR* mRNA levels were higher in the CRC group than in the control group (**Figure 1C**) which in turn correlated negatively with 25(OH)D (r=-0.268; p=0.008) (**Figure 1D**). This correlation was maintained using a partial correlation analysis corrected by gender (r=-0.273, p=0.01). Accordingly, mRNA levels were translated to higher VDR protein levels in the CRC group with regard to the control group (**Figure 1E** and **1F**).

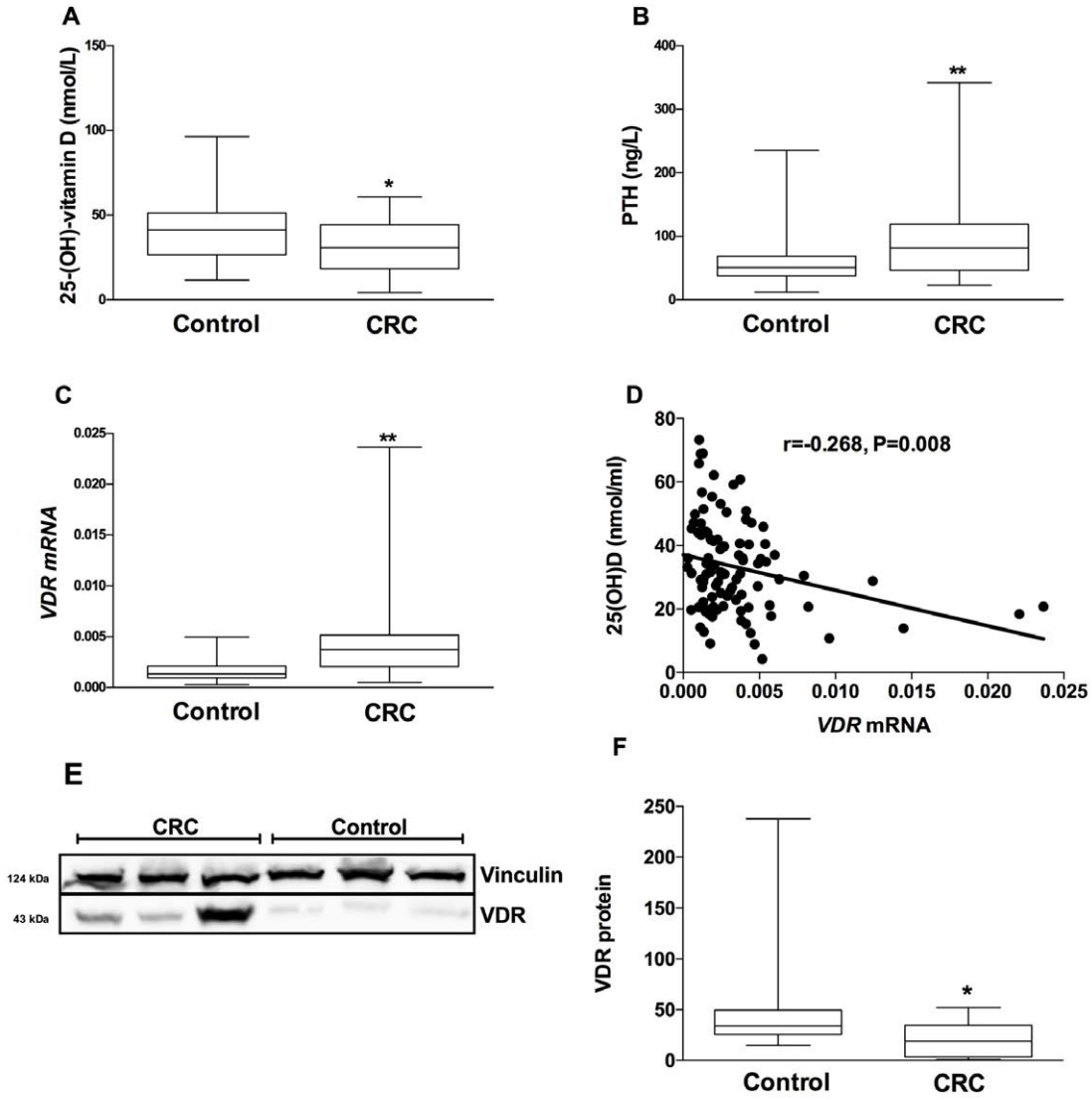

Figure 1. Comparison of serum 25(OH)D and PTH levels and adipose tissue VDR mRNA and protein in CRC patients and controls. Comparisons were performed using Student T-test (for 25(OH)D) and Mann-Whitney-U test (for serum PTH, and adipose tissue *VDR* mRNA and VRD protein). Serum levels of **(A)** 25(OH)D and **(B)** PTH was measured by ELISA in both, control and CRC group. **(C)** Adipose tissue VDR mRNA expression was measured by qPCR (n=107) and Spearman's correlation **(D)** between serum 25(OH)D and adipose tissue VDR mRNA in the whole study population was performed. Comparison for adipose tissue VDR protein (**E** and **F**) analyzed by western blot (n=18). * and ** mean $p<0.05$ and $p<0.01$ respectively. Parathyroid hormone (PTH); Vitamin D receptor (VDR); Colorectal Cancer (CRC).

Inflammatory status and relationship with circulating vitamin D

We checked the systemic inflammatory status by measuring serum CRP, as well as the inflammatory status of adipose tissue by determining mRNA levels of *NFκB1*, *IL6* and *IL1B* gene expression. We found higher levels of adipose tissue *NFκB1*, *IL6* and *IL1B* mRNA levels (**Figure2 B-D**) in the CRC group with regard to the control group.

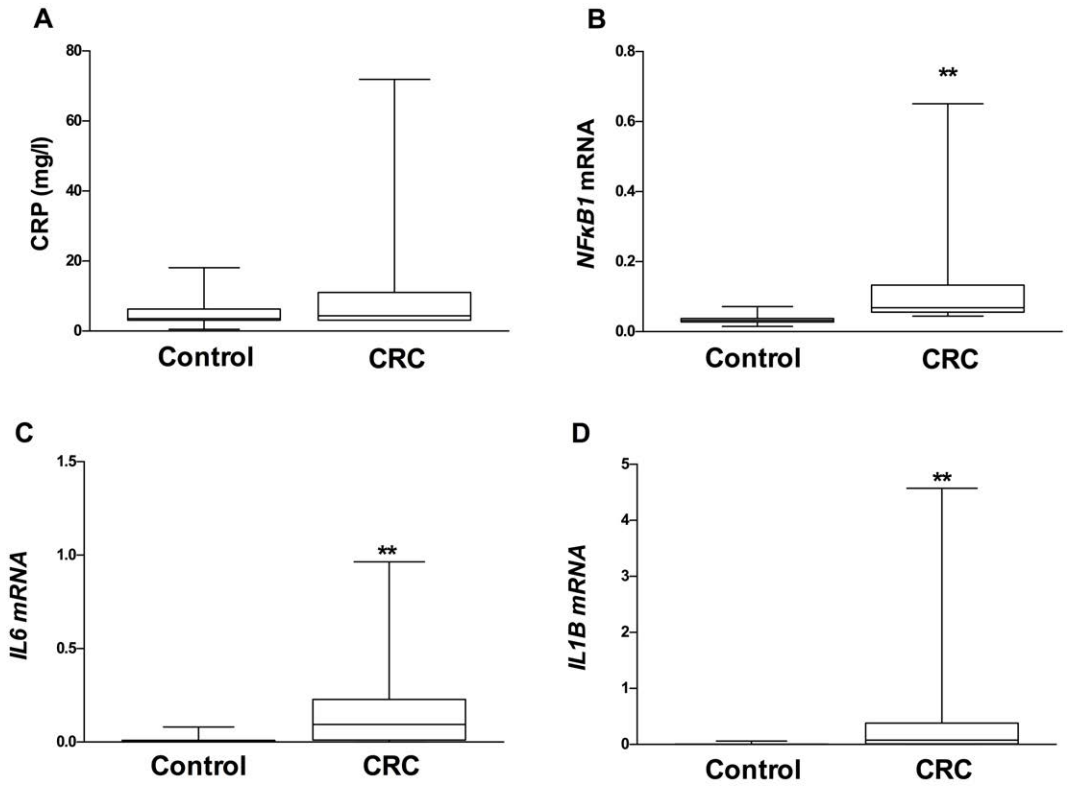

Figure 2. Serum and adipose tissue inflammatory markers. Comparisons were performed using non-parametric test (Mann-Whitney-U test). Serum CRP levels **(A)**, adipose tissue *NF κ B1* **(B)**, *IL6* **(C)** and *IL1B* **(D)** gene expression in control and CRC groups. ** means p<0.01..C-reactive protein (CRP); Nuclear Factor Kappa B subunit 1 (*NF κ B1*); Interleukin 6 (*IL6*); Interleukin 1 beta (*IL1B*); Colorectal Cancer (CRC).

Besides, 25(OH)D correlated negatively with adipose tissue NFκB1 mRNA (Table 2).

Table 2. Partial correlation between serum 25(OH)D and adipose tissue *NF κ B1* mRNA, *IL6* mRNA and *IL1B* mRNA corrected by gender in the whole population.

	25(OH)D	
	r	**p**
Log(*NFκB1* mRNA)	-0.232	0.041
Log(*IL6* mRNA)	-0.125	0.251
Log(*IL1B* mRNA)	-0.106	0.339

Abbreviations: 25-hydroxy-vitamin D (25(OH)D), Nuclear Factor Kappa B subunit 1 (*NF κ B1*), Interleukin 6 (*IL6*), Interleukin 1 beta (*IL1B*).

Concordantly, we observed that serum CRP levels were negatively correlated with serum 25(OH)D levels and positively correlated with both VDR and *NFκB1* gene expression in adipose tissue (**Table 3A**). In turn, there was a positive correlation between *NFκB1* mRNA and both, *IL6* and *IL1B* mRNA levels (**Table 3B**). Furthermore, a positive correlation was found between *NFκB1* mRNA and *VDR* (**Table 3B**).

Table 3. Partial correlations of C-reactive protein (A) with serum 25(OH)D, adipose tissue *VDR* mRNA, *NFκB1* mRNA, *IL6* mRNA, and *IL1B* mRNA corrected by gender. Partial correlations corrected by gender of adipose tissue *NF κ B1* mRNA (B) with IL 6 mRNA, *IL1B* mRNA and *VDR* mRNA.

A	**Log(C-reactive protein)**	
	r	**p**
Log(25(OH)D)	-0.270	0.011
Log(*VDR* mRNA)	0.219	0.049
Log(*NFκB1* mRNA)	0.284	0.016
Log(*IL6* mRNA)	0.245	0.029
Log(*IL1B* mRNA)	0.272	0.016

B	*NFκB1* mRNA	
	r	**p**
Log(*IL6* mRNA)	0.688	0.000
Log(*IL1B* mRNA)	0.778	0.000
Log(*VDR* mRNA)	0.761	0.000

Abbreviations: 25-hydroxy-vitamin D (25(OH)D), vitamin D receptor (*VDR*), Nuclear Factor Kappa B subunit 1 (*NF κ B1*), Interleukin 6 (*IL6*), Interleukin 1 beta (*IL1B*).

VDR and *NFκB1* methylation and association between the epigenetic factor *DNMT3A* and 25(OH)D

The DNA methylation status of the *VDR* promoter was determined by pyrosequencing, but no differences between the control and CRC groups were found (Figure 3A). When individual *VDR* CpG positions were compared, significant lower *VDR* methylation at position 4 (*VDR* P4) was found in the CRC group when compared with the control group (**Figure 3B**). *NFκB1* global methylation was lower in the CRC group than in the control group. A comparative analysis at each *NFκB1* CpG analyzed showed that *NFκB1* at position 3 (*NFκB1* P3) presented a lower methylation level in CRC with regard to the control group. Moreover, a negative trend (r=-0.252, p=0.061) was observed between *NFκB1* mRNA levels and *NFκB1* P3 and between *VDR* mRNA and the global *NFκB1* methylation (r=-0.228; p=0.064). Additionally, a negative and significant correlation was found between *VDR* mRNA and *NFκB1* at position 3 (*NFκB1* P3) (r=-0.296; p=0.015) and at position 4 (*NFκB1* P4) (r=-0.327; p=0.007). Furthermore, we analyzed *DNMT3A* gene expression in adipose tissue, which was higher in the CRC group than in the control group but without getting statistic significance (Figure 3E). We also found a negative correlation (corrected by gender) between the gene expression of the epigenetic factor *DNMT3A* and serum 25(OH)D levels (r=-0.264, p=0.013). There was a positive correlation between adipose tissue

DNMT3A gene expression and adipose tissue *VDR* DNA methylation in a partial correlation corrected by gender (r=0.256, p=0.034). We also found a positive correlation (Spearman's correlation) between *DNMT3A* mRNA and *NFκB1* mRNA (r=0.279, p=0.009).

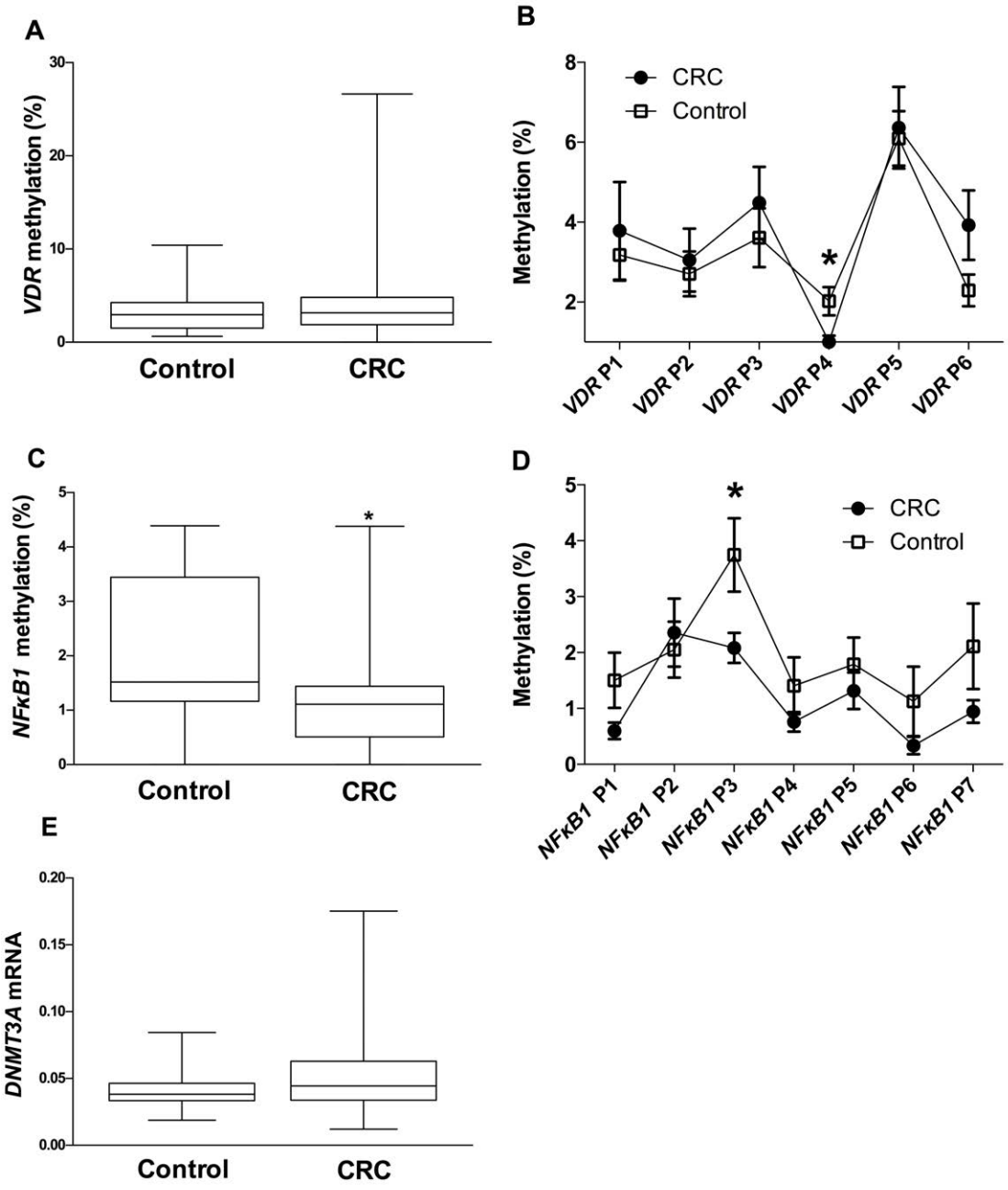

Figure 3. Methylation analyses at specific CpG dinucleotides for *VDR* and *NFκB1*promoters and gene expression of the epigenetic factor *DNMT3A* were performed to compare both, control

and CRC groups. Comparisons of the global VDR methylation **(A)** and among the CpG dinucleotides analyzed **(B)** by Mann-Whitney-U test. Non-parametric (Mann-Whitney-U test) comparison for the global *NFκB1* methylation **(C)** and at specific CpG dinucleotides **(D)**, as well as for the methytransferase *DNMT3A* gene expression **(E)**. * means $p<0.05$. Vitamin D receptor (VDR); DNA methyltransferase 3a (*DNMT3A*); Colorectal Cancer (CRC).

GENERAL DISCUSSION

In this PhD dissertation, we have proven that DNA methylation and H3K4m3 modifications at the promoter of several genes related to adipogenesis, metabolism and inflammation are related to MetS and obesity. We have also demonstrated that DNA methylation deregulation in AT is related to VD and inflammatory factors in the context of CRC.

Remarkably, we have described that LPL promoter is hyper-methylated in MetS subjects, which caused a lower LPL mRNA and protein expression. LPL methylation was positively associated to metabolic deterioration, and importantly with both, basal and postprandial TG levels. Since the role of LPL in AT is the withdrawal of TG, the fact that higher LPL methylation was related to higher TG levels and worse postprandial TG withdrawal, points out the importance that DNA methylation in this gene could have in MetS etiology.

In addition, we showed a negative correlation between DNA methylation of LPL and LPL mRNA expression, suggesting a possible epigenetic regulation of this gene. In fact, the studied CpG dinucleotides are pinpointed inside several transcription factor binding site sequences such as MYC proto-oncogene (MYC), MYC associated factor X (MAX), enhancer of Zeste 2 polycomb repressive complex 2 subunit (EZH2), SUZ12 polycomb repressive complex 2 subunit (SUZ12) and E2F transcription factor 6 (E2F6), which can be involved in the transcriptional regulation of LPL (191). For instance, DNA methylation levels at these CpGs could affect MYC binding, which has been reported to activate lipid metabolism factors or to promote adipogenesis (192,193). Interestingly, EZH2 and SUZ12 are part of the PRC2 complex, that is in charge of methylation at H3K27 (which is a repressive modification) and that can play a regulatory role in the interplay between DNA methylation-H3K27 methylation (194,195). Another factor, E2F6 has already been associated in mice to chromatin regulation, especially promoting

DNA methylation through DNMT3B recruitment (196). Whether these factors, EZH2, SUZ12 or E2F6, could be involved or not in the methylation levels shown in this study is unknown.

As it was previously indicated, epigenetics plays an important role in cell differentiation (137). Besides, LPL mRNA expression is known to increase during adipocyte differentiation, while impairment in adipocyte tissue-derived mesenchymal stem cells differentiation into adipocyte has been related to MetS (59,67). Thus, it might be possible that the increase in LPL mRNA expression during adipogenesis could be the result of epigenetic changes (as DNA methylation). Likewise, the highest LPL DNA methylation in MetS subjects could be a sign for impairment in adipocyte tissue-derived mesenchymal stem cells differentiation. However, additional studies would be necessary to confirm this hypothesis.

An increased LPL methylation in AT, with lower LPL mRNA and protein levels, could be translated into higher plasma triglycerides in MetS patients. In this sense, LPL activity in AT has been related to hypertriglyceridemia (68). In turn, increased concentrations of plasma TG can lead to lipid accumulation in peripheral tissues causing insulin-resistance (69), which would be in line with our results as the MetS subjects had greater insulin resistance, and therefore less capacity to incorporate glucose. Furthermore, high postprandial triglyceride concentrations have been associated with MetS (15). Increased postprandial TG concentrations have already been associated with cardiovascular failure, and is a risk factor for mortality after cardiovascular events (197). We have demonstrated that DNA methylation of the LPL promoter in AT is related to postprandial TG, being LPL DNA methylation levels higher in patients with a higher level of serum postprandial triglycerides.

On the other hand, other DNA methylation marks were found to be related to MetS and obesity parameters, although no differences in DNA methylation levels were found between both, MetS and Non MetS groups. LINE-1 DNA methylation levels have been proposed as a measurement of the global DNA methylation of an individual (198). A negative association between LINE-1 DNA methylation and MetS worsening and especially to glucose levels has been previously shown only in obese subjects (199). However, we expand the relationships existing between LINE-1 DNA methylation levels with the MetS worsening and glucose status to a population with a different degree of obesity and metabolic deterioration. In this line, it has been shown that lower LINE-1 methylation in blood cells is related to an impaired glucose metabolism improvement after a physical activity intervention study in subjects with glucose metabolic disorders (200). All these data suggest that global DNA methylation levels, determined by LINE-1 DNA methylation, could be affected by glucose metabolism and the insulin resistance state more than by the obesity degree itself. Other factors related to adipogenesis, lipid and glucose metabolism such as PPARα, PPARγ, RXRα, SREBF2, LRP1 or SCD did not present differences in DNA methylation attending to the presence or not of MetS, although diverse associations were found with biochemical and anthropometrical parameters related to MetS. For instance, PPARα DNA methylation, which is stimulated by the TG-lowering drugs called fibrates (75), was positively associated to TG, HOMA-IR and MetS worsening. It is known that PPARγ mRNA levels are negatively associated to BMI, which it has been associated to adipose tissue dysfunction (201). PPARγ down-regulation leads to impaired capacity of adipose tissue to accumulate lipids, leading to ectopic lipid accumulation, insulin resistance and other obesity-associated comorbidities (202). Thus, this association observed between PPARγ DNA methylation and BMI is in accordance with previous studies (201,202).

Moreover, as it has been described, AT dysfunction has been widely related to a low-grade inflammatory state, which in turn can trigger to a deterioration of the AT and a worsening of the metabolic state, which as consequence reinforces the inflammatory process (203). Thus, AT inflammation can be related to MetS parameters. We have also studied the DNA methylation of important inflammatory factors in AT: C3, TNF and LEP in a population of MetS and Non MetS subjects. We did not find differences for C3 and LEP DNA methylation levels, while TNF DNA methylation levels were lower in MetS patients. TNF DNA methylation was also negatively related to the worsening of the metabolic state and to metabolic and anthropometric parameters such as TG, glucose, LDL-cho and DBP. By contrast, TNF methylation was positively associated with HDL-cho. We showed that TNF DNA methylation is an epigenetic mark closely related to the metabolic state, which could be contributing to the etiology of metabolic disorders.

AT TNF is almost entirely produced by the macrophage fraction, especially by the pro-inflammatory M1 phenotype (37,204). Moreover, as it has been described before, obesity and AT dysfunction is related to a higher macrophage infiltration and polarization of the M2 phenotype to the pro-inflammatory M1 phenotype (37,47). This polarization from M2 to M1 phenotypes is thought to be regulated by epigenetics in a process in which the metabolic status can modulate it (204–207). Though, the lowest TNF DNA methylation levels in AT of MetS patients might be due to a polarization of M2 to M1 phenotype, although this deserves further investigation.

Contrary to TNF, C3 DNA methylation is not altered in MetS according to our data. Nevertheless, we demonstrated that C3 DNA methylation levels are decreased in extremely obese people. While C3 mRNA expression was related to glucose metabolism parameters, C3 DNA methylation was closely associated to BMI and

adiposity. However, neither significant associations were found between C3 DNA methylation nor mRNA levels nor ASP in serum. It is known that AT C3 is stimulated after CH addition, and that a peak of ASP occurs during the postprandial state and at AT-environment level (102–104,208–210). ASP has been shown to stimulate LPL activity (that is otherwise inhibited by its own product, free fatty acids) and to activate adipocyte lipid storage (209). Our study was carried out using AT samples from patients in starvation previous to surgery intervention. Thus, it could happen that basal gene expression of C3 in AT under this starvation state did not let us observe a relationship between C3 DNA methylation and C3 mRNA, but this correlation could rise during the postprandial state. Thus, the lowest C3 DNA methylation observed in extremely obese subjects could be a physiological response trying to improve the lipid storage in the AT, which as a secondary result could trigger AT inflammation that is characteristic of obesity (211).

Aside from DNA methylation, epigenetics involve a complex regulatory landscape in which histone modifications play an important role. As it has been described, both mechanism, DNA methylation and histone modifications work in complete harmony, and both are necessary to the correct function of gene regulation, DNA repair, or to establish a correct epigenetics pattern (150–152). However, there is a scarce knowledge about histone modifications in AT, being investigated mainly in mice AT and cellular experiments (184,186,212). AT manipulation implies dealing with a tricky tissue, which has an enormous amount of lipid content, hindering tissue manipulation and down-stream experimental procedures (213). Thus, we have developed an improved chromatine inmunoprecipitation (ChIP) protocol for its usage in small pieces of frozen AT. This will allow to research about AT-histone modification in large human sample banks. The development of this protocol determined the usage of a lower concentration

of formaldehyde (0.5%) solution to fixe the whole tissue (previous to AT homogenization), higher volumes of buffers previous to nuclei recovery (fixation buffer, cell lysis buffer and wash buffers), the Ultraturrax-type homogenization method instead the traditional pestle and mortar method and a de-crosslinking step carried out at a moderate temperature during 5 hours instead of a short period of time at a high temperature (with addition of chelex-100), among other improvements to the traditional method.

Once established the procedure, we studied H3K4me3 enrichment at the promoter of several genes related to adipogenesis, AT-metabolism and inflammation in a population of healthy lean and obese subjects with a different degree of glucose metabolism impairment. Thus, we observed an increase of H3K4me3 at the promoter of E2F1, LPL, SREBF2, SCD, PPARγ and IL6 in pre-diabetic obese subjects compared to healthy lean subjects. Besides, positive correlations with BMI, glucose, insulin, HOMA-IR and MetS worsening were found. It is known that histone methylation relies on substrate disposal, for instance, SAM levels (169). It has been shown that SAM levels increases with BMI, and that this increase is associated to adiposity (172). On the other hand, de-methylation processes involves FAD/FADH2, α-ketoglutarate and succinate levels (all of them part of the TCA cycle) affected by nutrition and metabolic state and that could be altered for the reduction in fatty acid oxidation and glucose incorporation observed in insulin resistance states (173–175). Moreover, hypoxia has also been reported to increase H3K4me3 marks through reduction of de-methylation (169). Hypoxia in AT is a factor that has been widely associated to metabolic disturbance and insulin resistance (214). Therefore, an increase in SAM associated to the BMI with a decrease of de-methylation processes trigger by hypoxia and/or FAD/FADH2 and α-ketoglutarate bioavailability could explain the increase in H3K4me3 that we have found in our study.

However, we did not find a significant positive correlation between promoter-H3K4me3 enrichments and mRNA expression for any of the study genes, except for E2F1. E2F1 has been shown to regulate cholesterol withdrawal in the liver (215,216). In AT, overexpression of E2F1 has been described in obesity, and has been related to inflammation and metabolic deterioration (217,218). Though, it might be possible that the increased H3K4me3 at E2F1 promoter could be regulating this gene, contributing to AT inflammation and deterioration in obesity and metabolic disease.

Systemic inflammation has also been related to CRC (115). Since AT deterioration can contribute to a low-grade inflammation state (112,116), it would be necessary to bear in mind the role of AT in the appearance, development or the outcome of CRC. As it has been described, CRC has been associated to low levels of VD (219), which we have confirmed in our work. VD is a potent anti-inflammatory hormone, carrying out its function through VDR (127,136). VDR acts as a heterodimer with RXRα as partner (220). Besides, RXRα is the partner for the adipogenic factor PPARγ, which has also been demonstrated to exert an anti-inflammatory effect (221,222). However, high levels of VDR without its ligand has been shown to inhibit PPARγ (223), fact that together with the lack of the anti-inflammatory effect consequence of the low VD levels could contribute to an pro-inflammatory state in AT of CRC subjects. Accordingly, we have observed a rise in AT of the pro-inflammatory master gene NfκB1 and of the interleukins IL6 and IL1B in the CRC group respect to the control group, as well as, a negative correlation between serum 25(OH)D and AT-NfκB1 mRNA expression. Besides, it has been demonstrated that TNF (a pro-inflammatory factor) can stimulate VDR expression (224). Thus, a pro-inflammatory state in AT could lead to the overexpression of VDR which in turn, together with the lack of VD, could cause a rise of inflammation in a positive loop.

Again, we observed that AT epigenetic marks could be in part contributing to AT deterioration in CRC subjects. Indeed, we observed lower levels of global NfκB1 DNA methylation and VDR DNA methylation at CpG position 4 in CRC, suggesting that the higher levels of VDR and NfκB1 mRNA could be controlled by DNA methylation at the promoter of both genes. Nevertheless, no statistical associations were found between mRNA and DNA methylation levels for both genes, although the associations were closed to the statistical significance. As it has been discussed before, epigenetic regulation is a complex landscape of regulation, an other CpG positions as well as histone modifications might shed light a more precise mechanism of regulation for NfκB1 and VDR. We found that 25(OH)D was negatively associated to DNMT3A expression, which could be leading to epigenetic modifications of AT. In this sense, we observed a positive correlation of DNMT3A mRNA with VDR and NfκB1 DNA methylation levels. VDR without VD has also been related to an opposite effect in gene expression regulation and could alter epigenetic marks (225,226). Accordingly, we observed a negative association between VDR mRNA and NfκB1 DNA methylation, although more studies would be necessary to clarify whether VDR without VD can increase inflammation by lowering NfκB1 DNA methylation.

In conclusion, we have shown that some epigenetic marks (DNA methylation and the H3K4me3 mark) in AT are related to metabolic deterioration and could be contributing to metabolic diseases through the regulation of adipogenic, lipid metabolism and inflammatory factors. Besides, we have shown that the lack of VD could be altering AT DNA methylation, which in turn could be related to AT inflammation, prompting AT to deliver pro-inflammatory cytokines that has been shown to be a favorable environment to CRC development and bad-prognosis.

CONCLUSIONS

1. LPL promoter methylation in adipose tissue is higher in MetS than Non-MetS subjects, and this DNA methylation could explain the mRNA expression and, in turn, the protein levels in these subjects. We found that methylation at the LPL promoter may be associated with MetS components and the metabolic profile, and that LPL DNA methylation is a variable that is directly related to the probability of being MetS. The hyper-methylation in the promoter of LPL could hinder triglyceride withdrawal in MetS, causing hypertriglyceridemia and disorders associated with MetS, such as insulin resistance and diabetes.
2. MetS subjects have higher and lower levels of adipose tissue DNA methylation at LPL and TNF promoter respectively. While LPL methylation was again associated to TG levels, TNF was strongly associated to the metabolic worsening. Global methylation, determined by LINE-1 is positively associated to serum glucose levels. Although there are no differences between MetS *versus* Non MetS for the rest of studied promoters, PPARα, PPARγ, RXRα, SREBF2, LRP1, SCD, LXRB, C3 and LEP DNA methylation levels are associated to variables related to the MetS. Altogether, these data support the idea that DNA methylation can be involved in the etiology of the MetS
3. DNA methylation of C3 is strongly associated to BMI, whereas C3 mRNA associates to the pathophysiology of related-obesity metabolic diseases.
4. Chromatin inmunoprecipitation was improved to be applied in small and frozen pieces of adipose tissue: It is recommended to fix the whole tissue before mincing it in 5 ml of PBS + 0.5% formaldehyde, after which we recommend several washing and cell lysis steps with abundant cold PBS+PIC. Furthermore, the use of the ultraturrax homogenizer improves nucleus pellet recovery. Afterwards, optimization of the chromatin shear is a key step to success in the

following IP. Finally, we recommend de-crosslinking the chromatin under a moderate temperature for 5 hours, adding PK in order to degrade the protein fraction and improve the DNA recovery.

5. We have applied the adipose tissue ChIP protocol to determine the histone H3K4 trimethylation modification in human VAT in a population with a different degree of BMI and glucose metabolism. We have observed an increase of H3K4me3 histone mark at the promoter of several adipogenic, lipid metabolism and inflammatory genes in adipose tissue as the BMI and HOMA-IR increase. Interestingly, we have shown that the metabolic regulator E2F1 could be susceptible of epigenetic regulation in adipose tissue. The fluctuation in the H3K4me3 mark observed in this work could be due to the metabolic status since energetic and metabolic intermediaries have been related to histone methylation, which could be related to adipose tissue dysfunction.

6. Adipose tissue may be a key factor in CRC development. The low 25(OH)D levels in CRC and high adipose tissue *VDR* expression may, at least in part, mediate this relationship by modifying adipose tissue DNA methylation and promoting inflammation.

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SUPPLEMENTAL DATA

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Adipose Tissue LPL Methylation is Associated with Triglyceride Concentrations in the Metabolic Syndrome.

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Author information

Abstract

BACKGROUND:

DNA methylation is one of the epigenetic mechanisms that regulate gene expression. DNA methylation may be modified by environmental and nutritional factors. Thus, epigenetics could potentially provide a mechanism to explain the etiology of metabolic disorders, such as metabolic syndrome (MetS). The aim of this study was to analyze the level of DNA methylation of several lipoprotein lipase (LPL)-promoter-CpG dinucleotides in a CpG island region and relate this to the gene and protein expression levels in human visceral adipose tissue (VAT) from individuals with and without MetS.

METHODS:

VAT samples were collected from laparoscopic surgical patients without and with MetS, and levels of LPL mRNA, LPL protein, and LPL DNA methylation were measured by qPCR, western blot, and pyrosequencing. Biochemical and anthropometric variables were analyzed. Individuals included in a subset underwent a dietary fat challenge test, and levels of postprandial triglycerides were determined.

RESULTS:

We found higher levels of DNA methylation in MetS patients but lower gene expression and protein levels. There was a negative association between LPL methylation and LPL gene expression. We found a positive association between LPL methylation status and abnormalities of the metabolic profile and basal and postprandial triglycerides, whereas LPL gene expression was negatively associated with these abnormalities.

CONCLUSIONS:

We demonstrate that LPL methylation may be influenced by the degree of metabolic disturbances and could be involved in triglyceride metabolism, promoting hypertriglyceridemia and subsequent associated disorders, such as MetS.

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Altered Adipose Tissue DNA Methylation Status in Metabolic Syndrome: Relationships Between Global DNA Methylation and Specific Methylation at Adipogenic, Lipid Metabolism and Inflammatory Candidate Genes and Metabolic Variables.

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Abstract

Metabolic syndrome (MetS) has been postulated to increase the risk for type 2 diabetes, cardiovascular disease and cancer. Adipose tissue (AT) plays an important role in metabolic homeostasis, and AT dysfunction has an active role in metabolic diseases. MetS is closely related to lifestyle and environmental factors. Epigenetics has emerged as an interesting landscape to evaluate the possible interconnection between AT and metabolic disease, since it can be modulated by environmental factors and metabolic status. The aim of this study was to determine whether MetS has an impact on the global DNA methylation pattern and the DNA methylation of several genes related to adipogenesis (PPARG, PPARA), lipid metabolism (RXRA, SREBF2, SREBF1, SCD, LPL, LXRb), and inflammation (LRP1 C3, LEP and TNF) in visceral adipose tissue. LPL and TNF DNA methylation values were significantly different in the control-case comparisons, with higher and lower methylation respectively in the MetS group. Negative correlations were found between global DNA methylation (measured by LINE-1 methylation levels) and the metabolic deterioration and glucose levels. There were associations among variables of MetS, BMI, and HOMA-IR with DNA methylation at several CpG positions for the studied genes. In particular, there was a strong positive association between serum triglyceride levels (TG) with PPARA and LPL methylation levels. TNF methylation was negatively associated with the metabolic worsening and could be an important factor in preventing MetS occurrence according to logistic regression analysis. Therefore, global DNA methylation and methylation at specific genes related to adipogenesis, lipid metabolism and inflammation are related to the etiology of MetS and might explain in part some of the features associated to metabolic disorders.

Genes (Basel). 2018 Aug 13;9(8). pii: E410. doi: 10.3390/genes9080410.

Complement Factor C3 Methylation and mRNA Expression Is Associated to BMI and Insulin Resistance in Obesity.

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Author information

Abstract

Epigenetic marks, and especially DNA methylation, are becoming an important factor in obesity, which could help to explain its etiology and associated comorbidities. Adipose tissue, now considered as an important endocrine organ, produces complement system factors. Complement component 3 (*C3*) turns out to be an important protein in metabolic disorders, via either inflammation or the *C3* subproduct acylation stimulating protein (ASP) which directly stimulates lipid storage. In this study, we analyze *C3* DNA methylation in adipose tissue from subjects with a different grade of obesity. Adipose tissue samples were collected from subjects with a different degree of obesity determined by their body mass index (BMI) as: Overweight subjects (BMI ≥ 25 and <30), obese class 1/2 subjects (BMI ≥ 30 and <40) and obese class 3 subjects (BMI ≥ 40). C3 DNA methylation was measured for 7 CpGs by pyrosequencition using the Pyromark technology (Qiagen, Madrid Spain). C3 messenger RNA (mRNA) levels were analyzed by pre-designed Taqman assays (Applied biosystems, Foster City, CA, USA) and ASP/C3a was measured using a ELISA kit. The data were analyzed using the statistic package SPSS. *C3* DNA methylation levels were lower in the morbid obese group. Accordingly, *C3* methylation correlated negatively with BMI and leptin. However, *C3*mRNA levels were more associated with insulin resistance, and positive correlations with insulin, glucose and homeostasis model assessment-estimated insulin resistance (HOMA-IR) existed. ASP correlated negatively with high density lipoprotein (HDL) cholesterol. *C3* methylation levels were associated to adiposity variables, such as BMI and leptin, while the *C3* mRNA levels were associated to glucose metabolism.

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Chromatin immunoprecipitation improvements for the processing of small frozen pieces of adipose tissue.

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Author information

Abstract

Chromatin immunoprecipitation (ChIP) has gained importance to identify links between the genome and the proteome. Adipose tissue has emerged as an active tissue, which secretes a wide range of molecules that have been related to metabolic and obesity-related disorders, such as diabetes, cardiovascular failure, metabolic syndrome, or cancer. In turn, epigenetics has raised the importance in discerning the possible relationship between metabolic disorders, lifestyle and environment. However, ChIP application in human adipose tissue is limited by several factors, such as sample size, frozen sample availability, high lipid content and cellular composition of the tissue. Here, we optimize the standard protocol of ChIP for small pieces of frozen human adipose tissue. In addition, we test ChIP for the histone mark H3K4m3, which is related to active promoters, and validate the performance of the ChIP by analyzing gene promoters for factors usually studied in adipose tissue using qPCR. Our improvements result in a higher performance in chromatin shearing and DNA recovery of adipocytes from the tissue, which may be useful for ChIP-qPCR or ChIP-seq analysis.

PloS One. Under Peer Review

Human Adipose Tissue H3K4me3 in Adipogenic, Lipid and Inflammatory genes are Positively Associated to BMI and HOMA-IR

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Abstract

Introduction: Adipose tissue is in charge of energy storage and can act in systemic homeostasis and inflammation. Epigenetics, for instance DNA methylation and histone-tail modifications, can be modified by environmental conditions. Obesity and metabolic disorders are closely related to lifestyle and nutrition. Thus, epigenetics could play an important role in the onset of these diseases although little is known about histone marks in human adipose tissue. We study, for the first time, H3K4 trimethylation (H3K4me3) (open chromatin) on the promoter of several adipogenic, lipid metabolism and inflammatory factors in visceral adipose tissue (VAT) from subjects with different degrees of BMI and metabolic disease.

Methodology: Frozen VAT samples (-80ºC) were fixed in formaldehyde, homogenized and the chromatin sheared. Chromatin inmunoprecipitation was performed with an antibody anti-H3K4me3 and promoter enrichment was analyzed by qPCR. mRNA extraction on the same samples was performed to quantify gene expression of these genes.

Results: Positive correlations existed for H3K4me3 enrichment at E2F1, LPL, SREBF2, SCD, PPARG and IL6 promoters with BMI and HOMA-IR. Regression analyses showed association of H3K4me3 at E2F1 and LPL promoters, LEP and SCD mRNA levels with BMI; and of H3K4me3 enrichment at SCD and IL6 promoters and with LEP and SCD mRNA levels with HOMA-IR. There was a positive correlation between E2F1-H3K4me3 and E2F1 mRNA, suggesting a possible role of this histone in the gene regulation of E2F1 in VAT.

Conclusions: H3K4me3 at LEP, LPL, SREBF2, SCD, PPARG, IL6, TNF and E2F1 promoter is directly associated with increasing BMI and metabolic deterioration.

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Adipose tissue inflammation and *VDR* expression and methylation in colorectal cancer.

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Author information

Abstract

BACKGROUND:

Lack of vitamin D (VD) has been associated with colorectal cancer (CRC). VD has anti-inflammatory effects and regulates several cellular pathways by means of its receptor, including epigenetic modifications. Adipose tissue dysfunction has been related to low-grade inflammation, which is related to diseases like cancer. The aim of this study was to explore the relationship between serum 25-hydroxyvitamin D (25(OH)D), adipose tissue gene expression of VD receptor (VDR), pro-inflammatory markers, and the epigenetic factor DNA methyltransferase 3a (DNMT3A) as well as VDR promoter methylation in CRC.

METHODS:

Blood and visceral adipose tissue from 57 CRC and 50 healthy control subjects were collected. CRC subjects had lower serum 25(OH)D levels and higher VDR gene expression, and these were negatively correlated in the CRC group.

RESULTS:

Adipose tissue *NFκB1*, *IL6*, and *IL1B* gene expression were higher in the CRC subjects than in the control subjects. 25(OH)D correlated negatively with *NFκB1* and CRP. In turn, CRP correlated positively with *NFκB1*, *IL6*, *IL1B*, and *VDR* gene expression as well as *NFκB1* that correlated positively with *IL6* and *IL1B*. *DNMT3A* mRNA was negatively correlated with serum 25(OH)D and positively correlated with *VDR* DNA methylation. *VDR* DNA methylation at position 4 had lower levels in the CRC group. Global *NFκB1* methylation at dinucleotide 3 was lower in the CRC group.

CONCLUSION:

Our results suggest that adipose tissue may be a key factor in CRC development. The low 25(OH)D levels and high adipose tissue *VDR* expression in CRC may, at least in part, mediate this relationship by modifying adipose tissue DNA methylation and promoting inflammation.