EPIGENETICS
2022, VOL. 17, NO. 6, 665–676
https://doi.org/10.1080/15592294.2021.1950991

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RESEARCH PAPER

DNA methylome in visceral adipose tissue can discriminate patients with and without colorectal cancer

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ABSTRACT

Adipose tissue dysfunction, particularly the visceral (VAT) compartment, has been proposed to play a relevant role in colorectal cancer (CRC) development and progression. Epigenetic mechanisms could be involved in this association. The current study aimed to evaluate if specific epigenetic marks in VAT are associated with colorectal cancer (CRC) to identify epigenetic hallmarks of adipose tissue-related CRC. Epigenome-wide DNA methylation was evaluated in VAT from 25 healthy participants and 29 CRC patients, using the Infinium HumanMethylation450K BeadChip. The epigenome-wide methylation analysis identified 170,184 sites able to perfectly separate the CRC and healthy samples. The differentially methylated CpG sites (DMCpGs) showed a global trend for increased methylated levels in CRC with respect to healthy group. Most of the genes encoded by the DMCpGs belonged to metabolic pathways and cell cycle, insulin resistance, and adipocytokine signalling, as well as tumoural transformation processes. In gene-specific analyses, involved genes biologically relevant for the development of CRC include *PTPRN2, MAD1L1, TNXB, DIP2C, INPP5A, HDCA4, PRDM16, RPTOR, ATP11A, TBCD, PABPC3,* and *IER2*. The methylation level of some of them showed a discriminatory capacity for detecting CRC higher than 90%, showing *IER2* to have the highest capacity. This study reveals that a specific methylation pattern of VAT is associated with CRC. Some of the epigenetic marks identified could provide useful tools for the prediction and personalized treatment of CRC connected to excess adiposity.

ARTICLE HISTORY
Received 21 April 2021
Revised 8 June 2021
Accepted 29 June 2021

KEYWORDS
DNA methylation; adipose tissue; cancer; obesity; microarray

Introduction

Obesity is considered an important risk factor for the onset and progression of several types of cancer. Hence, targeting adipose tissue dysfunction has been proposed to provide a promising strategy for cancer prevention and therapy [1,2]. In obesity, adipose tissue becomes dysfunctional and secretes several carcinogenic factors [3–5]. Over the last decade, obesity rate has been gradually increasing globally [6]. This metabolic disorder is associated with several pathological conditions including cancer [7,8]. Obesity-associated cancer types have been estimated to account for up to 40% of newly diagnosed cancers [9].

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Supplemental data for this article can be accessed here.

Several studies have pointed out that being overweight/obese is a well-established risk factor for the development of colorectal cancer (CRC) [10], and visceral adipose tissue (VAT) has been identified as a risk factor for colorectal adenomas [11] and carcinomas [12,13]. Metabolomic, lipidomic, and transcriptomic analyses have identified differences between visceral adipose tissue (VAT) and subcutaneous adipose tissue in CRC. The major difference was found to be increased levels of inflammatory markers in VAT, which supports inflammation in CRC [13,14]. According to this hypothesis, our group provided several evidences describing the role of VAT in CRC [15,16]. Indeed, *LINE1* (marker for DNA global methylation) methylation in VAT was hypomethylated in the CRC patients than in the control subjects [17], as well as altered expression of adipogenic genes [18]. Therefore, a thorough understanding of the crosstalk between CRC and the epigenetic regulation of visceral adipose microenvironment may reveal novel pathways and therapeutic targets for CRC diagnosis and prognosis.

Epigenetic modifications, particularly DNA methylation, can respond to environmental conditions and increase the risk of cancer [19]. Currently, a massive amount of research is being conducted to provide better insights into tissue-specific epigenetic alterations and their roles in the development of metabolic diseases [20,21]. Previous studies from our group have demonstrated a specific epigenetic profile associated with the adiposity state in breast [22] and CRC [23]. We proposed that the VAT secreted factors-induced effect on obesity-related diseases could be mediated by epigenetic mechanisms [24–27] that can be remodelled after weight loss interventions [28,29]. Moreover, the epigenetic marks associated to obesity were also proposed as a potential tool for precision management of obesity [30–32]. Nevertheless, the epigenome-wide DNA methylation profile of VAT from CRC patients is currently unknown, despite the growing evidence of the role of VAT in cancer development. Therefore, the aim of the present study was to explore the global DNA methylation profile in VAT from healthy and CRC patients through an epigenome-wide association study, to identify the epigenetic hallmarks of adipose tissue-related CRC and to provide an epigenetic landscape of CRC-related obesity.

Material and methods

Study subjects

The study included participants from the University Hospital 'Virgen de la Victoria,' (Malaga, Spain) between 2012 and 2013 who were divided into two groups. The tumour-free (TF) group included 25 healthy subjects (control), who underwent hiatus hernia surgery or cholecystectomy. The second group included 29 patients with CRC who were diagnosed by clinical investigations and positive colonoscopy and was confirmed by pathology results from biopsies. CRC patients underwent surgery with curative intent, by hemicolectomy, lower anterior resection with ileostomy (caused by a carcinoma of the CRC), followed by a total mesocolorectal excision. Patients with inflammatory bowel disease (Crohn's disease or ulcerative colitis), familial polyposis coli, or who received neo-adjuvant chemotherapeutic or radiotherapeutics treatment, were excluded from the study [16,17]. We also excluded patients diagnosed with cardiovascular diseases, arthritis, acute or chronic inflammatory diseases, infections or renal diseases, or patients who were receiving treatment known to alter the lipid and glucose profiles, calcium, or vitamin D supplements. All the participants gave written informed consent (0311/PI7) and the study was conducted in accordance with the guidelines laid down in the Declaration of Helsinki. The study was reviewed and approved by the Ethics and Research Committee of Virgen de la Victoria Hospital. The height and weights of the patients were recorded before surgery.

Laboratory measurements

VATs were collected during surgery, washed in physiological saline solution, and immediately frozen in liquid nitrogen. Biopsy samples were maintained at −80°C until analysis. Blood samples were obtained from the antecubital vein and collected in vacutainer tubes (BD vacutainer™). The serum was separated by centrifugation for 15 min at

4000 rpm and immediately frozen at −80°C until further analysis. Serum glucose, cholesterol, triglycerides, and HDL cholesterol (HDL-C) were measured in a Dimension Autoanalyzer (Dade Behring Inc.) using enzymatic methods (Randox Laboratories Ltd.). The concentration of LDL cholesterol (LDL-C) was calculated using the Friedewald equation [33]. Insulin was quantified by a radioimmunoassay supplied by BioSource International Inc., Camarillo, CA, USA. The homoeostasis model assessment of insulin resistance (HOMA-IR) was calculated with the following equation: HOMA-IR = fasting insulin (μIU/mL) × fasting glucose (mmol/L)/22.5 [34].

DNA Methylation analysis in visceral adipose tissue

Total genomic DNA was extracted from VAT with the Qiamp DNA Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's guidelines. DNA integrity was further assessed with the Pico Green dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA). High-quality genomic DNA samples (500 ng) were subjected to bisulphite treatment using the EZ-96 DNA Methylation kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. Subsequently, DNA methylation was analysed by microarrays assays using Infinium Human Methylation 450 K bead chip technology (Illumina, San Diego, CA). DNA quality checks, bisulphite modification, hybridization, data normalization, statistical filtering, and value calculation were performed as described elsewhere [23]. Whole-genome amplification and hybridization were then performed using BeadChip, followed by single-base extension and analysis using HiScan SQ module (Illumina) to assess the cytosine methylation states. The annotation of CG islands (CGIs) used the following categorization: (1) shore, for each of the 2-kb sequences flanking a CGI; (2) shelf, for each of the 2-kb sequences next to a shore; and (3) open sea, for DNA not included in any of the previous sequences or in CGIs [23]. The transcription start site, TSS200 and TSS1500, indicated the regions either 200 or 1500 bp upstream of the transcription start site, respectively. DNA methylation for each CpG site was represented by beta values ranging from 0 to 1, corresponding to fully unmethylated and fully methylated, respectively.

Statistical analyses

The results for parametric variables are presented as mean ± standard deviation. Welch's two sample tests were used to determine differences between anthropometric and biochemical variables. For DNA methylation analysis, the *ChAMP* pipeline from Bioconductor with default settings and *minfi* package were used [35,36]. Quality check and normalization of β values were performed using the BMIQ method. Probes with *p*-values above 0.01 were excluded. Probes with single nucleotide polymorphisms at CpG or single-base extension sites and sex chromosomes were removed. Principal component analysis (PCA), heatmaps, differentially methylated CpGs (DMCpGs) and regions (DMRs) between healthy subjects and CRC patients were obtained from *minfi* package, using the Benjamini-Hochberg and False Discovery Rate (FDR) adjustment, in which significant results were set up as <0.05. The MEAL package (v.1.4.2) was used to generate Manhattan and Volcano plots. DMCpGs and DMRs were adjusted by the presence of co-variables, such as age, sex, and body mass index (BMI), by comparing lean *versus* overweight/obese. The gene–protein interaction network was constructed according to STRING analysis and database [37] and most significant methylated genes. Gene ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis was conducted to explore the function of the genes related to CRC and VAT using the *gometh* function in *missMethyl* package [38]. The predictor effect of the candidate genes that were differentially methylated was calculated as the agreement percentage using receiver operating characteristic (ROC) curve analyses, to discriminate between CRC patients and healthy indivi-

duals. These results are interpreted as negligible efficiency (<35%), minimal efficiency (35–50%), moderate efficiency (50–75%), good efficiency (75–90%), and excellent efficiency (>90%). All statistical analyses were performed using the R v.3.5.1 software [39].

Results

Characteristics of participants

Significant differences were not observed in sex, BMI, and HOMA-IR between the CRC patients and healthy subjects. However, the CRC group had significantly higher age, and glucose and triglycerides levels ($p< 0.05$), whereas the levels of insulin, total cholesterol, LDL, and HDL were significantly lower in the CRC patients than in the healthy participants ($p< 0.05$). The biochemical and anthropometric data from healthy participants (N = 25) and CRC patients (N = 29) are summarized in **Supplemental Table 1**.

DNA methylation analysis in VAT from CRC patients compared with healthy subjects

Comparison of samples with global PCA clearly distinguished two clusters or groups (healthy subjects and CRC patients) by the methylation levels (Figure 1a). Similar results were observed after performing a heatmap analysis of the methylation levels of 1,000 DMCpG (Figure 1d).

The differential analysis, adjusted by age, sex, and BMI identified 170,184 DMCpGs between the CRC and control groups after FDR adjustment (FDR < 0.05) (see detailed list, **Supplemental Table 2**). The Manhattan and Volcano plots illustrate the significant DMCpGs around the genome and the most hyper- and hypomethylated CpGs (Figure 1b, Figure 1c). Overall, the CRC-associated DMCpGs in VAT showed a statistically significant ($p< 0.001$) trend for hypermethylation in both global and specific promoter region in the CRC group with respect to the control group (Figure 1e, figure 1f). In fact, 58.8% (89,917 DMCpGs) of the

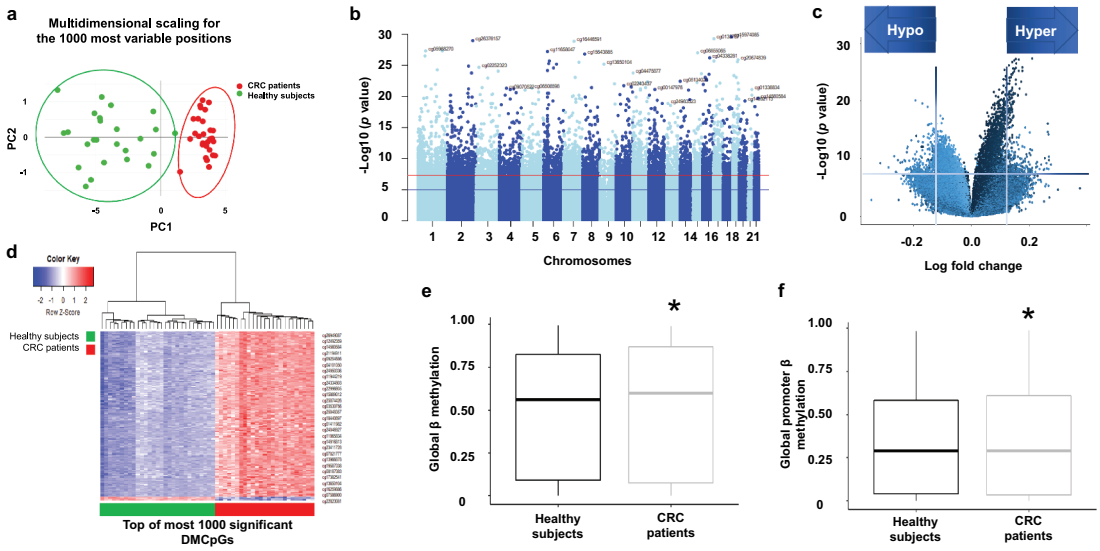

Figure 1. Profile of DNA methylation in visceral adipose tissue from colorectal cancer patients compared with healthy participants. (a) Principal component analysis for DNA methylation levels of 1,000 most variable CpGs between colorectal cancer and tumour free visceral adipose tissue (VAT) samples. (b) . Manhattan plot showing epigenome-wide *p*-values of association. The y axis shows the – log10(*p*) values of 455,267 valid CpGs, and the x axis shows their chromosomal position. The horizontal discontinuous line represents the threshold of $p< 0.05$ for selecting differentially methylated CpG sites. (c). Volcano plot of differences in DNA methylation between colorectal cancer and tumour free VAT samples. Each point represents a CpG site (n = 455,267) with mean differences (fold change) in DNA methylation between groups on the x-axis and -log10 of the corrected *p* value on the y-axis. Negative methylation differences indicate hypomethylation and positive differences show hypermethylation in the CRC patients compared to the healthy participants. (d) Supervised clustering of the 1,000 DMCpGs that were found to be differentially methylated between CRC patients and healthy subjects (e) Global and (f) promoter region differences in methylation levels between both groups. Asterisk indicates differences statistically significant according to the Wilcoxon test ($p< 0.001$). Abbreviations: **PC**, principal component, **DMCpG**, differentially methylated CpGs.

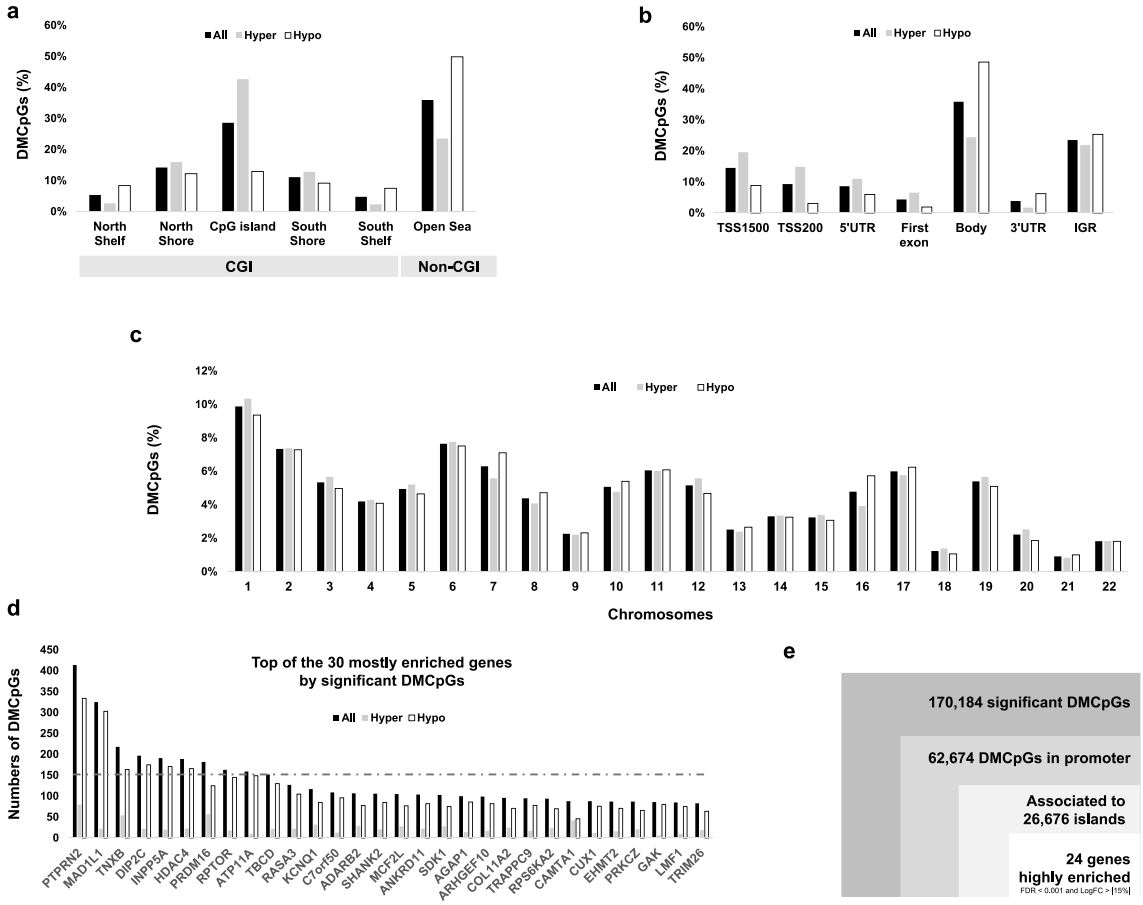

Figure 2. Characterization of the visceral adipose tissue-related colorectal cancer DMCpGs. (a) Genomic distribution of the differentially methylated CpG (DMCpGs) and their respective locations regarding the broader CpG context, (b) gene region and (c) chromosome. (d) List of the top of 30 genes mostly enriched by significant CpGs. Grey intermittent line represents a cut-off point of 150 DMCpGs with lower methylation in the CRC than healthy group. (e) Genes represented by filtered criteria, which DMCpGs are in promoter and islands, follow a false discovery rate (FDR) lower than 0.001 and a difference in β-values at least of 15% in absolute values. Abbreviations, **DMCpG**, differentially methylated CpGs, **CGI**, CpG island, **TSS**, transcription start site, **UTR**, untranslated region, **IGR**, intergenic region, **FDR**, false discovery rate, **FC**, fold change.

DMCpGs showed increased methylation levels in the CRC patients than in the healthy participants. A list of the most top 20 CRC-associated DMCpGs are represented in **Supplemental Table 3**.

The DMCpGs were mainly distributed in CpG island (CGI), open sea regions (non-CGI) (Figure 2a) and the body region (Figure 2b). Moreover, 36.8% of the DMCpGs were in the promoter region (the promoter region includes transcription start site (TSS)1500, TSS200, 5 ′UTR (untranslated region) and the first exon) (Figure 2b). With regard to the chromosomal distribution, most of the identified DMCpGs were in chromosomes 1, 2, 6, 7, 11, and 17 (Figure 2c).

Identification of genes with numerous differentially methylated CpG sites related to CRC in VAT

To identify genes epigenetically regulated in VAT and with a strong association with CRC, we evaluated the number of genes mostly enriched with significant CpGs. Figure 2d represents the top 30 genes mostly enriched with significant CpGs. Among them, *PTPRN2, MAD1L1, TNXB, DIP2C, INPP5A,*

HDCA4, PRDM16, RPTOR, ATP11A, and *TBCD* showed more than 150 DMCpGs with a lower methylation status in the CRC group than the healthy group. In a more stringent analysis, we selected those genes represented by differentially methylated regions located in promoter and islands and with a difference in β values ≥ |15|% and an FDR < 0.001. Thus, a total of 24 unique genes containing 28 DMCpGs were identified. Among them, *PABPC3* and *IER2* genes were the most represented with four and two DMCpGs, respectively (Figure 2e). The majority of these CpGs were found to be hypomethylated in CRC patients, when compared to healthy subjects (**Supplementary Figure 1**).

Functional enrichment analysis of CRC-associated DMCpGs in VAT

To further evaluate the biological significance of the 18,613 genes encoded by sequences containing the 170,184 CRC-associated DMCpGs in VAT, a GO analysis was performed. A GO analysis was conducted using the significant DMCpGs that were located at the promoter region. As shown in Figure 3a, we found significant biological processes, such as biosynthetic and metabolic processes (cellular component), DNA binding activity (molecular function), and intracellular organelle activity (biological processes). To assess the biological relevance, a KEGG analysis was conducted, using the most significant probe located at the promoter region (Figure 3b). Interestingly, key identified pathways are related to mammalian target of rapamycin (mTOR) signalling, AMP-

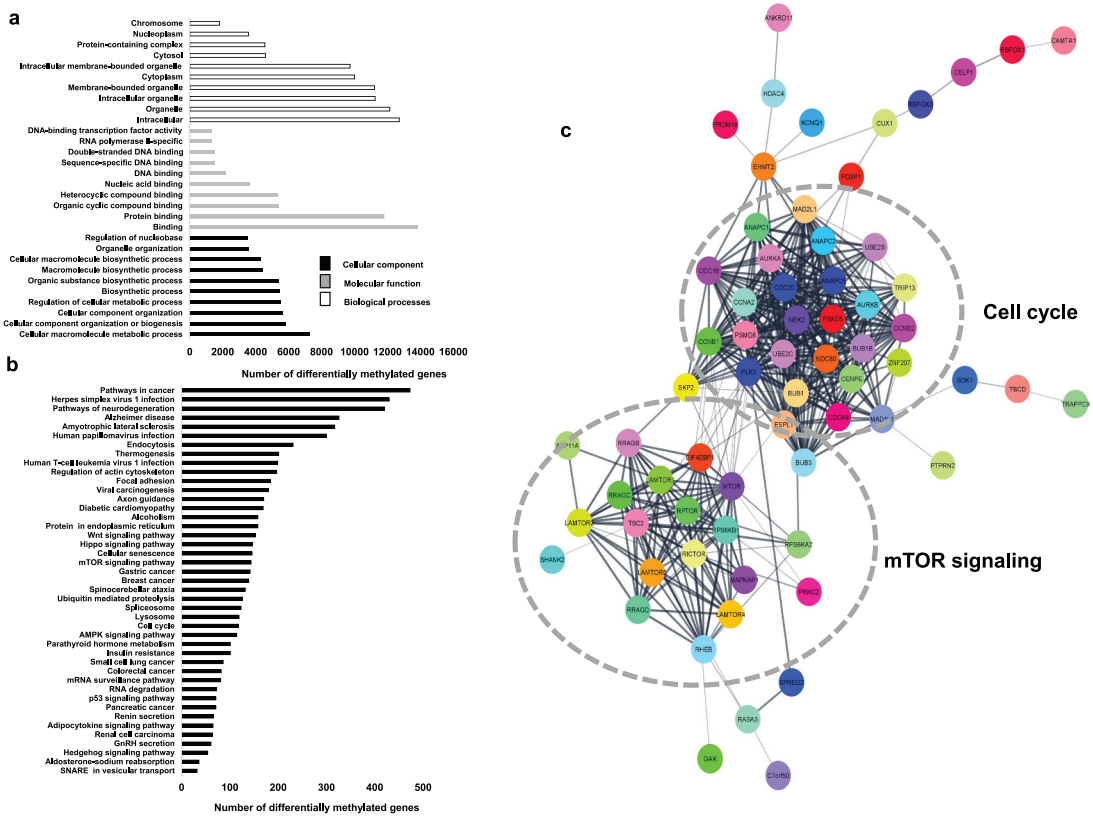

Figure 3. (A) **Biological implications of the visceral adipose tissue-related CRC DMCpGs.**(a) Summary of the GO analysis of the biological process categories representing the differentially methylated genes located at the promoter region. (b) KEGG pathway analysis of DMCpGs located at the promoter region. (c) Gene–protein interaction network-STRING analysis. Most of the genes regulated by methylation belonged to a network significantly enriched in protein interactions ($p < 0.001$) according to STRING analysis. Abbreviations: **DMCpGs**, differentially methylated CpGs; **GO**, gene ontology, **KEGG**, Kyoto Encyclopaedia of Genes and Genomes.

activated protein kinase (AMPK) signalling pathway, tumour protein 53 (p53) signalling pathway, insulin resistance, adipocytokine signalling pathway, or cancer pathways.

When the analysis was focused on those genes represented the 30 genes mostly enriched with significant DMCpGs and those genes that are in the promoter regions with more than 15% difference in methylation levels between CRC and healthy groups, with an FDR < 0.001 (54 unique genes), the STRING analysis revealed the existence of a statistically significant protein–protein network, indicating that the proteins are at least partially biologically connected, as a group (Figure 3c). This network included 818 nodes, 1010 edges, and 68 seeds genes with the genes, *FYN, ESR1*, and *PSMD14* showing the highest number of interactions. The *HDAC4, MAD1L1, TBCD, and PTRPN2* genes connect with the first cluster that belongs to cell cycle. Such process is closely linked to other cellular activities like growth and cell division. The genes *RPTOR* and *ATP11A* connect with the second cluster, which belongs to the mTOR signalling pathway, which is significantly associated with the genes epigenetically regulated in CRC-associated VAT.

Analysis of differential methylated regions related to CRC in VAT

To identify highly correlated CpG regional clusters, we studied the DMRs. DMR analysis is a clustering method that transforms single CpGs into regional clusters of highly correlated CpGs [40]. DMRs are extended segments of the genome that show a quantitative and significant difference in DNA methylation level between tumour and healthy groups. Our DMR analysis identified 21,293 DMRs, differentially methylated between the analysed groups according to the harmonized FDR, suggesting the presence of differential methylated regions, related to CRC in VAT. The top 20 significantly hyper- and hypomethylated DMR sites and their DMR locations are listed in the **Supplemental Table 4**, and the complete list in the **Supplemental Table 5**, ranked by statistical significance, showing that significantly hypomethylated DMRs are more common in adipose tissue derived from CRC patients.

A GO analysis was conducted using significant DMRs that were located at the promoter region. As shown in the **Supplementary Figure 2**, we found significant top biological processes related to organelle and lumen activities, such found in the analysis of DMCpGs. Regarding KEGG, which was conducted, using the most significant DMR located at the promoter region. The pathways found in this analysis are related to spliceosome, CRC, cell cycle, and tumour protein 53 (p53) signalling pathway. These results are matched with the KEGG and the STRING analyses are conducted with the DMCpGs. While the analysis was focused on those genes located at the top 20 DMRs, the STRING analysis revealed the existence of a statistically significant protein–protein network. Accordingly, two clusters were identified, which were related to cell cycle and ribosome biogenesis.

Analysis of predictor effect of the differentially methylated CpGs

ROC curves were used to evaluate the ability of the methylation levels of the selected DMCpGs to discriminate between CRC patients and healthy participants (Figure 4). Most of the identified CpGs showed a statistically significant AUROC > 70% (Figure 4a-i). The most significant AUROC was found in the cg17700633 from the *IER2* gene (Figure 4i). It was followed by cg10983056 from ATP11A (AUROC = 99%; Figure 4e), cg15376007 from HDAC4 (AUROC = 99%; Figure 4d), cg11025793 from IER2 (AUROC = 96%; Figure 4i), cg26983835 from MAD1L1 (AUROC = 93%; Figure 4b) and cg25152368 from PRDM16 (AUROC = 91%; figure 4f).

Discussion

In our study, we carried out an epigenome-wide analysis to evaluate the methylation profile of VAT in CRC. Our results revealed significant differentially methylated genes and a specific DNA methylation profile in VAT between healthy subjects and CRC patients. This analysis demonstrated that the potential link between VAT and CRC could be mediated by epigenetic mechanisms and hence provides a new approach to understand the role

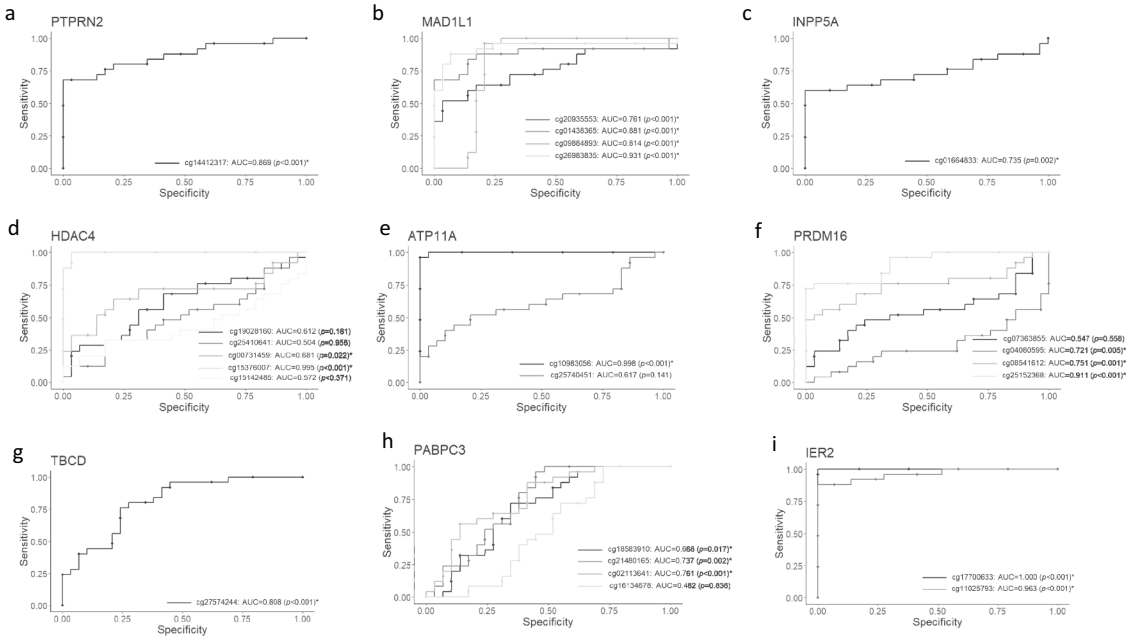

Figure 4. Receiver operating characteristic (ROC) curves for the methylation levels of the CRC-related differentially methylated CpGs in visceral adipose tissue. (a) Differentially methylated CpG site (DMCpGs) from PTPRN2. (b) DMCpGs from MAD1L1. (c) DMCpGs from INPP5A. (d) DMCpGs from HDAC4. (e) DMCpGs from ATP11A. (f) DMCpGs from PRDM16. (g) DMCpGs from TBCD. (h) DMCpGs from PABPC3. (i) DMCpGs from IER2. Abbreviations: AUC, area under the ROC curve.

of VAT and the effect of obesity on CRC development. To the best of our knowledge, this is the first study that provides an epigenetic signature of CRC in VAT. In this context, our findings offer several methylated candidate genes, which may be further considered in the epigenetic regulation processes of VAT in CRC.

DNA methylation was proposed as a mechanism by which adipose tissue can become dysfunctional [41–43]. Interestingly, in this study, a high number of CpGs were found to be differentially methylated with a trend for hypermethylation, which could be involved in colorectal carcinogenesis. Most of the genes encoded by the DMCpGs belong to metabolic pathways and thermogenesis, insulin resistance, and adipocytokine signalling, as well as tumoural transformation processes. The genes, *PTPRN2, MAD1L1, TNXB, DIP2C, INPP5A, HDCA4, PRDM16, RPTOR, ATP11A, TBCD, PABPC3,* and *IER2* were identified to be significantly enriched by DMCpGs and were among the most representative epigenetic signatures of CRC in VAT.

PTPRN2 gene is encoded for a receptor-like protein, tyrosine phosphatase and may be involved in the regulation of insulin secretion [44]. *PTPRN2* gene is considered to be a metabolic gene whose differentially methylated levels were previously associated with metabolic diseases, such as obesity [45]. Parallel to its role in metabolic processes, the expression of *PTPRN2* gene was also observed in cancer-related traits [46]. *PTPRN2* gene is also found to be epigenetically regulated in various cancer cells [47,48], which suggest that *PTPRN2* gene is a promising biomarker to be considered in the VAT-obesity-insulin pathway-CRC axis. In addition, *PRDM16* gene is a zinc finger transcription factor, largely involved in obesity, adipogenesis regulation, as well as reactive oxygen species production [49], and it also serves as a promising candidate in CRC [50]. *DIP2C* gene is one of the members of DISCO interacting protein 2 (DIP2) family, which is involved in the fatty-acid metabolism, obesity [51] as well as cancer [52,53]. Similarly, the expression of *DIP2C* [53] and *MALDL1* [54] was related to the development of carcinogenesis.

Additionally, *PABPC3* gene is tightly involved in nucleic acid binding, which is related to mRNA stability and deadenylating-dependent mRNA. A recent study related *PABPC3* gene to prognosis

for CRC, being , *PABPC3* expression significantly correlated with overall survivial [55]. IER2 gene is a transcription factor that is involved in tumour cell motility and metastasis, and predicts poor survival of CRC patients [56]. In this study, most of the identified genes are related to obesity, metabolism, and CRC, which corroborates previous studies reporting the relationship between obesity and CRC [23]. We also found several DMRs in association with CRC, showing a strong connection between the epigenetic regions around the genome. The DMR analysis revealed that these DMRs were associated with several genes related to cell cycle and p53 signalling pathway, which could provide additional information regarding colorectal carcinogenesis, metastasis, and invasiveness.

VAT has been well proposed to play a key role in the development of several diseases, including cancer [57] and it is associated with a higher rate of mortality [58]. One explanation is that dysfunctional adipose tissue secretes proinflammatory factors that promote disease development [59,60]. The differential epigenetic markers identified in the current study support the idea that tumour cells are highly metabolically active and that VAT is a source of energy that promotes tumour growth [1]. The impact of dysfunctional adipose tissue in promoting carcinogenesis manifests itself particularly as a state of obesity. Recently, our group showed that secreted factors of VAT from obese individuals are able to induce proliferation of non-tumoural epithelial breast cells and the expression of early carcinogenesis-related genes [3]. A similar relationship between adiposity and carcinogenesis-related genes has been observed in non-tumoural liver cells [61]. In addition, we found a higher expression of the oncogene *survivin/BIRC5* in VAT from individuals with obesity and this overexpression is restored after a weight loss intervention [5]. We proposed that epigenetic mechanisms could be the bridge between obesity and its related-diseases [24–27]. It has been reported that obesity-related diseases epigenetic marks can be reversed after weight loss therapies, such as bariatric surgery [28] and other weight loss therapies [62]. This same hypothesis could be applied to the effect of VAT on the promotion of carcinogenesis, as it was demonstrated in the current study together with a recent study that also demonstrated a differential methylome of periprostatic adipose tissue in obese/overweight compared to normal weight patients with prostate cancer [63].

The relatively small sample size could be a limitation of this study; however, the cohort of patients in both CRC and healthy groups was homogenous enough to avoid possible biases in the analyses. Moreover, stringent filters were applied to produce consistent results. In addition, we adjusted both DMCpGs and DMRs by age, sex, and BMI, to eliminate the confusion effect of these parameters in the differential analysis. Another potential limitation of this study is that it is an association study and, therefore, the nature of the found differences should be further explored.

In conclusion, this study demonstrates that DNA methylation patterns in VAT are strongly associated with CRC, identifying key epigenetic modifications. This epigenetic mark may be regulated to facilitate the development and maintenance of a tumoural phenotype in patients with high susceptibility, such as patients with obesity. Therefore, this study proposes several candidate genes, to further validate in longer cohorts with clinical parameters related to the main outcome of CRC, such as early diagnosis, prognosis, and tumour site detection, as well as the efficacy of neoadjuvant treatment.

Acknowledgments

The authors thank all the participants of this study and the research group involved in the project, as well as the individuals who performed the field work.

Disclosure statement

The authors declare that no competing interests exist.

Funding

This work was supported by grants from the Fondo de Investigacion Sanitaria such as PI17/01287, CP17/00088 and PI18/01399 research projects and CIBERobn from the Instituto de Salud Carlos III (ISCIII)-Subdireccion General de Evaluacion y Fomento de la Investigación; Fondo Europeo de Desarrollo Regional (FEDER), as well as Educación e Ordenación Universitaria, Xunta de Galicia (IN607B2020/09). Ana B Crujeiras received funding by a research contract

'Miguel Servet' (CP17/00088) and Angel Diaz-Lagares by a research contract 'Juan Rodes' (JR17/00016) from ISCIII, co-financed by the European Regional Development Fund (FEDER). M.M.G. was the recipient of the Nicolas Monardes Program from the 'Servicio Andaluz de Salud, Junta de Andalucía,' Spain (RC-0001-2018 and C-0029-2014).

Author contributions

ABC, MMG, AGI, and HB designed and performed experiments, analysed data, and wrote the manuscript; ADL contributed to the analysis and interpretation of data; AIS performed the recruitment of participants; ME, FJT, and FFC contributed to the interpretation of data and discussion. MMG, ABC, ME, FJT, and FFC obtained funding for the study. MMG and ABC are the guarantors of this work and, as such, have full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. All the authors read and approved the final manuscript.

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References

[1] Wang YX, Zhu N, Zhang CJ, et al. Friend or foe: multiple roles of adipose tissue in cancer formation and progression. J Cell Physiol. 2019;234:21436–21449.
[2] Quail DF, Dannenberg AJ. The obese adipose tissue microenvironment in cancer development and progression. Nat Rev Endocrinol. 2019;15:139–154.
[3] Crujeiras AB, Cabia B, Carreira MC, et al. Secreted factors derived from obese visceral adipose tissue regulate the expression of breast malignant transformation genes. Int J Obes. 2016;40(3):514–523. .
[4] Spyrou N, Avgerinos KI, Mantzoros CS, et al. Classic and Novel Adipocytokines at the Intersection of Obesity and Cancer: diagnostic and Therapeutic Strategies. Curr Obes Rep. 2018;7:260–275.
[5] Izquierdo AG, Carreira MC, Rodriguez-Carnero G, et al. Weight loss normalizes enhanced expression of the oncogene survivin in visceral adipose tissue and blood leukocytes from individuals with obesity. Int J Obes. 2021;45(1):206–216. .
[6] Bentham J, Di Cesare M, Bilano V, et al. Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128·9 million children, adolescents, and adults. Lancet. 2017;390(10113):2627–2642.
[7] Lauby-Secretan B, Scoccianti C, Loomis D, et al. Body fatness and cancer - Viewpoint of the IARC working group. N Engl J Med. 2016;375:794–798.
[8] Avgerinos KI, Spyrou N, Mantzoros CS, et al. Obesity and cancer risk: emerging biological mechanisms and perspectives. Metabolism 2019;92:121–135.
[9] Steele CB, Thomas CC, Henley SJ, et al. Vital Signs : trends in Incidence of Cancers Associated with Overweight and Obesity — United States, 2005–2014. MMWR Morb Mortal Wkly Rep. 2017;66 (39):1052–1058. .
[10] Murphy N, Jenab M, Gunter MJ. Adiposity and gastrointestinal cancers: epidemiology, mechanisms and future directions. Nat Rev Gastroenterol Hepatol. 2018;15:659–670.
[11] Nagata N, Sakamoto K, Arai T, et al. Visceral abdominal fat measured by computed tomography is associated with an increased risk of colorectal adenoma. Int J Cancer. 2014;135(10):2273–2281. .
[12] Choe EK, Kim D, Kim HJ, et al. Association of visceral obesity and early colorectal neoplasia. World J Gastroenterol. 2013;19(45):8349–8356.
[13] Liesenfeld DB, Grapov D, Fahrmann JF, et al. Metabolomics and transcriptomics identify pathway differences between visceral and subcutaneous adipose tissue in colorectal cancer patients: the ColoCare study. Am J Clin Nutr. 2015;102(2):433–443. .
[14] Mirza RE, Koh TJ. Contributions of cell subsets to cytokine production during normal and impaired wound healing. Cytokine. 2015;71(2):409–412.
[15] Boughanem H, Cabrera-Mulero A, Hernández-Alonso P, et al. Association between variation of circulating 25-OH vitamin D and methylation of secreted frizzled-related protein 2 in colorectal cancer. Clin Epigenetics. 2020;12(1):83. .
[16] Castellano-Castillo D, Morcillo S, Clemente-Postigo M, et al. Adipose tissue inflammation and VDR expression and methylation in colorectal cancer. Clinical Epigenetics. 2018;10(1):1. .
[17] Castellano-Castillo D, Morcillo S, Crujeiras AB, et al. Association between serum 25-hydroxyvitamin D and global DNA methylation in visceral adipose tissue from colorectal cancer patients. BMC Cancer. 2019;19(1):93.
[18] Boughanem H, Cabrera-Mulero A, Hernández-Alonso P, et al. The expression/methylation profile of adipogenic and inflammatory transcription factors in adi-

pose tissue are linked to obesity-related colorectal cancer. Cancers (Basel). 2019;11:11.

[19] Heyn H, Esteller M. DNA methylation profiling in the clinic: applications and challenges. Nat Rev Genet. 2012;13(10):679–692.

[20] Allum F, Grundberg E. Capturing functional epigenomes for insight into metabolic diseases. Mol Metab. 2020;38:100936.

[21] Huang YT, Chu S, Loucks EB, et al. Epigenome-wide profiling of DNA methylation in paired samples of adipose tissue and blood. Epigenetics. 2016;11(3):227–236.

[22] Crujeiras AB, Diaz-Lagares A, Stefansson OA, et al. Obesity and menopause modify the epigenomic profile of breast cancer. Endocr Relat Cancer. 2017;24(7):351–363.

[23] Crujeiras AB, Morcillo S, Diaz-Lagares A, et al. Identification of an episignature of human colorectal cancer associated with obesity by genome-wide DNA methylation analysis. Int J Obes. 2019;43(1):176–188.

[24] Izquierdo AG, Crujeiras AB. . Role of epigenomic mechanisms in the onset and management of insulin resistance. Rev Endocr Metab Disord. 201920(1):89–102.

[25] Crujeiras AB, Diaz-Lagares A, Moreno-Navarrete JM, et al. Genome-wide DNA methylation pattern in visceral adipose tissue differentiates insulin-resistant from insulin-sensitive obese subjects. Transl Res. 2016;178(13–24.e5).

[26] Crujeiras AB, Casanueva FF. Obesity and the reproductive system disorders: epigenetics as a potential bridge. Hum Reprod Update. 2015;21(2):249–261.

[27] Crujeiras AB, Pissios P, Moreno-Navarrete JM, et al. An epigenetic signature in adipose tissue is linked to nicotinamide N-methyltransferase gene expression. Mol Nutr Food Res. 2018;62:14.

[28] Izquierdo AG, Crujeiras AB. Obesity-related epigenetic changes after bariatric surgery. Front Endocrinol (Lausanne). 2019;10:232.

[29] Crujeiras AB, Izquierdo AG, Primo D, et al. Epigenetic landscape in blood leukocytes following ketosis and weight loss induced by a very low calorie ketogenic diet (VLCKD) in patients with obesity. Clin Nutr. 2021;0:0.

[30] Salas-Pérez F, Cuevas-Sierra A, Cuervo M, et al. Differentially methylated regions (DMRs) in PON3 gene between responders and non-responders to a weight loss dietary intervention: a new tool for precision management of obesity. Epigenetics 202125:1–12.

[31] Crujeiras AB, Campion J, Díaz-Lagares A, et al. Association of weight regain with specific methylation levels in the NPY and POMC promoters in leukocytes of obese men: a translational study. Regul Pept. 2013;186:1–6.

[32] Nicoletti CF, Pinhel MS, Noronha NY, et al. Association of MFSD3 promoter methylation level and weight regain after gastric bypass: assessment for 3 y after surgery. Nutrition. 2020;70:110499.

[33] Bairaktari ET, Seferiadis KI, Elisaf MS. Evaluation of methods for the measurement of low-density lipoprotein cholesterol. J Cardiovasc Pharmacol Ther. 2005;10(1):45–54.

[34] Matthews DR, Hosker JP, Rudenski AS, et al. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28(7):412–419.

[35] Tian Y, Morris TJ, Webster AP, et al. ChAMP: updated methylation analysis pipeline for Illumina BeadChips. Bioinformatics. 2017;33(24):3982–3984.

[36] Aryee MJ, Jaffe AE, Corrada-Bravo H, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics. 2014;30(10):1363–1369.

[37] Szklarczyk D, Franceschini A, Wyder S, et al. STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res. 2015;43(D1):D447–52.

[38] Phipson B, Maksimovic J, Oshlack A. MissMethyl: an R package for analyzing data from Illumina's HumanMethylation450 platform. Bioinformatics. 2016;32(2):286–288.

[39] RStudio Team .(2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA (Computer Software v0.98.1074). http://www.rstudio.com/

[40] Sofer T, Schifano ED, Hoppin JA, et al. A-clustering: a novel method for the detection of co-regulated methylation regions, and regions associated with exposure. Bioinformatics. 2013;29(22):2884–2891.

[41] Kerr AG, Sinha I, Dadvar S, et al. Epigenetic regulation of diabetogenic adipose morphology. Mol Metab. 2019;25:159–167.

[42] Ali MM, Naquiallah D, Qureshi M, et al. DNA methylation profile of genes involved in inflammation and autoimmunity correlates with vascular function in morbidly obese adults. Epigenetics. 2021;25:1–17.

[43] Huang RC, Melton PE, Burton MA, et al. Adiposity associated DNA methylation signatures in adolescents are related to leptin and perinatal factors. Epigenetics 20218;1–18.

[44] Tonks NK. Protein tyrosine phosphatases: from genes, to function, to disease. Nat Rev Mol Cell Biol. 2006;7(11):833–846.

[45] Lee S. The association of genetically controlled CpG methylation (cg158269415) of protein tyrosine phosphatase, receptor type N2 (PTPRN2) with childhood obesity. Sci Rep. 2019;9:1.

[46] Ghashghaei M, Niazi TM, Aguilar-Mahecha A, et al. Identification of a radiosensitivity molecular signature induced by enzalutamide in hormone-sensitive and

hormone-resistant prostate cancer cells. Sci Rep. 2019;9:1.

[47] Sengelaub CA, Navrazhina K, Ross JB, et al. PTPRN 2 and PLC β1 promote metastatic breast cancer cell migration through PI (4,5)P 2 -dependent actin remodeling. EMBO J. 2016;35(1):62–76.

[48] Gentilini D, Scala S, Gaudenzi G, et al. Epigenome-wide association study in hepatocellular carcinoma: identification of stochastic epigenetic mutations through an innovative statistical approach. Oncotarget. 2017;8(26):41890–41902.

[49] Chuikov S, Levi BP, Smith ML, et al. Prdm16 promotes stem cell maintenance in multiple tissues, partly by regulating oxidative stress. Nat Cell Biol. 2010;12 (10):999–1006.

[50] Burghel GJ, Lin WY, Whitehouse H, et al. Identification of candidate driver genes in common focal chromosomal aberrations of microsatellite stable colorectal cancer. PLoS One. 2013;8:12.

[51] Li WD, Jiao H, Wang K, et al. A genome wide association study of plasma uric acid levels in obese cases and never-overweight controls. Obesity. 2013;21:9.

[52] Larsson C, Ali MA, Pandzic T, et al. Loss of DIP2C in RKO cells stimulates changes in DNA methylation and epithelial-mesenchymal transition. BMC Cancer. 2017;17:1.

[53] Li J, Ping JL, Ma B, et al. DIP2C expression in breast cancer and its clinical significance. Pathol Res Pract. 2017;213(11):1394–1399.

[54] Cui C, Lu Z, Yang L, et al. Genome-wide identification of differential methylation between primary and recurrent hepatocellular carcinomas. Mol Carcinog. 2016;55 (7):1163–1174.

[55] Wang Y, Chen Y, Xiao S, et al. Integrated Analysis of the Functions and Prognostic Values of RNA-Binding Proteins in Colorectal Cancer. Front Cell Dev Biol. 2020:595605.

[56] Neeb A, Wallbaum S, Novac N, et al. The immediate early gene Ier2 promotes tumor cell motility and metastasis, and predicts poor survival of colorectal cancer patients. Oncogene. 2012;31(33):3796–3806.

[57] Cozzo AJ, Fuller AM, Makowski L. Contribution of adipose tissue to development of cancer. Compr Physiol. 2018;8(1):237–282.

[58] Stefan N. Causes, consequences, and treatment of metabolically unhealthy fat distribution. Lancet Diabetes Endocrinol. 2020;8(7):616–627.

[59] Carobbio S, Pellegrinelli V, Vidal-Puig A. Adipose tissue function and expandability as determinants of lipotoxicity and the metabolic syndrome. Adv Exp Med Biol. 2017;960:161–196.

[60] Unamuno X, Gómez-Ambrosi J, Rodríguez A, et al. Adipokine dysregulation and adipose tissue inflammation in human obesity. Eur J Clin Invest. 2018;48 (9):e12997.

[61] Izquierdo AG, Carreira MC, Amil M, et al. An energy restriction-based weight loss intervention is able to reverse the effects of obesity on the expression of liver tumor-promoting genes. FASEB J. 2020;34 (2):2312–2325.

[62] Samblas M, Milagro FI, Martínez A. DNA methylation markers in obesity, metabolic syndrome, and weight loss. Epigenetics. 2019;14(5):421–444.

[63] Cheng Y, Monteiro C, Matos A, et al. Epigenome-wide DNA methylation profiling of periprostatic adipose tissue in prostate cancer patients with excess adiposity-a pilot study. Clin Epigenetics. 2018;10:1.