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PH.D. THESIS

UNDERSTANDING OBESITY: NEW INSIGHTS FROM *ANKRD26*

UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II - Dottorato Di Ricerca In Medicina Clinica Sperimentale - DIPARTIMENTO DI SCIENZE MEDICHE TRANSLAZIONALI
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List of Publications

Campitelli M, Desiderio A, Cacace G, Nigro C, Prevenzano I, Leone A, de Simone S, Campiglia P, Formisano P, Raciti GA, Beguinot F, Miele C. *Citrus aurantium* L. Dry Extracts Ameliorate Adipocyte Differentiation of 3T3-L1 Cells Exposed to TNFα by Down-Regulating *miR-155* Expression. Nutrients. 2020 May 28;12(6):1587.

Desiderio A, Longo M, Parrillo L, Campitelli M, Cacace G, de Simone S, Spinelli R, Zatterale F, Cabaro S, Dolce P, Formisano P, Milone M, Miele C, Beguinot F, Raciti GA. Epigenetic silencing of the ANKRD26 gene correlates to the pro-inflammatory profile and increased cardio-metabolic risk factors in human obesity. Clin Epigenetics. 2019 Dec 4;11(1):181.

Abstract

Mounting evidence sustains the role of DNA methylation in determining obesity as well as the down-stream adverse responses to increased BMI. Thus, identifying new players and mechanisms relevant to obesity and its related endo-phenotypes and understanding whether and how DNA methylation changes may affect these targets is of particular importance. My colleagues and I have recently recognized the *Ankyrin repeat domain 26* (*Ankrd26*) gene as an interesting and proper target to study.

This work aims to *i.* provide clearer evidence in humans of the cause-effect relationship between *ANKRD26* gene expression and DNA methylation and investigate the correlation of these changes to obesity-related endo-phenotypes, unhealthy metabolic states and cardio-metabolic risk. Also, I aim to *ii.* establish *in vitro* the obesity-induced hypothalamic regulation of *Ankrd26* gene in terms of DNA methylation changes, and clarify the role of the Ankrd26 protein on the hypothalamic regulation of anorexigenic signals *in vitro*.

i. Hyper-methylation at three specific CpG sites within the *ANKRD26* promoter causes down-regulation of its gene expression and represents a common abnormality in obese patients, particularly if metabolically unhealthy. Furthermore, these mRNA and DNA methylation changes of *ANKRD26* gene correlate to increased Body Mass Index (BMI), and raised levels of both pro-inflammatory molecules and cardio-metabolic risk-related factors in humans.

ii. Down-regulation of *Ankrd26* mRNA and protein expression occur in the hypothalamus of diet-induced obese mice compared to lean control mice. These changes are associated to hyper-methylation of a specific CpG site in the gene promoter. Furthermore, Ankrd26 protein is up-regulated by the treatments with both the hormones, insulin and leptin, and the drug Exendin-4 in murine hypothalamic mHypoE-N46 cells. Also, over-expression of Ankrd26 modulates MAPK signaling and neuropeptide gene expression, by increasing the mRNA of the anorexigenic *POMC* and *CART* and decreasing the mRNA of the orexigenic *AgRP*, in mHypoE-N46-*Ankrd26* cells.

In conclusion, the results showed in my PhD thesis demonstrated that down-regulation of the *ANKRD26* gene and hyper-methylation at specific CpGs of its promoter are common abnormalities in obesity and mark adverse health outcomes. Also, my data demonstrate that Ankrd26 protein might play a pivotal role in the regulation of energy homeostasis *in vitro,* acting as down-stream effector of anorexigenic signals in hypothalamic neuronal cells.

1.Introduction

1.1 Obesity: general overview

Obesity is a major worldwide threat to human health and was defined by the World Health Organization (WHO) as a global epidemic of this century [1]. According to WHO data, in 2014 more than 2.1 billion adults have been classified as overweight or obese globally, of which 1.5 billion were overweight, and 640 million were obese [2]. In this same year, in the adult population, the age-standardized prevalence of obesity was estimated to 10.8 % in men and 14.9 % in women [3]. Noteworthy, obesity today is not just a problem for adults. In 2013-2014, the worldwide number of children and adolescents (2-19 years of age) with obesity was estimated to be 110 million; this number has doubled since 1980 [1]. Currently, overweight and obesity in adults is determined by the measure of the body mass index (BMI), an indirect measure of body fat, where the individual's weight in kilograms is divided by the square of his/her height in meters (kg m^{-2}) [2,4]. According to this parameter, subjects are thus classified as overweight (BMI $\geq$ 25 and < 30), and Class I (BMI $\geq$ 30 and < 34.9), Class II (BMI $\geq$ 35 and < 39.9) and Class III (BMI > 40) obese [5].

Obesity *per se* is a chronic disorder characterized by excess fat storage, arising from an imbalance between energy intake and energy expenditure [6]. Multiple factors interact to produce a state of positive energy contributing to excessive adiposity, such as environmental factors, low diet quality, physical inactivity, gut microbiota, endocrine disruptors, drugs, etc. [4,7]. When the energy intake exceeds the energy expenditure, the excess energy is stored as fat in the white adipose tissue (WAT). Compared to other tissues in humans, WAT has indeed the unique peculiarity to remodel itself by changing its dimensions in response to nutritional demands. Remodelling of WAT can be accomplished or by increasing the size of adipose cells (hypertrophy) or by recruiting new adipocytes from the resident pool of precursor cells (hyperplasia) or by both processes [8]. In response to caloric overload, WAT depots usually expand by hypertrophy until a critical threshold is reached. Then, hypertrophy is followed by cell death and by the secretion of several mediators that increase cell proliferation and/or differentiation of pre-adipocytes [8]. In particular, the increase of visceral white adipose tissue (VAT), rather than subcutaneous white adipose tissue (SAT), is associated with a so-called metabolically unhealthy phenotype in obese individuals (MUO), a higher risk of diseases such as insulin resistance, Type 2 Diabetes (T2D) and hypertension, and closely correlates with the development of various metabolic abnormalities, commonly referred to as the metabolic syndrome (Figure 1) [9].

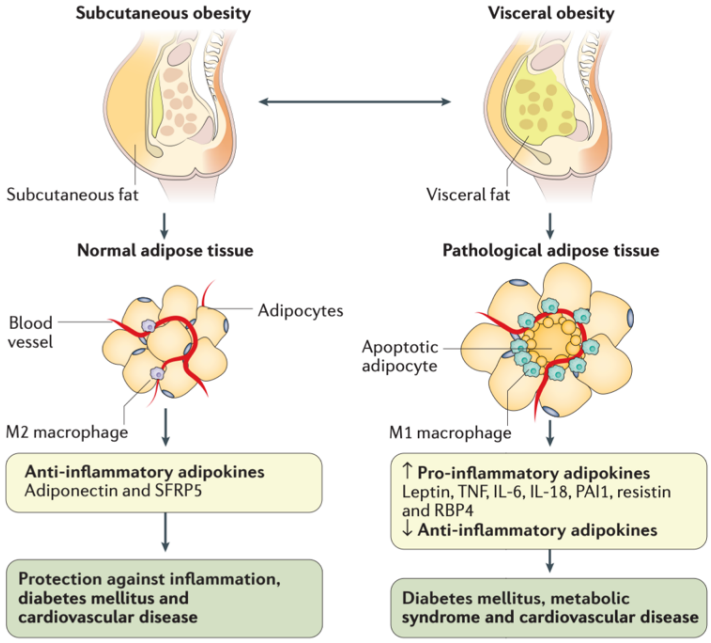

Figure 1. Pathological changes in adipose tissue. A large-scale study revealed that the absolute value of visceral fat was correlated with obesity-associated cardiovascular risks, but cardiovascular risks did not increase with subcutaneous fat. Accordingly, adipose tissues in subcutaneous fat obesity might generally function with the expected release of anti-inflammatory adipokines, whereas adipose tissues in visceral fat obesity release an increased amount of pro-inflammatory adipokines and suppress the secretion of anti-inflammatory adipocytokines, thereby creating low-grade inflammation, which contributes to systemic metabolic and cardiovascular impairment that is associated with obesity-related disorders. Pathological changes in visceral adipose tissue show higher levels of adipocyte necrosis, owing in part to abnormal oxygen tension in the expanded fat depots and the recruitment of macrophages with an inflammatory phenotype (M1 macrophages) that are arranged around dead cells in crown-like structures. PAI1, prothrombin activator inhibitor 1; RBP4, retinol-binding protein 4; SFRP5, secreted frizzled-related protein 5; TNF, tumour necrosis factor. Adapted from Nat Rev Dis Primers. 2017; 3:17034.

1.2 Obesity and comorbidities

Obesity has a profound impact on quality of life, even in seemingly healthy individuals [10]. Obese individuals have indeed high risks of adverse health outcomes [11]. The prevalence of obesity is associated with the developing features of metabolic syndrome. Other common comorbidities related to obesity are T2D, respiratory problems (*e.g.*, sleep apnoea), dyslipidemia, hypertension, cardiovascular diseases (CVD; *e.g.*, atherosclerosis and heart attack) and cancers (*e.g.*, endometrial, liver and kidney cancers) [12]. Moreover, obesity has negative impacts on psychological and mood issues and cognitive function [13].

In particular, the association between obesity and several unfavourable prognostic factors, including T2D and CVD, is well documented [3]. Obese subjects have a 10-40 fold higher risk of developing T2D [7].

Furthermore, studies conducted in the United States indicate that African Americans exhibited a higher prevalence of extreme obesity and higher risk of future onset of T2D than other ethnicities [14]. Also, though Asians have a lower BMI than white individuals, they showed to be prone to visceral fat deposition, making this population more susceptible to developing T2D at a lower BMI than white individuals [9]. Likewise, also the incidence of heart failure increases progressively with the BMI increase [2].

It needs to be mentioned that a small percentage of obese subjects do not develop obesity-related complications. It is indeed recognized that among the obese people, one out of three is "metabolically healthy" (MHO), which means that his/her fasting glucose, triglycerides (TG), and other metabolic markers are normal. This obese population is at no higher risk of T2D or CVD than their metabolically healthy lean (MHL) counterparts [15]. How this is possible is still unclear, but several studies have shown that body fat distribution (*e.g.*, expansion of SAT rather than VAT in response to caloric overload) more than BMI *per se* is crucial in T2D or CVD risk assessment [9].

In recent years, much interest has been raised on the potential role of regional adipose tissue distributions in contributing to obesity-related complications [16]. Follow-up studies on healthy subjects at baseline revealed higher levels of cardiovascular risk factors such as BMI, low-density lipoprotein cholesterol (LDL-C), TG and blood pressure in individuals who have developed T2D compared to those who have not develop diabetes [16]. This led to the hypothesis of the "ticking clock", which means that the risk factors for ischemic heart disease are probably operative before diabetes onset [17]. Fasting or post-prandial hyper-glycaemia, overweight/obesity, elevated blood pressure and dyslipidaemia have been defined as cardio-metabolic risk factors because of their close relation to both diabetes and CVD [9, 18]. In addition to the above, the assessment of visceral obesity through both the waist to hip circumference ratio (WHR) and the visceral adiposity index (VAI) can indirectly represent an excellent index of cardio-metabolic risk, as a reflection of both insulin sensitivity and cardiovascular risk [9]. Several other studies have also shown that the TG/HDL-C ratio, which is high in patients with visceral obesity, represents a good index of cardio-metabolic risk and insulin resistance [9]. In a case-control study in patients with coronary artery disease, high TG/HDL-C ratio levels relate to 16 times increase risk of myocardial infarction compared to patients with low TG/HDL-C ratio [19].

Furthermore, abdominal obesity is also associated with chronic low-grade inflammation. In fact, the inflammatory state allows the possibility of establishing a link between obesity, insulin resistance, hypertension and adverse cardiovascular events [16]. The C-Reactive Protein (CRP), a non-glycosylated polymer, consisting of five identical subunits, produced by liver when antigens need to be fought, is a good marker of inflammation and cardio-metabolic risk [20]. Liver secretes CRP in response to cytokines' production by inflammatory cells, particularly Interleukin 6 (IL-6) [20]. Many studies have shown the relationship between serum CRP levels and cardiovascular morbidity and mortality. It has been also hypothesized that CRP has a pro-inflammatory role, stimulates complement activation and can exacerbate tissue damage. Also, elevated CRP levels may reflect increased atherosclerotic plaque formation and an increased tendency for plaque to thrombosis and rupture.

Although the markers described above allow to well-assess the risk of cardio-metabolic disease in obese, discovery of new markers, whether they are genetic, epigenetic or biochemical, is necessary for improving the detection of high-risk individuals in the obese population.

1.3 Central and peripheral aspects of feeding behavior and energy homeostasis in healthy and obese humans

As above mentioned, obesity results from the dysregulation of energy metabolism [21]. The central nervous system (CNS) plays a crucial role in sensing and controlling the organism's energy status [22]. The hypothalamus and, in particular, the arcuate nucleus (ARC) within the hypothalamus are particularly important for regulating feeding and metabolism [22]. The ARC, which is located close to the median eminence (ME), a fenestrated capillary-rich circumventricular organ leading to a "leaky" blood-brain barrier (BBB), indeed, combines hormonal and nutritional metabolic signals from the bloodstream and central neuronal inputs to generate coordinated feedback response. Within ARC, there are two distinct and functionally antagonistic subtypes of neurons: the orexigenic (appetite-stimulating) neurons, which express the neuropeptide Y (NPY) and the agouti-related peptide (AgRP), and the anorexigenic (appetite-suppressing) neurons, which express the pro-opiomelanocortin (POMC) [23, 24].

POMC neurons project to second-order neurons mainly into the paraventricular hypothalamic nucleus (PVN), but also into the dorsomedial hypothalamus (DMH), the lateral hypothalamus (LH) and the ventromedial hypothalamus (VMH) [25]. In turn, these second-order neurons project multiple neuro-circuits outside of the hypothalamus (extra-hypothalamic response) [26].

E.g., neurons within the PVN control sympathetic outflow to peripheral organs [28] and secrete various regulatory neuropeptides [26]. POMC, which is cleaved to α-melanocyte-stimulating hormone (α-MSH) after nutrient absorption, is released from POMC axons to stimulate melanocortin 3 and 4 receptors (MC3/4R) on downstream neurons, resulting in a decrease in food intake and an increase in energy expenditure [27].

AgRP/NPY neurons also project to second-order neurons within the PVN and the LH [29]. Fasting induces their activation and determines the co-release of NPY and AgRP. NPY directly stimulates food intake via NPY Y1 and Y5 receptors activation [30,31]. Furthermore, NPY reduces the energy expenditure by reducing tyrosine hydroxylase expression into the PVN and the brainstem via Y1-receptor, leading to a decreased sympathetic output to the brown adipose tissue (BAT) and concomitantly reducing BAT activity [32]. AgRP, instead, acts as an inverse agonist of MC3/4R, thereby preventing the anorexigenic effect of α-MSH on second-order neurons [33]. Furthermore, AgRP/NPY neurons directly inhibit POMC neurons via inhibitory γ-aminobutyric acid (GABA) action at the ARC level (Figure 2) [34].

In addition to the highly complex neuropeptide signalling in the hypothalamus, several peripheral hormones act directly on these neurons to alter neuropeptide secretion. These effectors include both anorexigenic and orexigenic hormones [35]. Insulin and leptin are the two main long-term regulators of food intake and energy balance [21, 22]. Their bloodstream levels are directly proportional to adipose mass, and CNS access occurs via saturable receptor-mediated processes. The primary CNS target for these "*adipostats*" is the ARC, where leptin and insulin receptors are highly expressed and where direct administration of either hormone has a potent effect on food intake and body weight [21].

Insulin, secreted from β-cells of the pancreas upon nutrient ingestion, plays an important role in the peripheral regulation of energy homeostasis [36]. Once insulin binds its receptors on POMC neurons, it causes membrane hyperpolarization, reduced firing of these neurons, and increases POMC mRNA expression [23,24]. Also, insulin receptor (IR) activation on AgRP neurons induces hyperpolarization and reduction in the firing rate of these neurons, thus decreasing the release of AgRP and other neurotransmitters [33].

Leptin, an adipose tissue-derived hormone, codified by the *LEP* gene, regulates food intake and energy homeostasis at several levels [25]. It directly excites POMC neurons inducing POMC expression, while it inhibits AgRP/NPY neurons and AgRP expression [28]. Interestingly, although insulin and leptin actions are interconnected, they act on different subpopulations of POMC

neurons, and both are required for a complete anorexigenic effect at the hypothalamic level [23].

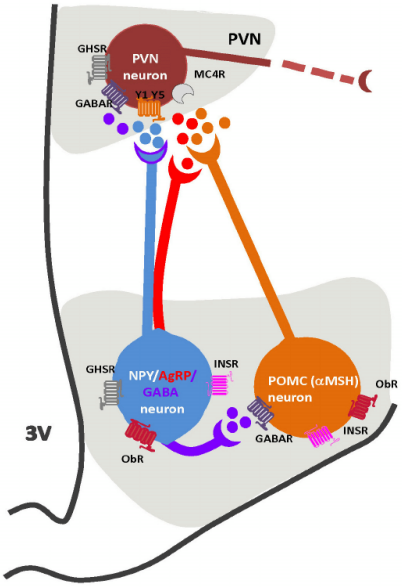

Figure 2. The melanocortin system (ARC-PVN) circuits controlling food intake and body weight regulation. Adapted from: *Hypothalamic control of food intake and energy metabolism; Reichenbach and Andrews, 2012*

Other molecules involved in the regulation of food intake are the glucagon-like peptide 1 (GLP1), the cholecystokinin (CCK), and the ghrelin.

GLP1 is primarily produced and secreted from entero-endocrine L-cells of the small intestine in response to nutrient stimuli and is a product of post-translational modification of pro-glucagon [37]. As an incretin, GLP1 directly acts on the pancreatic β-cells to enhance glucose stimulation of insulin secretion, but also exerts anorexigenic activity. Indeed, intracerebroventricular (ICV) administration of GLP1 suppresses appetite; also, more impressively, ICV administration of GLP1 antagonist increases food intake [38]. GLP1 has been proposed as potential drug for weight loss treatment. However, because of its short circulating half-life, due to rapid proteolysis by dipeptidyl peptidase IV (DPP-IV), its therapeutic utility in obese patients was limited [39]. This pharmacokinetic limitation has been now abolished by employing GLP1 analogues resistant to DPP-IV degradation, such as liraglutide and exenatide [40]. Similarly to GLP1, even CCK exerts anorexigenic activity [42]. CCK is produced and released by gut in response to proteins in meal and has long been

thought to be an endogenous satiety factor [41,42]. To note, administration of a CCK antagonist stimulate food intake [43]. Differently from GLP1 and CKK, ghrelin is the only known circulating orexigenic hormone. Ghrelin derives from the cleavage of pre-proghrelin and is mainly produced and secreted in the gastric fundus [44]. In rodents, ICV or peripheral administration of ghrelin induces a dose-dependent increase in food intake and bodyweight [45, 46]. Ghrelin also regulates long-term energy homeostasis. It has been reported that ghrelin induces hunger and feeding by activating NPY/AgRP neurons in the ARC [47].

However, into pathological conditions such as obesity, the mechanisms above described resulted to be altered. Obesity *per se* is characterized by chronic low-grade inflammation and resistance to leptin and insulin action not only in the periphery but also centrally in the CNS [48]. As an early event in obesity development, but even within a few days of ingesting calorie-dense diet, an increased amount of free fatty acids (FFAs) from the periphery crosses the BBB and induces inflammatory response in hypothalamic neurons [49]. Local inflammation in turns promotes endoplasmic reticulum stress in hypothalamic neurons, leading to insulin and leptin resistance [50]. It has been recently reported that constitutive activation of the pro-inflammatory c-Jun N-terminal kinase 1 (JNK1) pathway in AgRP neurons induces an increased spontaneous firing in these cells, along with leptin resistance, resulting in hyperphagia, increased weight gain, and adiposity [51]. Also, hypothalamic inflammation triggers the constitutive activation of inhibitor of nuclear factor kappa-B kinase 2 (IKK2) pathway, blunting the response of the AgRP neurons to insulin and thus impairing systemic glucose homeostasis [51]. Although POMC and AgRP neurons in the ARC nucleus develop insulin resistance in response to calorie-dense diet, the steroidogenic factor 1 (SF-1) neurons in the VMH improve their insulin sensitivity, leading to an impairment of the glutamatergic innervation of SF-1 neurons to POMC neurons, which in turn affects POMC activation [48,52]. Also, in obesity, leptin sensitivity is retained in the DMH and increased leptin action on these neurons contributes to obesity-related hypertension [53]. Thus, obesity is associated with selective insulin and leptin resistance at the CNS level, whereby leptin and insulin action are attenuated in specific neuronal populations. In contrast, other neurons become more leptin- and insulin-sensitive, thus further promoting hyperphagia, weight gain, and dysregulation in glucose homeostasis [48].

Leptin and insulin resistance in the CNS can also result from over-activation of signal transducer and activator of transcription 3/suppressor of cytokine signaling 3 (STAT3/SOCS3). Chronic activation of STAT3, *e.g.* due to elevated leptin resulting from growing adiposity, leads to increased SOCS3 activation,

which in turn inhibits STAT3 signaling in a negative feedback manner resulting in resistance, not only to leptin, but also to insulin [54].

However, obesity and calorie-dense diet are not only associated with a central resistance to insulin and leptin. Indeed, there is also evidence for the impaired ability of ghrelin to induce food intake at the level of the ARC AgRP/NPY neurons. This event could be an adaptive response to prevent further food intake in obese individuals [55]. Also, lower circulating ghrelin levels are found in obese subjects. Again, this is presumably an adaptive mechanism [56].

1.4 Genetic basis of obesity

Genetics has always been considered a "fertile ground" for the identification of disease risk factors. Obesity was not an exception. The role of genetics in obesity has been supported by several populations, families, and twin-based studies [57]. Studies in families have revealed that obesity is a risk factor for childhood obesity [58]. Trends of obesity prevalence related to different ethnicity have instead confirmed a significant contribution of genes to obesity heredity [58].

Furthermore, studies in twins have demonstrated more identical body fat acquisition in monozygotic compared to dizygotic twins, suggesting that this feature is severely impacted by genotype [58,59]. Finally, studies in adopted children furtherly supported the genetic aspect of obesity. Indeed, the BMI of these children corresponded more with the BMI of their biological parents rather than the BMI of their adopted parents [58].

Today, it is estimated that genetic factors contribute to the rise of obesity, with inheritance rates across populations varying from 50 to 70% [60,61,62]. However, while heritability studies have been essential in understanding the genetics of obesity, the analysis of these studies needs more attention since certain estimates of heritability could be exacerbated by an unrecognized shared environment [57].

The completion of the human genome sequence, together with scientific and methodological advances, has deeply contributed to discovering of a variety of genes associated with syndromic monogenic, non-syndromic monogenic, oligogenic, and polygenic obesity [58].

Syndromic monogenic obesity is exceptionally uncommon and is characterized by mental disease, dysmorphic highlights, and organ-specific anomalies, in addition to obesity. More than 30 syndromes have been identified [58]. The *Bardet-Biedl Syndrome* (BBS), the first recorded obesity syndrome, is a genetically and clinically heterogeneous ciliopathy with autosomal recessive inheritance. The 72-92% of BBS patients feature hyperphagia, which results

from central and peripheral obesity. Retinal degeneration, developmental deficiency, polydactyly, genital and renal disorders are included in clinical features. Approximately, 19 BBS genes, encoding proteins responsible for both primary cilia formation and function, have been reported to date [58]. Mutations within these genes causes cilia dysfunction, which in turn impairs both food intake regulation and adipocyte differentiation [59]. Non-syndromic monogenic obesity means a single gene disorder, which results in a highly penetrating disease [58]. Recent studies have shown that the majority of mutations have been found in just ten genes, eight of which can explain only the 10% of cases of extreme obesity. These genes are *leptin* (*LEP*)*, leptin receptor* (*LEPR*)*, MC4R, POMC* and other protein-coding genes that are part of the leptin-melanocortin signalling in the hypothalamus. Homozygous or compound heterozygous loss-of-function mutations within these genes contribute to unusual forms of fully penetrant obesity in humans. On the contrary, heterozygous deleterious coding mutations of these genes may result in non-fully penetrant oligogenic obesity [58]. Defects in multiple genes may instead cause polygenic form of obesity with modest effects [58].

Linkage/positional cloning, candidate gene and genome-wide association study (GWAS) have been used to identify genetic loci for obesity traits [62]. The first significant achievement of GWAS identified a cluster of common non-coding variants in *the fat mass and obesity-associated* (*FTO*) locus, which was highly correlated with the risk of obesity in children and adults [58,62]. The *FTO* genotype contributes to body weight by controlling appetite, thermogenesis, browning of adipocytes and obesity-related epigenetic mechanisms [65,66]. After *FTO*, more than 135 additional variants of other genetic loci linked with BMI, adult obesity, childhood obesity, and waist-to-hip ratio (WHR) have been identified (Figure 3) [58,62,63,64].

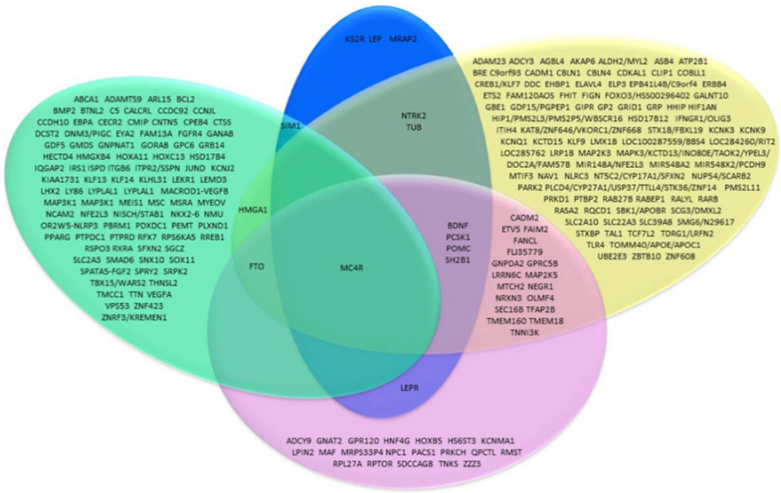

Figure 3. Venn diagram of genes involved in monogenic, oligogenic and polygenic obesity. Monogenic/oligogenic obesity genes are showed in blue, polygenic BMI-related genes in yellow, overweight or obesity-related genes in purple and fat distribution-related genes in green. Marie Pigeyre et al. Clin. Sci. 2016;130:943-986

However, GWAS have some limitations. *E.g.*, rare mutations can not be identified because SNP genotyping arrays are normally intended to identify common SNPs [57]. Recent developments in next-generation sequencing (NGS) technologies will allow researchers to rapidly evaluate the contribution of rare variants to disease predisposition by expanding their GWAS beyond common SNPs. Another source of heritability often missed by GWAS is the contribution of copy number variants (CNVs), which are chromosomal segments encompassing large replications or deletions in genetic loci. Common bi-allelic CNVs, identified in more than 5% of the population, have been reported in association with BMI and obesity. However, similarly, to common SNPs, these common CNVs have small effect sizes on obesity risks. Also, some CNVs associated with obesity are difficult to replicate across populations because they can have ethnicity-specific impacts [58]. Finally, there is no doubt that, in addition to genetic variants, other mechanisms are involved into the pathogenesis of obesity. Several evidences indeed sustain that gene-environment interactions and epigenetic information contribute to obesity.

1.5 DNA methylation and obesity

The genetic information in the DNA sequence is largely the same in every cell group of a multicellular eukaryotic organism [67]. However, considering the same genomic sequence, cells in various tissues and organs display significantly

different gene expression profiles and unique functions [68]. Also, complex gene expression patterns need to be properly triggered and retained and to respond to changing developmental and environmental stimuli [69]. Therefore, much of the distinctions between specialized cells are epigenetic and non-genetic [67]. Today, epigenetics has been described as "a study of changes in gene function that are mitotic and/or meiotically hereditary and do not involve a change in DNA sequence" [70]. The main epigenetic mechanisms include DNA methylation, small interfering RNA (*miRNAs*), and histone post-translational modifications (PTMs). To date, however, only DNA methylation has been shown to be stably inherited.

DNA methylation occurs mostly in cytosine residues, primarily in cytosine-guanine nucleotide (CpG) sites along the vertebrates' DNA chain. It is a covalent modification resulting in the addition of the methyl group (CH_3) to the cytosine ring's 5' carbon position. Usually, it leads to transcriptional silencing of the genes [71]. Many findings support DNA methylation as the main epigenetic mechanisms involved in obesity. Data from mouse models have revealed that obesity development induced by environmental exposures, such as diet, exercise, parental nutrition and intrauterine environment, is associated with DNA methylation changes at genes involved in the regulation of adipogenesis, energy homeostasis, appetite and satiety [72]. Similarly, increasing evidence in humans indicates an association between environmental-induced changes of global or gene-specific DNA methylation and the development of or susceptibility toward obesity. Hypo-methylation at the *tumor necrosis factor α* (*TNFα*) in peripheral blood leukocytes (PBLs), *pyruvate dehydrogenase kinase 4* (*PDK4*) in skeletal muscle, and *LEP* in whole blood, as well as, hyper-methylation at the *POMC* in whole blood, *PPARγ coactivator 1 alpha* (*PGC1α*) in skeletal muscle, and *Circadian Locomotor Output Cycles Kaput* (*CLOCK*) in PBLs were among the marks reported to be differentially methylated in obese compared with lean individuals [73]. Moreover, associations of BMI, adiposity, and waist circumference with DNA methylation changes at *PDK4* in skeletal muscle, *melanin-concentrating hormone receptor 1* (*MCHR1*) in whole blood, and *serotonin transporter* (*SLC6A4*), *androgen receptor* (*AR*), and *glucocorticoid receptor* (*GR*) in PBL have also been reported [73]. Interestingly, a direct association between increased DNA methylation of a region straddling the Intron2/Exon 3 of the *POMC* locus and childhood obesity has been reported [74]. Also, the hyper-methylation of this region functionally impairs the binding activity of the histone acetyltransferase/transcriptional coactivator p300 and thus causes *POMC* gene silencing [74].

The development of high-throughput sequencing technologies and

epigenome-wide association study (EWASs) has opened a new window for identifying a large number of differentially methylated genes and CpGs. *E.g.*, *Dick et al.* have reported in a study conducted on cohorts of European that increased BMI in adults is associated with specific hyper-methylation at the *hypoxia-inducible factor 3 alpha* (*HIF-3α*) locus in blood cells and adipose tissue [75]. *Ronn et al.* have instead demonstrated an association between age, BMI, and/or HbA1c levels and DNA methylation and expression in several genes, including *ELOVL Fatty Acid Elongase 2* (*ELOVL2*), *Four and a half LIM domains protein 2* (*FHL2*), *Kruppel Like Factor 14* (*KLF14*), *Glycine Receptor Alpha 1* (*GLRA1*) and *Ankyrin repeat domain 26* (*ANKRD26*) genes, by analyzing whole-genomic DNA methylation and gene expression in human adipose tissue from European individuals [76]. Furthermore, *Crujeiras et al.*, by using DNA samples isolated from SAT and circulating leukocytes from obese and lean patients, have recently recognized new DNA methylation biological markers of obesity-related adipose tissue dysfunction in blood sample [77]. Collectively, all these EWAS studies have furnished evidence that obesity is associated with modification of DNA methylation status of several metabolically relevant genes, including both obesity candidate genes and genes regulating adipose tissue function, cell differentiation, immune response, and transcription. Importantly, they have also demonstrated that CpG methylation changes in blood can mirror epigenetic signatures in target tissues for metabolic diseases such as adipose tissue. It is important to note that in addition to CpG methylation recent evidence are revealing that other epigenetic mechanisms, including miRNAs and histone modifications, might be also directly involved in obesity [78].

Overall, important progress has been made in the field of epigenetics and obesity. Nevertheless, there is still much to be learned before we fully understand the (causal) function of the epigenetic processes in the development of complex disorders such as obesity, and evaluating the epigenome at the right time in life and in the relevant tissues is a major barrier for studies in humans.

1.6 *ANKRD26* gene and obesity

Our laboratory has recognized the *Ankyrin Repeat Domain 26* (*ANKRD26*) gene as an interesting and proper target to study [79].

Through a methylated DNA immuno-precipitation sequencing (MeDIP-seq) approach, we have indeed demonstrated that high-fat feeding/obesity triggers a DNA methylation reprogramming of the *Ankrd26* gene [79]. High-fat exposure/obesity indeed causes a specific hyper-methylation of the *Ankrd26* promoter at the - 436 and - 431 bp CpG sites, which is dependent upon enhanced

binding of the *de novo* DNA methyltransferases 3a and 3b to the same *Ankrd26* promoter region. These changes are followed by down-regulation of *Ankrd26* expression in the white fat depots [79] (Figure 4).

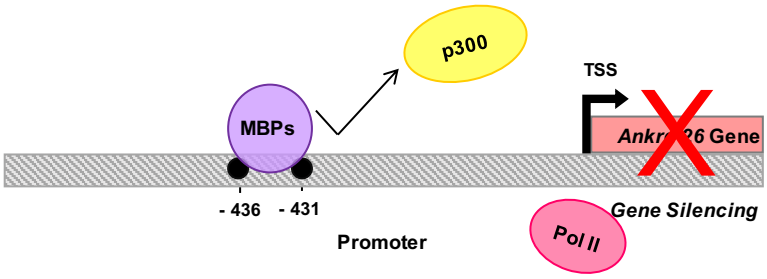

Figure 4. Schematic representation of the epigenetic regulation at the *Ankrd26* promoter gene in mice. The strong reduction of the *Ankrd26* mRNA expression in VAT of HFD fed mice is due to an epigenetic regulation that involve the hyper-methylation of two specific CpG sites by Dnmts 3a and 3b, the bind of Mbd2 to the methylated CpG sites that in turn crowd out the HAT p300 recruitment to the *Ankrd26* promoter, resulting in hypo-acetylation of histone H4 and reduced binding of RNA Pol II on the TSS of the *Ankrd26* gene.

In addition to ours, other groups have further described *Ankrd26* as a gene involved in the regulation of feeding behavior and in the development of both obesity and diabetes in animal models [80]. In mice, Ankrd26 protein indeed resulted to be highly abundant in the hypothalamus and other regions of the brain, known to play a crucial role in the regulation of feeding behaviour, as well as in many tissues and organs, including insulin target tissues like liver, skeletal muscle and WAT. Furthermore, mutant mice with a partial inactivation of this gene (*Ankrd26* MT) show obese and diabetic phenotypes which result from marked hyperphagia rather than reduction in energy expenditure or activity (Figure 5) [80]. Also, it has been reported that the C-terminal deletion of this gene, *in vivo*, in the *Ankrd26* MT mice, leads to defects in primary cilia formation in CNS areas which regulate both food intake and energy homeostasis [81].

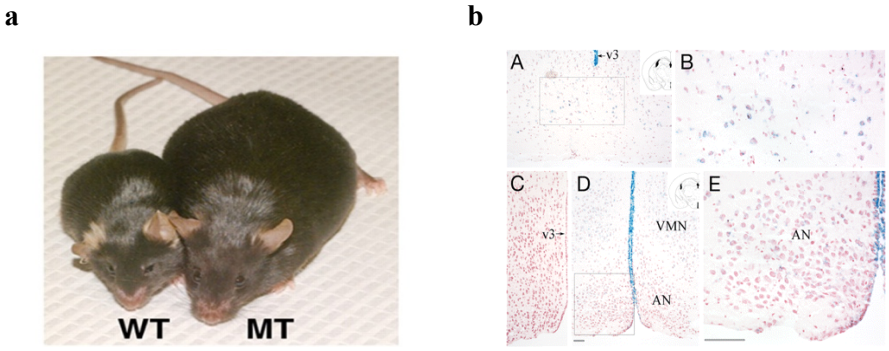

Figure 5. Photomicrograph of a homozygous *Ankrd26* mutant (MT) and wild-type (WT) mice; *LacZ* staining of Ankrd26-expressing cells in the mouse medial basal hypothalamus. Adapted from *Proc. Natl. Acad. Sci. U S A.* 2008; 105:270-275.

The Ankrd26 protein (190 kDa) is located in the cytosol, close to the inner aspect of the cell membrane, where it could act as scaffold protein regulating the function of signalling proteins and effectors. Indeed, it contains two conserved domains: ankyrin repeats and spectrin regions known to mediate protein-protein interactions [82]. To date, four proteins, the triple functional domain protein (Trio), the G protein pathway suppressor 2 (Gps2), the delta-interacting protein A (Dipa) and the hyaluronan-mediated motility receptor (Hmmr), have been identified as Ankrd26 interactors and have been reported to cooperate with Ankrd26 in adipocyte regulation [82]. Indeed, the specific silencing of *Ankrd26*, *Trio*, *Gps2*, *Dipa* or *Hmmr* enhanced adipocyte differentiation upon induction in 3T3-L1 cells, suggesting that *Ankrd26* may exert its pro-adipogenic function by modulating the localization and/or function of these interacting partners [82]. These findings also indicate that the C-terminus domain of Ankrd26 is responsible for its protein-protein interactions and its biological functions [82]. In addition to this, the role of Ankrd26 in the regulation of adipocyte differentiation has been confirmed in mouse embryonic fibroblasts from *Ankrd26* mutant mice (MEFs *Ankrd26*$^{-/-}$) [83]. Indeed, the partial disruption of the *Ankrd26* gene enhanced adipogenesis, both at the level of pre-adipocyte commitment and early and late differentiation steps, in these cells [83].

In humans, the *ANKRD26* gene is located at chromosome 10p12, a locus previously associated with maternally inherited obesity in humans [84]. To note, two rare SNPs at the *ANKRD26* gene, the rs139049098 (minor allele frequency, MAF: *C* = 0.0004/2) and the rs191015656 (MAF: *A* = 0.0004/2), have been also associated with severe obesity in humans [85]. Furthermore, very recently, the *ANKRD26* gene SNP, rs7081476 (MAF: *C* = 0.0541/271), has been associated across multiple studies to a variety of diseases, including diabetes and CVDs,

and to different endo-phenotypes, such as BMI, HDL-C, TG, blood glucose, and HbA1c [86]. Additionally, computational data from a genome-wide DNA methylation analysis in SAT revealed that the *ANKRD26* gene is included in a list of 2825 genes where DNA methylation and mRNA expression levels correlate significantly with BMI in humans [86]. Very recently, we found that down-regulation of *ANKRD26* mRNA and hyper-methylation of a specific region of the *ANKRD26* promoter, embedding the CpG dinucleotides - 689, - 659, and - 651 bp, occur in PBL from obese compared with the lean subjects (Figure 6). These data suggest that epigenetic regulation of *ANKRD26* gene may represent a pathogenic mechanism by which environmental exposures to nutrients contribute to disease susceptibility through epigenetic modifications.

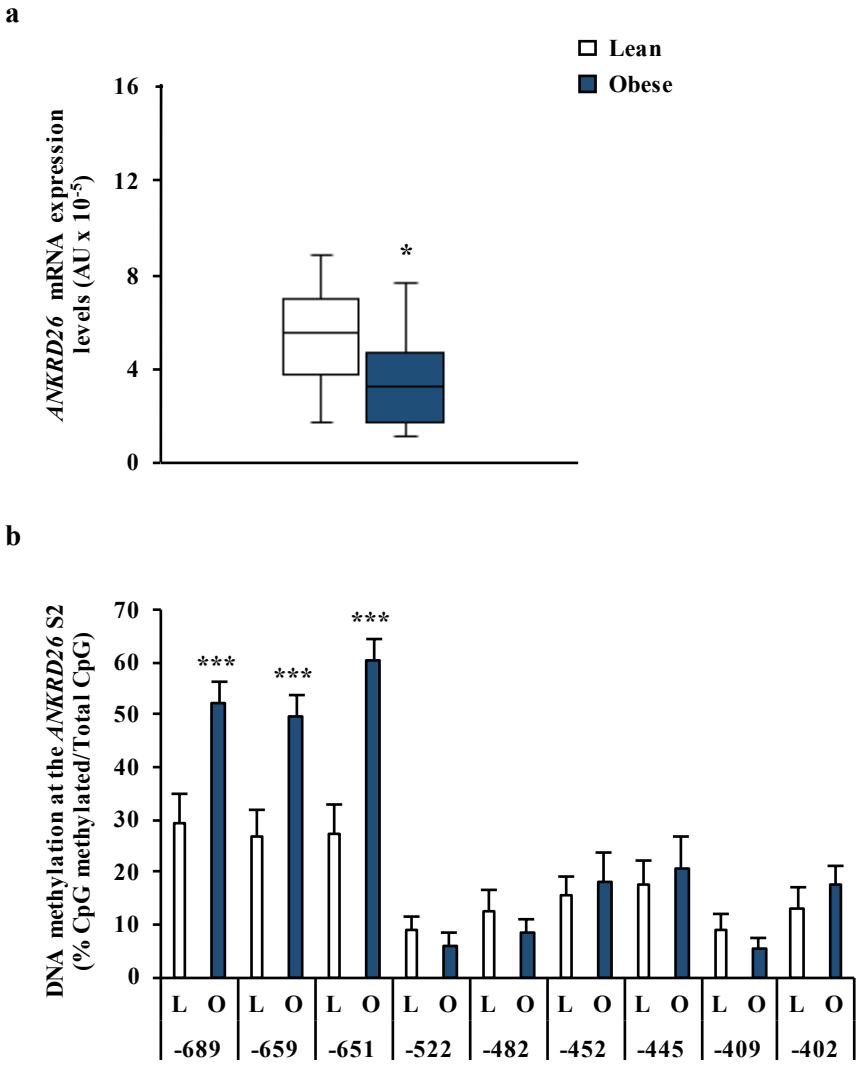

Figure 6. *ANKRD26* mRNA levels and *ANKRD26* promoter methylation in PBL of lean and obese subjects. a) *ANKRD26* mRNA levels were determined in lean (n = 14) and obese (n = 20) subjects. Values are expressed in absolute units (AU) and their distribution within each group is represented by box plots. Box plots show median (line within the box), quartiles (upper and lower box boundaries), and extreme values (whiskers). Statistical differences between the two groups were tested using nonparametric quantile regression, with inference based on median, to adjust for age. $p = 0.027$ *vs* lean. b) Bisulfite sequencing methylation analysis of the CpG sites, - 689, - 659, - 651, within *ANKRD26* promoter in converted genomic DNA from PBL of 14 lean and 20 obese subjects. Results are means ± SD. Statistical difference between the means of the two groups was assessed by classical OLS regression model to adjust for age. *$p < 0.5$ and ***$p < 0.001$ *vs* lean % CpG methylation.

2. Aims of the study

Obesity is now considered to be a major threat to human health and well-being worldwide. This makes the need of achieving a better understanding of its pathogenesis a priority. Furthermore, recent clinical, epidemiological and experimental evidence points out that epigenetic modifications have an extraordinary impact on the natural history of this disorder and may explain predisposition for obesity in cases of familial aggregation or as effects of environmental exposure. Thus, identifying new players relevant to the regulation of feeding behavior and understanding how epigenetic changes on these targets may contribute to human obesity is of particular importance.

My colleagues and I have recognized the *Ankyrin repeat domain 26* (*Ankrd26*) gene as an interesting and proper target. The *Ankrd26* is a newly described gene implied in the feeding behavior regulation and in the development of both obesity and diabetes in mice. Indeed, mice with a partial inactivation of this gene are diabetics and show an obese phenotype resulting from a marked hyperphagia. More, we have recently identified *Ankrd26* as a target sensitive to environmental-induced epigenetic modifications (DNA hyper-methylation) in a mouse model of diet-induced obesity. Noteworthy, we have also reported that changes of the *ANKRD26* gene expression and DNA methylation at specific CpG sites within the *ANKRD26* gene promoter occur in the PBLs from the obese subjects.

This work aims to *i.* provide clearer evidence of cause-effect relationship between *ANKRD26* gene expression and DNA methylation and *ii.* investigate the correlation of these changes to obesity-related endo-phenotypes, unhealthy metabolic states and cardio-metabolic risk. Also, I aim to *iii.* establish *in vitro* the obesity-induced hypothalamic regulation of *Ankrd26* gene in terms of DNA methylation changes, and *iv.* clarify the role of the Ankrd26 protein on the hypothalamic regulation of anorexigenic signals *in vitro*.

3. Materials and methods

Section 3.1

Subject enrollment

Thirty-four individuals, 14 lean (7 males and 7 females) undergoing cholecystectomy and 20 obese (10 males and 10 females) undergoing sleeve gastrectomy, were recruited at the Federico II University of Naples, and defined, respectively, based on BMI < 25 kg m^{-2} and BMI ≥ 30 kg m^{-2} [1]. Participants were selected as follows. Inclusion criteria are as follows: age between 25 and 50 years and Caucasian ethnicity. Exclusion criteria are as follows: not knowledge of a verifiable medical treatment period; psychotic disorders; severe depression; personality and eating behavior disorders assessed by a dedicated psychiatrist or psychologist; alcoholism and drug addiction; diseases related to reduced life expectancy; inability to take care of him/herself; inadequate family and social support; obesity secondary to endocrinopathies; gastrointestinal inflammatory diseases; risk of upper gastrointestinal tract bleeding; previous or current tumors; and use of drugs that can influence epigenetic status. The study adhered to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and has been reviewed and approved by the Ethic Committee of the Federico II University of Naples (Ethics Approval Number: No. 254/17). Informed consent was obtained individually from all of the subjects enrolled in the study. Obese subjects have been also classified as metabolically healthy obese (MHO) or metabolically unhealthy obese (MUO) based on *Wildman et al.* [87]. In particular, MUO is defined in subjects by the presence of two or more of these 6 criteria: blood pressure systolic ≥ 130 mmHg and/or diastolic ≥ 85 mmHg or use of anti-hypertensive drugs; fasting triglycerides ≥ 150 mg dL^{-1} or use of lipid-lowering drugs; fasting HDL-C ≤ 40 mg dL^{-1} in men and ≤ 50 mg dL^{-1} in women or use of lipid-lowering drugs; fasting glucose ≥ 100 mg dL^{-1} or use of anti-diabetic drugs; homeostatic model assessment of insulin resistance (HOMA-IR) > 90^{th} percentile; and CRP > 90^{th} percentile, while MHO subjects are defined by the presence of 0 or 1 of the previous criteria. Subjects have been also classified as high or low cardio-metabolic risk based on PCR *cut-off* 3.0 mg/L [87] and TG/HDL-C ratio *cut-off* 3.0 [88]

Sampling and management

Blood and sera samples were collected from fasted patients the day before surgery, and successively handled, processed, and stored by the same investigator. In detail, 1 whole blood vacuum tube was collected from each subject (lean, n = 14; obese, n = 20) enrolled in the study for PBL isolation. Also,

1 serum vacuum tube was collected from each subject (lean, n = 14; obese, n = 20) enrolled in the study for the determination of biochemical parameters, while a further serum vacuum tube was collected from 30 patients out of 34 for the determination of inflammatory parameters through Bioplex multiplex analysis. Blood cell count was evaluated for each individual patient and no significant differences were reported within subjects. PBL isolation was performed on each sample by using the following procedure. Whole blood samples were incubated on ice for 15 min with 5 volume of erythrocyte lysis buffer (KHCO3 10 mM, NH4Cl 155 mM, EDTA 0.1 mM), and PBL was recovered by centrifugation at 400×g for 10 min, and then stored at - 80° C in RNA Later stabilization reagent (Qiagen, Hilden, Germany). At the end of the recruitment, PBL obtained from each patient were lysed in RLT buffer 1× and total RNA and genomic DNA were then isolated using the AllPrep DNA/RNA/miRNA Universal kit (Qiagen) and following the manufacturer's instructions.

Determination of biochemical and inflammatory parameters

Plasma glucose and serum levels of TC, HDL-C, LDL-C, TG, and CRP were determined with an ABX Pentra 400 (Horiba Ltd., Kyoto, Japan). Serum samples have been also used for the dosage of the following secreted inflammatory mediators by Bioplex multiplex Human Cytokine, Chemokine and Growth factor kit (Bio-Rad Laboratories, Hercules, CA): IL-1β, IL-6, IL-7, IL-9, IL-12, IL-17, Interferon γ (IFNγ), TNFα, IL-8, eotaxin, IP-10, MCP1, MIP1α, MIP1β, and RANTES.

Cloning, *in vitro* methylation, and luciferase assay

ANKRD26 promoter (- 716/- 597 bp) was amplified by PCR. The purified PCR fragment was cloned into the firefly luciferase reporter pCpG-free-promoter-Lucia vector (Invivogen, Toulouse, France). This vector is completely void of CpG dinucleotides in all the elements required for replication and selection of the plasmid in *E. Coli* and gene expression in mammalian cells. Also, it is devoid of all Dam methylation sites (GATC) to prevent prokaryotic methylation [89]. The pCpG-*ANKRD26* vector was amplified in *E. Coli* GT115 cells (Invivogen). *In vitro* methylation was performed using the *M.SssI* CpG methyltransferase following manufacturer's protocol (New England BioLabs, Ipswich, MA). Un-methylated DNA was obtained in the absence of *M.SssI.* Methylation was confirmed by digestion with *MspJI* (New England BioLabs). HEK-293 cells were transfected with the CpG methylated or un-methylated pCpG-*ANKRD26* vector and Renilla control vector (Promega, Madison WI) by lipofectamine (Life Technologies), following manufacturer's instructions. Firefly luciferase activity

of each transfection was normalized for transfection efficiency against Renilla luciferase activity.

Statistical procedures

For symmetrically distributed variables, data are shown as mean ± SD, while for skewed distributions, data are presented as median (first quartile-Q1; third quartile-Q3). Statistical differences between groups were tested using parametric or nonparametric method, as appropriate. Two-tailed unpaired Student's t test, or classical OLS regression model to adjust for potential confounders, was used as parametric methods, while Mann-Whitney U test, or quantile regression model with inference based on median to adjust for potential confounders, was used as nonparametric methods. For luciferase assay, comparison between groups was determined by one-way analysis of variance (ANOVA) and Bonferroni correction *post-hoc* tests were carried out to detect significant differences between specific groups. Relationships between variables were assessed through an extension of Spearman's rank correlations [90], which also allowed to adjusted correlations for age using probability-scale residuals. For all the statistical tests, $p < 0.05$ was considered statistically significant. R software environment for statistical computing, Version 3.6.0 (http://www.R-project.org), was used for all statistical analysis.

Section 3.2

Animals and diets

Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85-23, revised 1996). Protocols were approved by the ethics committee of the "Federico II" University of Naples. Six-week-old C57BL/6 J male mice (n = 18) from Charles River Laboratories International, Inc. (Wilmington, MA) were housed in a temperature-controlled (22° C) room with a 12 h light/dark cycle. Two weeks after arrival, mice were randomly divided into two groups. DIO mice (n = 12) were fed with High fat diet (60 kcal% fat content; Research Diets formulas D12331; Research Diets, Inc., New Brunswick, NJ) control mice (n = 6) were fed with a standard chow diet (11 kcal% fat content; Research Diets formulas D12329; Research Diets, Inc.) for 22 weeks. Body weight was recorded weekly throughout the study. Mice were killed by cervical dislocation. Hypothalamus was collected from each mouse, snap frozen in liquid nitrogen and stored at - 80 °C.

DNA, RNA and proteins isolation from tissues and cells

Hypotalami from DIO mice (n = 12) and lean control mice (n = 6) were homogenized by TissueLyser LT (Qiagen, Hilden, Germany) following manufacturer's protocol. RNA and DNA were isolated from each mouse started from 5 mg of tissues using AllPrep DNA/RNA/miRNA Universal kit (Qiagen) following manufacturer's protocol. Proteins from each mouse were isolated starting from 10 mg of tissues using RIPA buffer [25 mM Tris (pH 7.5), 10 mM EDTA, 10 mM EGTA, 1% Nonidet P-40, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 20 µg/ml PMSF, 100 µM NaF, 50 µM NaPPi, 10 µM Na_3VO_4]. The lysates were incubated on ice 30 min and then clarified by centrifugation at 15,000 g for 30 min at 4° C. RNA from mHypoE-N46 cells was isolated by QIAzol Lysis Reagent (QIAGEN - Sample to Insight) according to the manufacturer's protocol. Proteins were isolated using RIPA buffer as described before. The protein concentration was determined using the Coomassie blue protein assay (Bio-Rad Laboratories, Hercules, CA).

Quantitative real-time PCR (qPCR)

cDNA synthesis was performed from total RNA (1 µg) isolated from hypothalamus/mHypoE-N46 cells by using the SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA) and following the manufacturer's instructions. cDNA from mice/cells, was then used as a template for quantitative real-time PCR assays. In detail, for each sample, reactions were performed in triplicates using iQ SYBR Green Supermix on an iCycler real-time detection system (Bio-Rad Laboratories) by using the following cycling condition and reaction protocol. Cycling condition: 10 min at 95° C for 1 cycle, and 15 s at 95° C and 1 min at 60° C repeated for 40 cycles. Reaction protocol: cDNA (25 ng), forward and reverse PCR primers (200 nM each), iQ SYBR Green Supermix 1X (Bio-rad laboratories) in a final volume of 10 µL. The absolute quantification of the *Ankrd26* mRNA expression levels for each sample was carried out by using *Vinculin,* as control reference. The following standard curves were used for the calculations: *Ankrd26* standard curve, y = -3,5188x + 31,148, r = 0,99972, and Vinculin standard curve, y = -3,3697x + 32,615, r = 0,99948. Primer sequences were as follow: *Vinculin* F: 5′-gagcagaaagctggtgaggt-3′; *Vinculin* R: 5′-tgtcattgcccttgctagacc-3′; *Ankrd26* F: 5′-ctttggacgcgagagtgcta-3′; and *Ankrd26* R: 5′-agcagtcctgtccttcttgtc-3′; *NPY* F: 5′-tggccagatactactccgt-3′; and *NPY* R: 5′-agggtcttcaagccttgttct-3′; *AgRP* F: 5′-gtgttctgctgttggcactg-3′; and *AgRP* R: 5′-gatctagcacctccgccaaa-3′; *POMC* F: 5′-caacctgctggcttgcatc-3′; and *POMC* R: 5′-cgtacttccgggggttttca-3'; *CART* F: 5′-ctggacatctactctgccg-3′; and *CART* R: 5′-gtagatcggaatgcgtttactc-3′.

Bisulfite sequencing

For bisulfite sequencing analysis, 350 ng of genomic DNA were isolated from hypothalami of DIO mice (n = 4) and lean controls (n = 4). Bisulfite conversion of DNA was performed with the EZ DNA Methylation Kit (Zymo Research, Orange, CA), following manufacturer's instructions. The *Ankrd26* promoter region S1, - 462/- 193 bp from transcription start site (TSS) was amplified by PCR using specific primers. PCR products were cloned into the pGEM T-Easy vector (Promega, Madison, WI) and 10 clones for sample were sequenced on AB 3500 genetic analyzer (Life Technologies). DNA methylation percentage at the - 436 and - 431 bp CpGs for each mouse was calculated using the formula: DNA methylation % = [methylated CpGs/ (methylated CpGs + unmethylated CpGs)]*100. Primers sequences: *Ankrd26* S1 F: 5′-taaattatttagtttaataaaatttttttt-3′; *Ankrd26* S1 R: 5′-tctttactattcaaaaaatcaaaac-3′;

Western blot analysis

Western blot analysis was performed as previously described. Thirty μg of protein from hypothalami or mHypoE-N46 cells were analyzed by SDS-PAGE and transferred to a PVDF membrane. Membranes were firstly probed with antibodies against ANKRD26 (#ab47984, abcam), pAKT (#sc-7985-R, Santa Cruz Biotechnology), AKT (#sc-8312, Santa Cruz Biotechnology), pERK1/2 (#9101, Cell Signaling TECHNOLOGY), ERK1/2 (#sc-514302, Santa Cruz Biotechnology), pAMPK (#2535, Cell Signaling TECHNOLOGY) and Vinculin (#sc-73614, Santa Cruz Biotechnology), then probed with secondary mouse or rabbit antibodies (Bio-Rad Laboratories) before detection of the signal with ECL plus (GE Healthcare, Chicago, IL).

Cell culture, treatments with anorexigenic stimuli and transfection

mHypoE-N46 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37° C in a humidified atmosphere of 5% CO_2. For treatments, mHypoE-46 cells were grown to 80-90% confluency, serum-starved for 16 h and then treated with insulin (100 nmol/L) or leptin (100 nmol/L) or Exendin-4 (100 nmol/L) or vehicle. The cells were harvested at 5, 15, 30, 45 and 60 min after treatments and proteins were isolated as described before. For Ankrd26 overexpression, mHypoE-N46 cells were grown and allowed to 80% confluence in 6 well plate. 1 μg of Ankrd26-overexpressing vector (pCMV-*Ankrd26*) or control vector pCMV (Origene technologies, Rockville, USA) was transfected using

lipofectamine (ThermoFisher Scientific), following manufacturer's instructions. RNA and proteins were isolated after 24 and 48 hours as described before.

Statistical procedures

All experiments were performed three times for each determination, and results are shown as mean ± SD. Statistical differences between groups were tested using two-tailed unpaired Student's t test. Significant p values are indicated as $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$. Correlation analysis was calculated using Pearson's correlation coefficient.

4. Results and Discussion

Section 4.1

My colleagues and I have previously demonstrated in a cohort of lean and obese Caucasian adults, recruited at the Federico II University of Naples, that changes of the *ANKRD26* gene expression and DNA methylation at specific CpG sites within the *ANKRD26* gene promoter occur in the PBLs from the obese subjects (Figure 6) [86]. Indeed, a 40% reduction of the *ANKRD26* mRNA levels was reported in PBLs from the obese compared to lean individuals (age-adjusted $p = 0.027$) (Figure 6a) [86]. Also, DNA methylation at the - 689, - 659, and - 651 bp CpG sites was increased by about 1.8-fold (age-adjusted $p < 0.001$), 1.9-fold (age-adjusted $p = 0.040$), and 2.2-fold (age-adjusted $p = 0.025$), respectively, in the obese compared with the lean (Figure 6b) [86].
However, the impact of this specific *ANKRD26* DNA methylation pattern on the gene expression and the relevance of these changes on phenotype of obese subjects needed to be explored.
In the first section of my PhD thesis I thus aimed to *i.* provide clearer evidence of cause-effect relationship between *ANKRD26* gene expression and DNA methylation, and *ii.* investigate the correlation of these changes to obesity-related endo-phenotypes, unhealthy metabolic states and cardio-metabolic risk factors.

4.1.1 Hyper-methylation at the *ANKRD26* promoter causes down-regulation of its gene expression in human obesity

To identify a potential cause-effect relationship between the DNA methylation at the *ANKRD26* gene promoter and its gene transcription, a DNA fragment (- 716/- 597 bp) containing the CpG sites, - 689, - 659, and - 651 bp, was cloned in a pCpG-free vector. Luciferase activities of either the *in vitro* CpG methylated or the un-methylated pCpG-*ANKRD26* luciferase reporter vectors were then assayed in human embryonic kidney HEK293 cells.

As shown in Figure 7a, the un-methylated *ANKRD26* promoter region induced a 2.2-fold increase of luciferase activity than the empty vector, indicating that this fragment features promoter activity. On the other hand, the methylation of the three selected CpG sites at the *ANKRD26* promoter causes a 30% reduction of the luciferase activity compared with the un-methylated pCpG-*ANKRD26* luciferase vector, indicating that methylation of one or more of these 3 CpG dinucleotides *in vitro* represses the *ANKRD26* gene transcription.

Also, when I related each to the other the DNA methylation and the *ANKRD26* mRNA data from the human lean and obese subjects, a negative

correlation was found between the combined methylation percentage at the cytosine residues - 689, - 659, and - 651 of the *ANKRD26* promoter and the expression levels of the *ANKRD26* gene in PBLs (n = 34, r = - 0.539, age-adjusted $p < 0.001$; Figure 7b). These last data suggest that a direct cause-effect relationship exists between these methylation events and *ANKRD26* gene expression in humans. Thus, hyper-methylation at the *ANKRD26* promoter and the accompanying down-regulation of its gene expression feature obesity in humans.

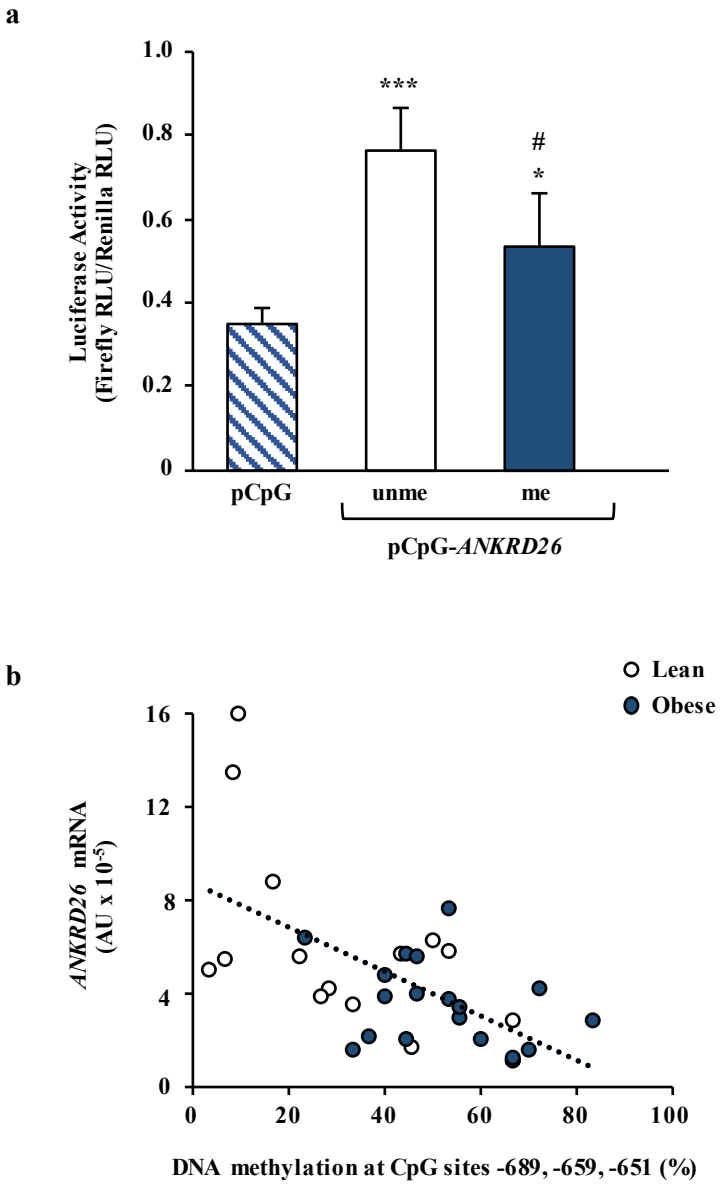

Figure 7. *ANKRD26* promoter methylation affects the promoter activity *in vitro* and correlates to A*NKRD26* gene expression in PBLs. a) Luciferase activity of *in vitro* CpG methylated (me), or un-methylated (unme) pCpG-*ANKRD26* constructs and the pCpG empty vector was assessed as described in the "Methods" section. Firefly luciferase activity was normalized to Renilla luciferase activity. Luciferase activity is displayed in relative light units (RLU). Results are means ± SD from three independent experiments. One-way ANOVA assessed statistical difference between the means of the groups. *$p < 0.05$ and ***$p < 0.001$ *vs* pCpG empty vector; #$p < 0.05$ *vs* pCpG-*ANKRD26*unme. b) Relationship between combined DNA methylation at the CpGs - 689, - 659, and - 651 and *ANKRD26* gene expression was assessed by covariate-adjusted Spearman's rank-order correlation adjusted for age (n = 34; r = - 0.494; $p = 0.002$).

4.1.2 Both *ANKRD26* mRNA expression and CpG methylation correlate to BMI, pro-inflammatory and increased cardio-metabolic risk factors

To investigate whether the observed changes in the *ANKRD26* gene expression and DNA methylation, which occur in human obese individuals, correlate to BMI and altered levels of metabolic and inflammatory mediators in obese subjects, the cohort of lean and obese Caucasian adults, previously recruited at the Federico II University of Naples, and used for the determination of gene expression and CpG methylation, was deeply phenotyped. The clinical features of the two groups are shown in Tables 1 and 2. Serum triglycerides (TG; $p < 0.001$) and low-density lipoprotein cholesterol (LDL-C; $p = 0.037$) concentrations were increased and the serum levels of high-density lipoprotein cholesterol (HDL-C; $p = 0.002$) were reduced in the obese compared with lean individuals. Also, the TG/HDL-C ratio, a described predictor of insulin resistance and cardiovascular metabolic risk [86,87], was increased in the obese subjects compared to lean controls. No difference in either fasting blood glucose and total serum cholesterol (TC) levels were found between the two groups (Table 1). Moreover, the obese individuals also featured an increase in serum level of C-reactive protein (CRP; $p < 0.001$; Table 1) as well as in several pro-inflammatory cytokines and chemokines, including Interleukin-6 (IL-6), IL-8, IL-12, interferon gamma-induced protein 10 (IP-10), monocyte chemotactic protein 1 (MCP1) and the regulated-on activation, normal T cell (RANTES), as showed in Table 2.

	Lean	Obese	*p* value
n (males/females)	14 (7/7)	20 (10/10)	
Age (years)	30.2 ± 2.6	37.0 ± 5.6	< 0.001
BMI (Kg m^{-2})	22.3 ± 2.7	46.7 ± 6.9	< 0.001
Glucose (mg dL^{-1})	87.9 ± 7.1	95.4 ± 37.3	n.s.
TC (mg dL^{-1})	176.4 ± 33.4	179.1 ± 37.3	n.s.
HDL-C (mg dL^{-1})	63.9 ± 20.4	46.5 ± 10.2	0.002
LDL-C (mg dL^{-1})	94.9 ± 31.6	117.8 ± 29.2	0.037
TG (mg dL^{-1})	58.6 (43.4; 83.6)	119.5 (90.2; 162.0)	< 0.001
TG/HDL-C ratio	0.9 (0.6; 1.9)	2.6 (1.8; 4.2)	< 0.001
CRP (mg L^{-1})	0.2 (0.1; 0.4)	7.7 (1.7; 14.4)	< 0.001

Table 1 Anthropometric and biochemical features of lean (n =14; 7 males and 7 females) and obese (n = 20; 10 males and 10 females) individuals. For symmetrically distributed variables, data are shown as mean ± SD, and a two-tailed unpaired Student's t-test tested statistical difference between the two groups. For skewed distributions, data are shown as median (first quartile-Q1; third quartile-Q3), and statistical differences between the two groups were tested using the Mann-Whitney U test. BMI, body mass index; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; CRP, C-reactive protein.

	Lean	Obese	*p* value
IL-1β (pg mL^{-1})	3.1 (2.7; 3.4)	3.4 (2.8; 4.5)	n.s.
IL-6 (pg mL^{-1})	4.1 (3.0; 5.3)	8.6 (6.0; 11.1)	< 0.001
IL-7 (pg mL^{-1})	9.0 (7.9; 12.0)	13.1 (8.8; 15.8)	n.s.
IL-9 (pg mL^{-1})	83.9 (59.0; 93.7)	83.9 (75.3; 96.7)	n.s.
IL-12 (pg mL^{-1})	25.6 (16.7; 40.1)	49.2 (34.9; 81.9)	0.006
IL-17 (pg mL^{-1})	125.9 (111.0; 149.4)	130.5 (116.9; 164.1)	n.s.
IFN-γ (pg mL^{-1})	101.2 (91.2; 113.9)	108.3 (85.4; 126.3)	n.s.
TNF-α (pg mL^{-1})	48.6 (39.7; 52.7)	47.2 (39.0; 54.7)	n.s.
IL-8 (pg mL^{-1})	16.5 (12.8; 18.2)	31.9 (22.0; 46.8)	< 0.001
Eotaxin (pg mL^{-1})	102.0 (63.8; 127.0)	116.3 (78.6; 157.6)	n.s.
IP-10 (pg mL^{-1})	313.3 (218.4; 850.3)	619.6 (528.5; 1095.1)	0.021
MCP-1 (pg mL^{-1})	7.0 (4.3; 14.5)	30.3 (18.3; 39.5)	0.001
MIP-1α (pg mL^{-1})	2.7 (2.6; 3.1)	3.2 (2.2; 4.5)	n.s.
MIP-1β (pg mL^{-1})	141.7 (90.5; 244.9)	202.6 (162.3; 424.4)	0.040
RANTES (pg mL^{-1})	21480.8 (15677.0; 24610.0)	28724.8 (26390.0; 36752.0)	< 0.001

Table 2 Pro-inflammatory cytokine and chemokine features of lean (n = 12; 6 males and 6 females) and obese (n = 18; 9 males and 9 females) individuals. Data are shown as median (first quartile-Q1; third quartile-Q3), and statistical differences between the two groups were tested using the Mann-Whitney U test. IL, interleukin; IFNγ, interferon γ; TNFα, tumor necrosis factor α; IP-10, interferon gamma-induced protein 10; MCP1, monocyte chemotactic protein 1; MIP1, macrophage inflammatory protein 1; RANTES, regulated on activation, normal T cell.

Firstly, I explored in these 34 individuals the relationship of the observed changes in the *ANKRD26* gene expression and DNA methylation with BMI and age. Interestingly, *ANKRD26* mRNA levels correlated negatively to BMI (n = 34; r = - 0.494; age-adjusted p = 0.002; Figure 8a), while no correlation was found between *ANKRD26* gene expression and age (n = 34; r = - 0.226; n.s). Also, the combined methylation of the 3 CpG sites positively correlated to BMI (n = 34, r = 0.736, age-adjusted p < 0.001; Figure 8b). On the contrary, no correlation was found between the combined DNA methylation at these 3 CpG sites and age (n = 34, r = - 0.223, n.s). It is noteworthy that similar results were obtained when these relationships with BMI, and age were performed on individual CpG methylation percentages (data not shown).

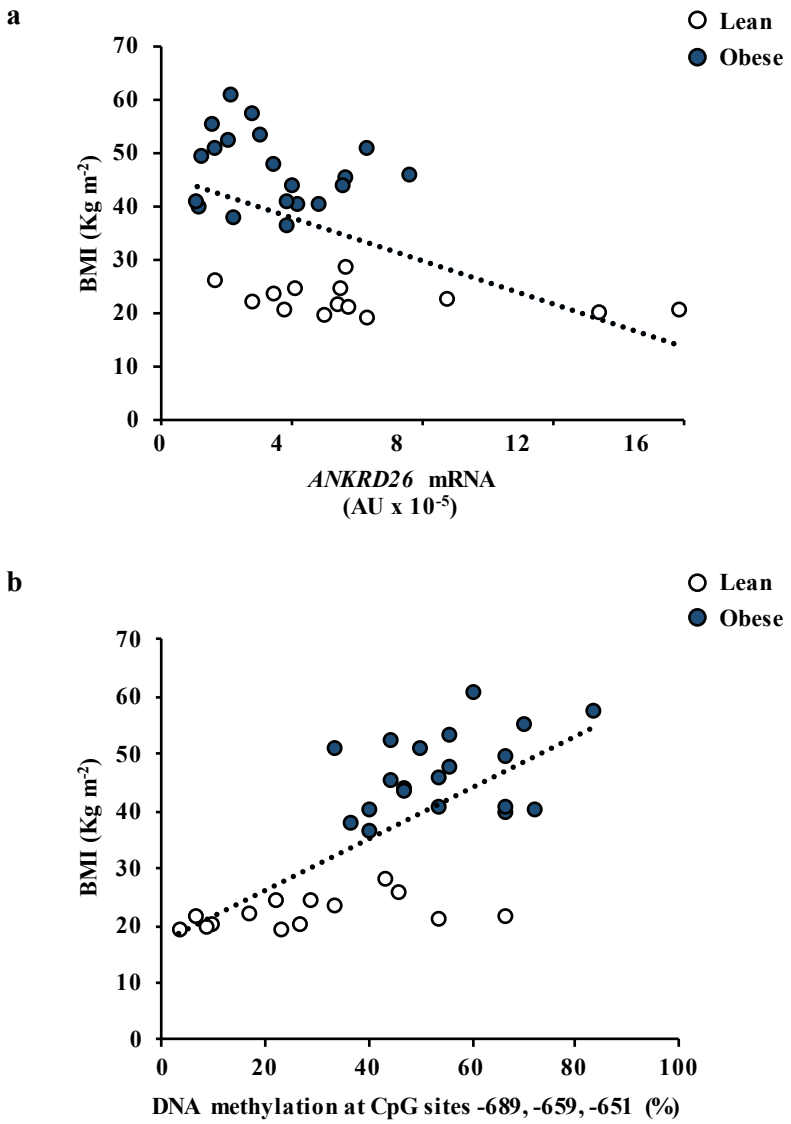

Figure 8. *ANKRD26* gene expression, promoter methylation, and BMI. a) Relationship between *ANKRD26* gene expression and BMI was assessed in lean (n = 14) and obese individuals (n = 20) by covariate-adjusted Spearman's rank-order correlation adjusted for age (n = 34; r = - 0.494; p= 0.002). b) Relationship between combined DNA methylation at the CpGs - 689, - 659, and - 651 and BMI was assessed in lean (n= 14) and obese individuals (n = 20) by covariate-adjusted Spearman's rank-order correlation adjusted for age (n= 34; r= 0.736; $p < 0.001$).

Secondly, I investigated in this same cohort of subjects the relationship of the *ANKRD26* gene expression and DNA methylation with metabolic and inflammatory mediators. I found that *ANKRD26* mRNA levels correlated inversely to serum TG concentration (n = 34, r = - 0.630, age-adjusted $p < 0.001$)

and directly to serum HDL-C concentration (n = 34, r = 0.647, age-adjusted $p <$ 0.001). In addition, the *ANKRD26* gene expression correlated negatively to serum levels of IL-1β (n = 30, r = - 0.498, age-adjusted $p = 0.010$), IL-6 (n = 30, r = - 0.401, age-adjusted $p = 0.027$), IL-12 (n = 30, r = - 0.506, age-adjusted $p =$ 0.002), IL-8 (n = 30, r = - 0.650, age-adjusted $p < 0.001$), IP-10 (n = 30, r = - 0.520, age-adjusted $p = 0.002$), macrophage inflammatory protein 1α (MIP-1α; n = 30, r = - 0.513, age-adjusted $p = 0.006$), MIP-1β (n = 30, r = - 0.601, age-adjusted $p < 0.001$), and RANTES (n = 30, r = - 0.476, age-adjusted $p = 0.002$), as well (Table 3).

Interestingly, as also reported in Table 4, the combined DNA methylation levels of the CpG dinucleotides - 689, - 659, and - 651 bp at the *ANKRD26* promoter correlated positively to serum TG concentration (n = 34, r = 0.593, age-adjusted $p < 0.001$) and negatively to serum HDL-C concentration (n = 34, r = - 0.539, age-adjusted $p < 0.001$). Also, the combined methylation of these 3 CpG sites correlate directly to serum levels of IL-6 (n = 30, r = 0.577, age-adjusted $p =$ 0.002), IL-12 (n = 30, r = 0.453, age-adjusted $p = 0.013$), IL-8 (n = 30, r = 0.698, age-adjusted $p = 0.001$), and RANTES (n = 30, r = 0.514, age-adjusted $p =$ 0.013). Very interestingly, these relationships were also confirmed when these biochemical and pro-inflammatory parameters were associated to individual CpG methylation percentages (data not shown).

Finally, in the obese and lean subjects I even investigated the relationship of the observed changes in the *ANKRD26* gene expression and DNA methylation in PBLs with cardio-metabolic risk factors, such CRP and TG/HDL-C ratio [86]. Interestingly, the *ANKRD26* gene expression negatively correlates both with the serum levels of CRP (n = 34, r = - 0.494, age-adjusted $p < 0.001$; Figure 9a) and TG/HDL-C ratio (n = 34, r = - 0.728, age-adjusted $p < 0.001$; Figure 9c). Also, the percentage of CpG dinucleotides methylation at - 689, - 659, and - 651 bp, taken as combined or as individual CpG methylation, of the *ANKRD26* promoter positively correlated to both the serum CRP (n = 34, r = 0.524, age-adjusted $p =$ 0.001; Figure 9b) and the TG/HDL-C ratio (n = 34, r = 0.666, age-adjusted $p <$ 0.001; Figure 9d).

Altogether these results indicate that both the down-regulation of *ANKRD26* mRNA and the hyper-methylation of the CpG dinucleotides - 689, - 659, and - 651 bp, taken as combined or as single CpG methylation percentage, correlate to a worse lipid profile, an increased pro-inflammatory state and an increased cardio-metabolic risk.

Parameters	Gene expression		
	n	r	*p* value
Glucose	34	−0.233	n.s.
TG	34	−0.630	<0.001
TC	34	−0.113	n.s.
HDL-C	34	0.647	<0.001
LDL-C	34	−0.315	n.s.
CRP	34	−0.494	0.001
IL-1β	30	−0.498	0.010
IL-6	30	−0.401	0.027
IL-7	30	−0.185	n.s.
IL-9	30	−0.123	n.s.
IL-12	30	−0.506	0.002
IL-17	30	−0.085	n.s.
IFN-γ	30	−0.112	n.s.
TNF-α	30	−0.037	n.s.
IL-8	30	−0.650	<0.001
Eotaxin	30	−0.116	n.s.
IP-10	30	−0.520	0.002
MCP-1	30	−0.206	n.s.
MIP-1α	30	−0.513	0.006
MIP-1β	30	−0.601	<0.001
RANTES	30	−0.476	0.002

Table 3. *ANKRD26* gene expression in relation to metabolic and inflammatory parameters. The relationship between metabolic or inflammatory parameters and *ANKRD26* gene expression was assessed in lean and obese individuals by covariate-adjusted Spearman's rank-order correlation adjusted for age. The number of individuals (n), correlation coefficient r, and age-adjusted *p* value are shown in the table. TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; IL, interleukin; IFNγ, interferon γ; TNFα, tumor necrosis factor α; IP-10, interferon gamma-induced protein 10; MCP1, monocyte chemotactic protein 1; MIP1, macrophage inflammatory protein 1; RANTES, regulated on activation, normal T cell expressed, and secreted.

Parameters	Combined % CpG methylation		
	n	r	*p* value
Glucose	34	0.081	n.s.
TG	34	0.593	< 0.001
TC	34	0.155	n.s.
HDL-C	34	− 0.539	0.004
LDL-C	34	0.376	0.044
CRP	34	0.524	0.001
IL-1β	30	0.242	n.s.
IL-6	30	0.577	0.002
IL-7	30	0.182	n.s.
IL-9	30	0.032	n.s.
IL-12	30	0.453	0.013
IL-17	30	− 0.001	n.s.
IFN-γ	30	− 0.025	n.s.
TNF-α	30	− 0.083	n.s.
IL-8	30	0.698	0.001
Eotaxin	30	0.001	n.s.
IP-10	30	0.337	n.s.
MCP-1	30	0.305	n.s.
MIP-1α	30	0.333	0.050
MIP-1β	30	0.377	n.s.
RANTES	30	0.514	0.013

Table 4. Combined DNA methylation at the CpG sites, - 689, - 659, and - 651 of the *ANKRD26* promoter in relation to metabolic and inflammatory parameters. Relationships were assessed in lean and obese individuals by covariate-adjusted Spearman's rank-order correlation adjusted for age. The number of individuals (n), correlation coefficient r, and age-adjusted p value are shown in the table. TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; IL, interleukin; IFNγ, interferon γ; TNFα, tumor necrosis factor α; IP-10, interferon gamma-induced protein 10; MCP1, monocyte chemotactic protein 1; MIP1, macrophage inflammatory protein 1; RANTES, regulated on activation, normal T cell expressed, and secreted.

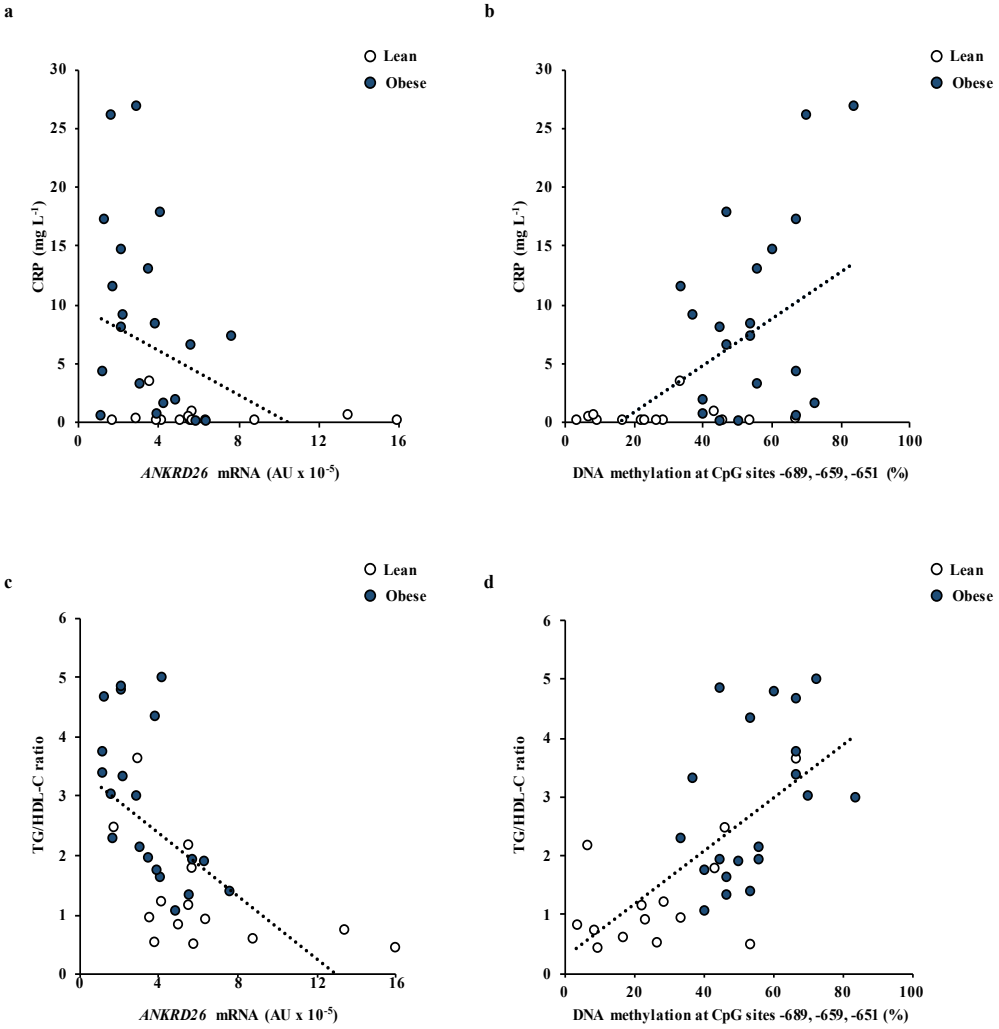

Figure 9. *ANKRD26* gene expression, promoter methylation, and cardio-metabolic risk factors. a) Relationship between *ANKRD26* expression and serum CRP was assessed in lean (n = 14) and obese individuals (n = 20) by covariate-adjusted Spearman's rank-order correlation adjusted for age (n = 34; r = - 0.494; $p < 0.001$). b) Relationships between CRP and the combined DNA methylation at the CpGs - 689, - 659, and - 651 in lean (n = 14) and obese individuals (n = 20) was assessed by covariate-adjusted Spearman's rank-order correlation adjusted for age (n = 34; r = 0.524; $p = 0.001$). c) Relationship between *ANKRD26* expression and TG/HDL-C ratio was assessed in lean (n = 14) and obese individuals (n = 20) by covariate-adjusted Spearman's rank-order correlation adjusted for age (n = 34; r = - 0.728; $p < 0.001$). d) Relationships between TG/HDL-C ratio and the combined DNA methylation at the CpGs - 689, - 659, and - 651 in lean (n = 14) and obese individuals (n = 20) was assessed by covariate-adjusted Spearman's rank-order correlation adjusted for age (n = 34; r = 0.666; $p < 0.001$).

4.1.3 Both *ANKRD26* mRNA expression and DNA methylation at the CpG site - 689 bp associate with MUO

Successively, I explored in the obese group the hypothesis that changes in the *ANKRD26* gene expression and DNA methylation might be related to a metabolically unhealthy status. To this aim, the obese subjects were stratified as MHO and MUO based on the Wildman criteria [87]. The clinical features used to define MHO and MUO groups are shown in Table 5.

Within the obese subjects, I found that the *ANKRD26* gene expression was much lower in the MUO compared with the MHO subjects ($p = 0.004$; Figure 10a and Table 5). Also, I found that DNA methylation at the CpG site - 689 bp was increased by about 1.6-fold in the MUO compared with the MHO subjects ($p = 0.009$; Figure 10b and Table 5). Noteworthy, no differences in CpG methylation were observed at the sites - 659 and - 651 bp (Figure 10b and Table 5).

Thus, these finding reveal a more pronounced silencing of *ANKRD26* and increased DNA methylation at the - 689 bp CpG in the MUO compared with the MHO individuals. This supports the hypothesis that the epigenetic regulation of the *ANKRD26* gene may be sensitive to the alteration of the metabolic and inflammatory profile occurring in the metabolically unhealthy obese.

	MHO	MUO	*p* value
Wildman criteria			
Glucose, mg dL^{-1}	82.8 ± 8.3	99.6 ± 42.3	n.s.
HDL-C, mg dL^{-1}	53.8 ± 13.6	44.4 ± 8.0	n.s.
SBP, mmHg	119.0 ± 10.2	135.3 ± 10.4	0.007
DBP, mmHg	84.0 ± 4.2	85.5 ± 6.0	n.s.
TG, mg dL^{-1}	70.0 (62.2; 119.5)	137.0 (104.0; 170.0)	0.036
CRP, mg L^{-1}	2.0 (0.5; 19.7)	9.1 (4.4; 17.2)	n.s.
HOMA-IR	1.6 (1.4; 2.2)	4.3 (3.0; 5.7)	0.006
***ANKRD26* mRNA, AU × 10^{-5}**	5.7 (4.4 - 7.0)	2.2 (1.6 - 3.8)	0.004
CpG − 689, % CpGmethylation	36.7 ± 4.7	57.8 ± 15.7	0.009
CpG − 569, % CpGmethylation	46.0 ± 8.9	51.2 ± 19.3	n.s.
CpG − 651, % CpGmethylation	54.0 ± 16.7	62.6 ± 17.7	n.s.

Table 5 Metabolic parameters in MHO (n = 5) and MUO (n = 15) individuals. Obese subjects have been classified as MHO or MUO based on Wildman et al. [87]. In particular, metabolically unhealthy obese subjects are defined by the presence of two or more of these 6 criteria: blood pressure systolic ≥ 130 mmHg and/or diastolic ≥ 85 mmHg or use of anti-hypertensive drugs; fasting triglycerides ≥ 150 mg dl^{-1} or use of lipid-lowering drugs; fasting HDL-C ≤ 40 mg dL^{-1} in men and ≤ 50 mg dL^{-1} in women or use of lipid-lowering drugs; fasting glucose ≥ 100 mg dl^{-1} or use of anti-diabetic drugs; HOMA-IR > 90th percentile; and CRP > 90th percentile, while metabolically healthy obese subjects are defined by the presence of 0 or 1 of the previous criteria. For symmetrically distributed variables, data are shown as mean ± SD and the statistical difference between the two groups was tested by two-tailed unpaired Student's t test. For skewed distributions, data are shown as median (first quartile-Q1; third quartile-Q3) and statistical differences between the two groups were tested using Mann-Whitney U test. HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; SBP, systolic blood pressure; DBP, diastolic blood pressure; CRP, C-reactive protein; HOMA-IR, homeostatic model assessment of insulin resistance.

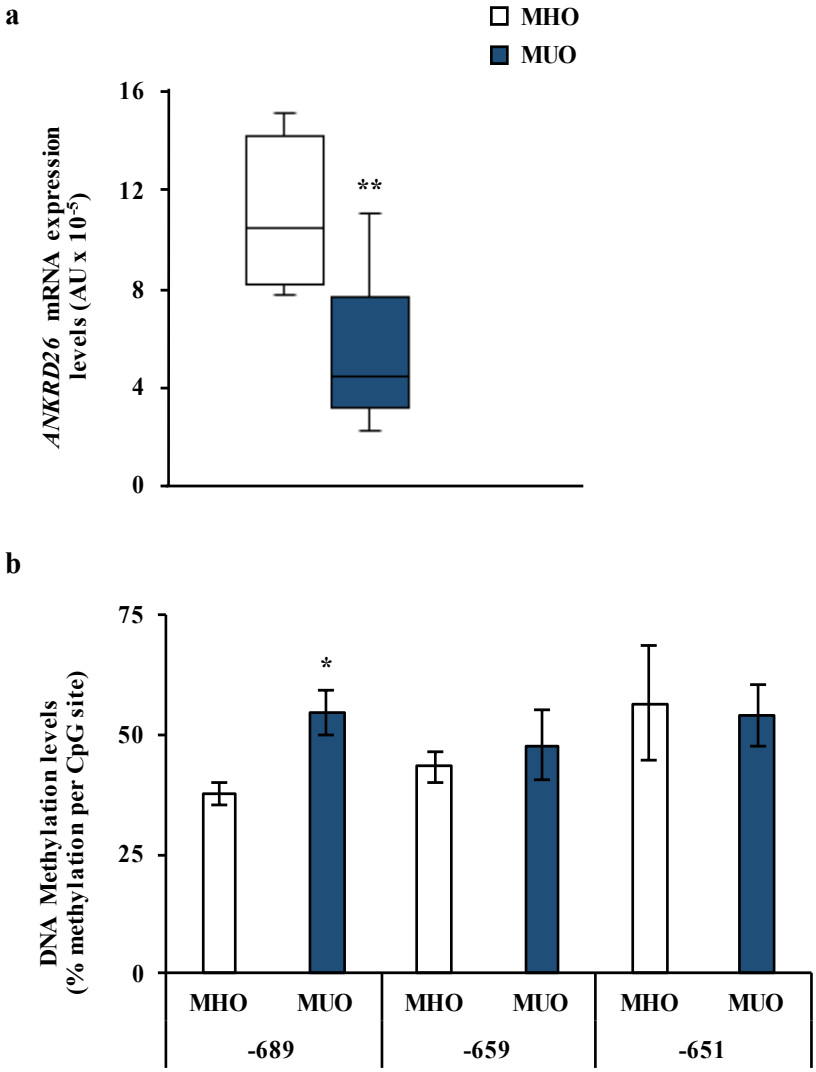

Figure 10. *ANKRD26* gene expression and promoter methylation in MHO (n = 5) and MUO (n = 15) individuals. a) *ANKRD26* mRNA levels in 15 MUO individuals and in 5 MHO individuals. Values are expressed in absolute units (AU), and box plots represent their distribution within each group. Box plots show median (line within the box), quartiles (upper and lower box boundaries), and extreme values (whiskers). Statistical differences between the two groups were tested using nonparametric quantile regression, with inference based on the median. p = 0.004 vs. MHO individuals. b) Bisulfite sequencing methylation analysis of the ANKRD26 CpG sites, - 689, - 659, and - 651 bp from the TSS in converted genomic DNA from PBL of 15 MUO individuals and in 5 MHO individuals. Results are means ± SD. The classical OLS regression model assessed the statistical difference between the means of the two groups to adjust for BMI. **$p < 0.01$ and *$p < 0.05$ vs. MHO individuals.

4.1.4 Both *ANKRD26* mRNA expression and DNA methylation at the CpG sites - 689, - 659, and - 651 bp associate with cardio-metabolic risk factors

Finally, I explored in all the 34 recruited subjects the hypothesis that changes in the *ANKRD26* gene expression and DNA methylation might be related to cardio-metabolic risk factors. To this aim, subjects were stratified into two groups at low and high cardio-metabolic risk based on their serum CRP (risk group cut-off ≥ 3.0 mg L^{-1}) and TG/HDL-C ratio (risk group cut-off ≥ 3.0). In the subjects stratified based on serum CRP, I found that the *ANKRD26* gene expression was significantly lower (BMI-adjusted $p < 0.01$) in the group with the higher cardio-metabolic risk (CRP > 3.0 mg L^{-1}; Figure 11a). Consistently, the methylation levels of the CpG sites at - 689 (BMI-adjusted $p < 0.01$) and - 651 (BMI-adjusted $p < 0.01$) from the *ANKRD26* TSS were significantly increased in the group with CRP values > 3.0 compared to the group at lower cardio-metabolic risk (Figure 11b). Furthermore, when the subjects were stratified based on the TG/HDL-C ratio [19], I found that, independent of BMI, the *ANKRD26* mRNA levels were reduced by about 60% in the individuals with the TG/HDL-C ratio > 3.0 compared with subjects with a TG/HDL-C ratio < 3.0 (BMI-adjusted $p = 0.016$; Figure 12a). Also, DNA methylation at the - 689, - 659, and - 651 bp CpG sites was increased by about 2.0-fold (BMI-adjusted $p < 0.001$), 1.7-fold (BMI-adjusted $p = 0.040$), and 1.8-fold (BMI-adjusted $p = 0.025$), respectively, in the individuals with a TG/HDL-C ratio > 3.0 compared with subjects featuring a TG/HDL-C ratio below the cut-off value (Figure 12b). Importantly, stratification of the study group based on serum CRP and TG/HDL-C index revealed association of higher levels of *ANKRD26* methylation and reduced gene expression with high-cardio-metabolic risk, independent of BMI, indicating that alterations of both the DNA methylation and mRNA expression at the *ANKRD26* gene correlate to increased risk of insulin resistance and cardiovascular disease in humans.

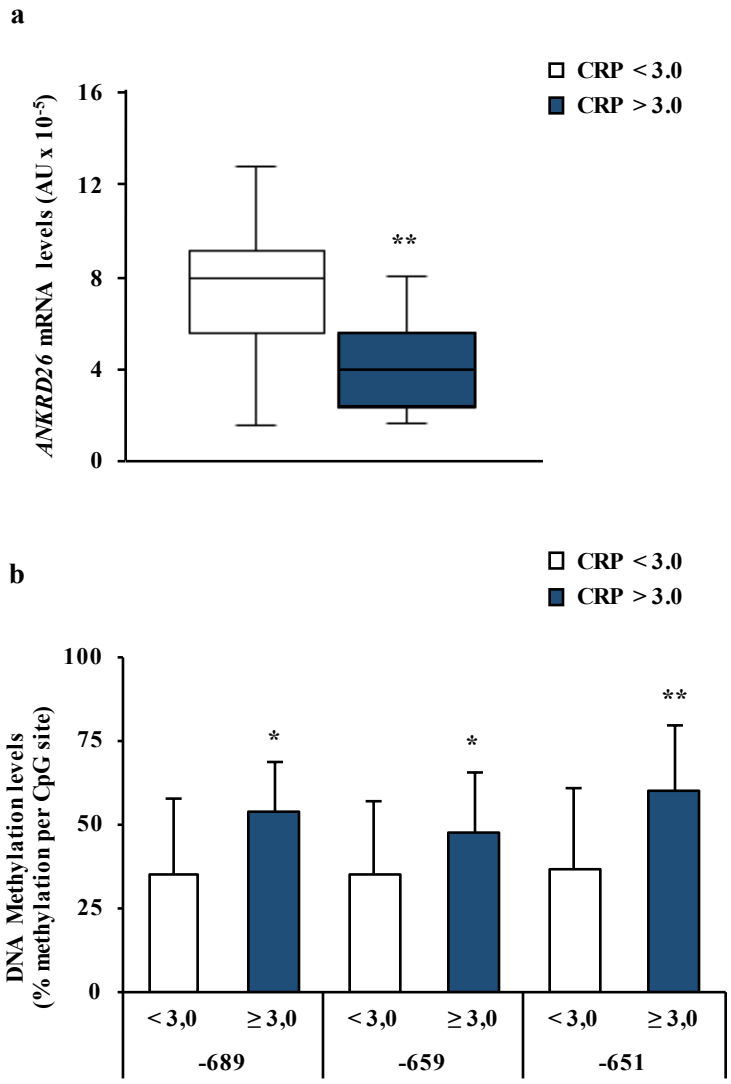

Figure 11. *ANKRD26* gene expression and promoter methylation in CRP > 3.0 mg L^{-1} (n = 15) and CRP < 3.0 mg L^{-1} (n = 19) individuals. a) *ANKRD26* mRNA levels in 15 individuals with the CRP > 3.0 mg L^{-1} and in 19 individuals with the CRP < 3.0 mg L^{-1}. Values are expressed in absolute units (AU), and box plots represent their distribution within each group. Box plots show median (line within the box), quartiles (upper and lower box boundaries), and extreme values (whiskers). Statistical differences between the two groups were tested using nonparametric quantile regression, with inference based on the median, to adjust for BMI. p = 0.016 vs. individuals with the CRP < 3.0 mg L^{-1}. b) Bisulfite sequencing methylation analysis of the ANKRD26 CpG sites, - 689, - 659, and - 651 bp from the TSS in converted genomic DNA from PBL of in 15 individuals with the CRP > 3.0 mg L^{-1} and in 19 individuals with the CRP < 3.0 mg L^{-1}. Results are means ± SD. The classical OLS regression model assessed the statistical difference between the means of the two groups to adjust for BMI. *p < 0.05 and **p < 0.01 vs. individuals with CRP < 3.0 mg L^{-1}.

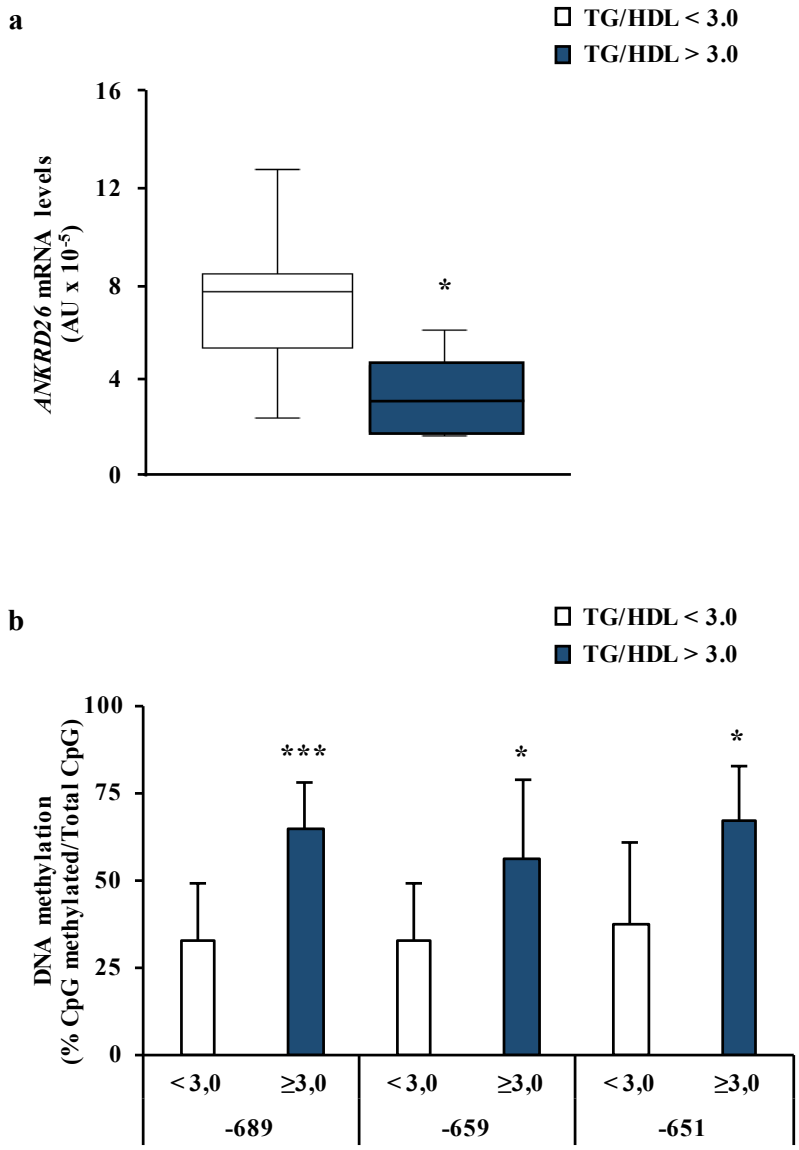

Figure 12. *ANKRD26* gene expression and promoter methylation in TG/HDL-C > 3.0 (n = 11) and TG/HDL-C < 3.0 (n = 23) individuals. a) *ANKRD26* mRNA levels in 11 individuals with the TG/HDL-C > 3.0 and in 23 individuals with the TG/HDL-C < 3.0. Values are expressed in absolute units (AU), and box plots represent their distribution within each group. Box plots show median (line within the box), quartiles (upper and lower box boundaries), and extreme values (whiskers). Statistical differences between the two groups were tested using nonparametric quantile regression, with inference based on the median, to adjust for BMI. p = 0.016 vs. individuals with the TG/HDL-C < 3.0. b) Bisulfite sequencing methylation analysis of the ANKRD26 CpG sites, - 689, - 659, and - 651 bp from the TSS in converted genomic DNA from PBL of 11 individuals with TG/HDL-C > 3.0 and in 23 individuals with the TG/HDL-C < 3.0. Results are means ± SD. The classical OLS regression model assessed the statistical difference between the means of the two groups to adjust for BMI. *$p < 0.05$ and ***$p < 0.001$ vs. individuals with TG/HDL-C < 3.0.

Section 4.2

The *Ankrd26* gene has been related to the regulation of feeding behavior and development of both obesity and diabetes in animal models (*Ankrd26* MT mice) [80]. Also, it has been reported that this gene is highly abundant in murine hypothalamus. Noteworthy, the *Ankrd26* gene is epigenetically regulated in VAT from a mouse model of diet-induced obesity (DIO mice). Indeed, the exposure to high fat/obesity onset determined a specific hyper-methylation of the *Ankrd26* promoter at the - 436 and - 431 bp CpG sites in obese mice. However, whether obesity may epigenetically modulate *Ankrd26* gene expression even in the hypothalamus is not known.

In the second section of my PhD thesis, I thus aimed to establish the obesity-induced hypothalamic regulation of *Ankrd26* gene in terms of DNA methylation changes. Also, I wondered to clarify the role of the Ankrd26 protein on the hypothalamic regulation of anorexigenic signals *in vitro*.

4.2.1 Obesity impairs *Ankrd26* expression and promoter DNA methylation in murine hypothalamus

To establish whether obesity modulates the *Ankrd26* expression *in vivo*, mRNA and protein levels were measured in the hypothalami of DIO mice (n = 12) and lean control mice (n = 6). As shown in Figure 13a, a 30 % decrease of the *Ankrd26* mRNA levels ($p < 0.001$) was observed in hypothalami from obese compared to lean mice. Also, *Ankrd26* mRNA levels correlated negatively to mice body weight (n = 18; r = - 0.599; age-adjusted $p = 0.009$). Similarly, obesity also led to a consistent decrease of the Ankrd26 protein expression ($p < 0.05$) (Figure 13b).

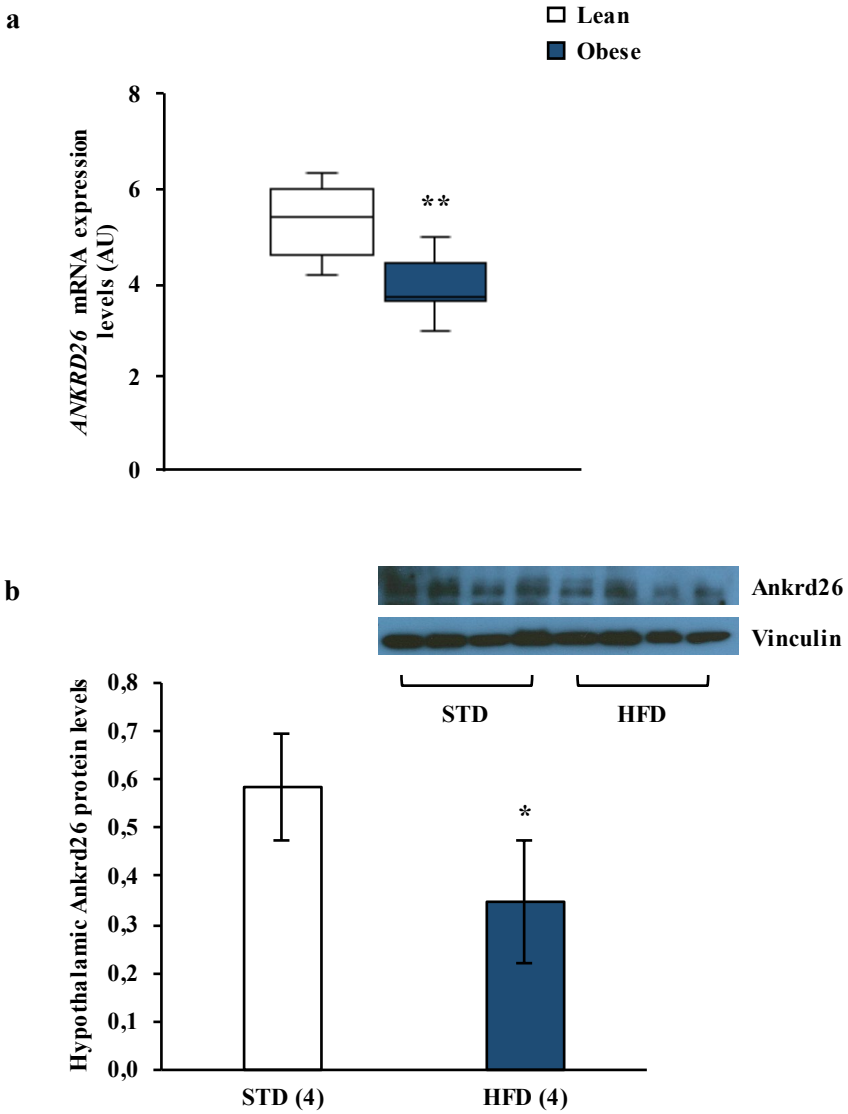

Figure 13. Hypothalamic *Ankrd26* gene and protein expression in obese mice. (a) qPCR of *Ankrd26* mRNA for DIO (n = 12) and lean control (n = 6) mice. mRNA levels are expressed in absolute units (AU). (b) Representative western blot for Ankrd26 and Vinculin proteins in DIO (n = 4) and control (n = 4) mice. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs lean.

Furthermore, to investigate whether obesity induces DNA methylation changes at the *Ankrd26* promoter, as previously identified in the VAT of obese mice [79], DNA methylation of 9 CpGs, located at - 436 and - 221 bp from the *Ankrd26* TSS, was analysed on bisulfite converted hypothalamic DNA from DIO mice (n = 4) and lean control mice (n = 4). From sequencing analysis, I found that a high methylation density at - 436 bp CpG site (1.8-fold; $p < 0.001$) was detected in obese compared with lean mice (Figure 14a); while no relevant

differences were among the other analyzed CpG sites (Figure 14a). Also, methylation at the - 436 bp CpG site was inversely related to the amount of *Ankrd26* mRNA (Figure 14b). All together, these data on obese and lean mice suggested that exposure to calorie overload (high fat diet) and/or obesity onset also affects the hypothalamic *Ankrd26* mRNA and protein expression. Detailed methylation analysis of the *Ankrd26* promoter also showed that the specific methylation at the - 436 bp CpG exerts a suppressive effect on *Ankrd26* gene.

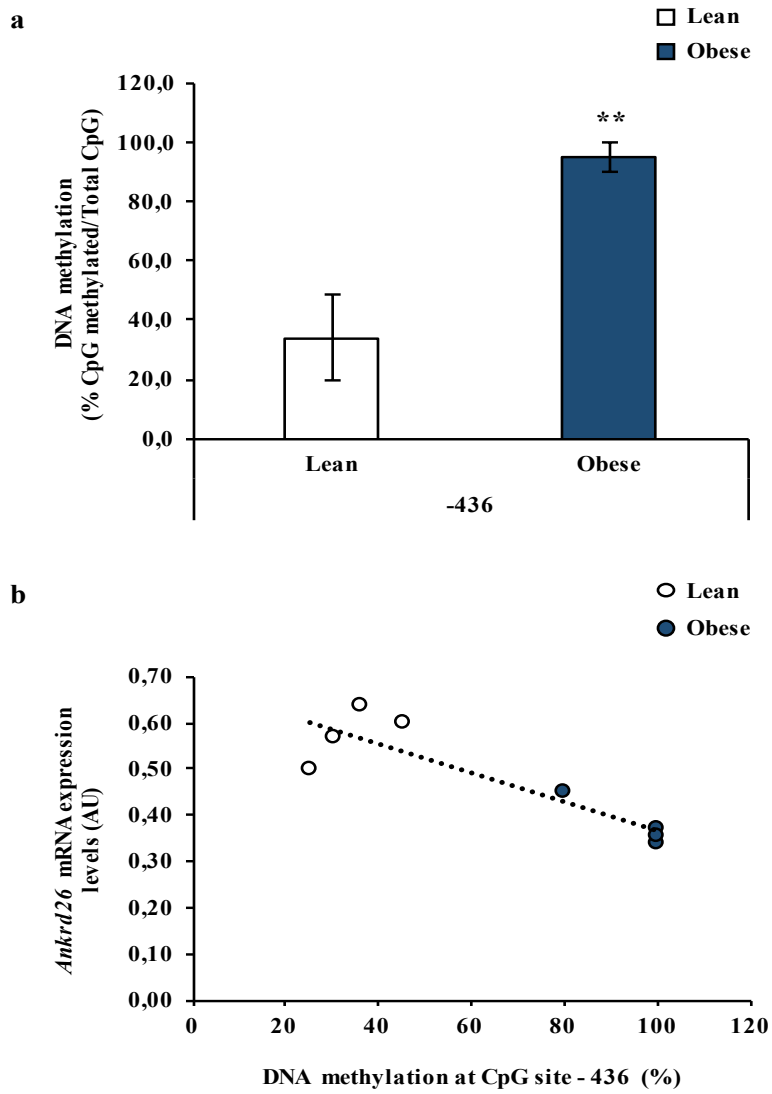

Figure 14. Hypothalamic *Ankrd26* DNA promoter methylation in obese mice. (a) Bisulfite sequencing of *Ankrd26* promoter region (- 436 bp/- 221 bp) in DIO (n = 4) and lean control (n = 4) mice. (b) Correlation between DNA methylation percentage at - 436 bp CpG and *Ankrd26* mRNA levels. r = - 0.890; $p = 0.003$. Results are mean ± SD from three independent experiments. **$p < 0.01$ vs lean.

4.2.2 Ankrd26 protein expression is modulated by anorexigenic signals in mHypoE-N46

To clarify the role of the Ankrd26 protein on the hypothalamic regulation of anorexigenic signals *in vitro*, I adopted the murine hypothalamic mHypoE-N46 cells as cellular model. These cells are immortalized hypothalamic neurons emerged as versatile tools in the study of hypothalamic function [92].

Firstly, I investigated whether the expression of the Ankrd26 protein is regulated by the anorexigenic responses to insulin and leptin stimuli. To this aim, mHypoE-N46 cells were treated with insulin (100 nmol/l) or leptin (100 nmol/l) over a one-hour time course.

Interestingly, insulin treatment up-regulates the Ankrd26 protein expression in the mHypoE-N46 cells already after 5 minutes, and these stimulatory effects were maintained up to 1 hour (Figure 15). Phosphorylation levels of Akt and ERK1/2 proteins were also evaluated as controls of insulin signaling induction. As shown in Figure 15, insulin activates both Akt and ERK1/2 already after 5 minutes, and its stimulatory effect persisted up to 1 hour.

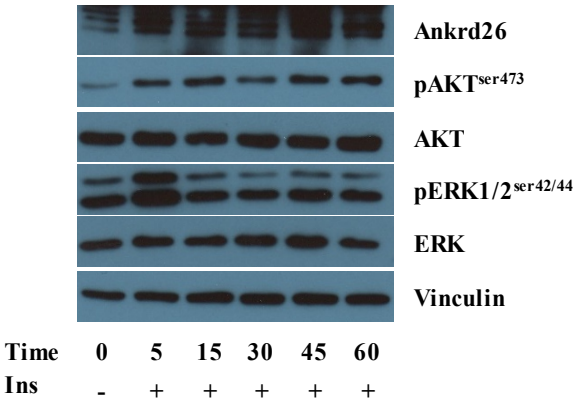

Figure 15. Insulin-mediated Ankrd26 protein expression in hypothalamic mHypoE-N46 cells. The mHypoE-N46 cells were starved over-night and then treated with insulin (100 nmol/l) for 5, 15, 30, 45 and 60 minutes. The representative western blots show the protein levels of Ankrd26, p-Akt (Ser^{473}), Total Akt, p-ERK1/2 ($Ser^{42/44}$), Total ERK1/2 and Vinculin (loading control).

Similarly to insulin, also leptin up-regulates the Ankrd26 protein expression already after 5 minutes and its stimulatory effect is maintained up to 1 hour (Figure 16). Phosphorylation levels of ERK1/2 protein was also evaluated as controls of leptin signaling induction. Leptin strongly activates ERK1/2 upon 5 minutes, and its phosphorylation levels remain higher than the basal up to 1 hour from the stimulation (Figure 16).

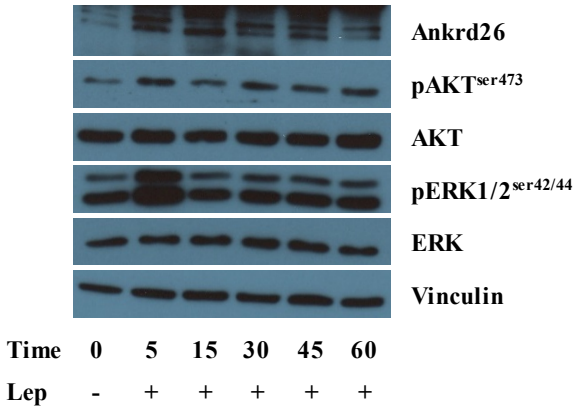

Figure 16. Leptin-mediated Ankrd26 protein expression in hypothalamic mHypoE-N46 cells. The mHypoE-N46 cells were starved over-night and then treated with leptin (100 nmol/l) for 5, 15, 30, 45 and 60 minutes. The representative western blots show the protein levels of Ankrd26, p-Akt (Ser^{473}), Total Akt, p-ERK1/2 ($Ser^{42/44}$), Total ERK1/2 and Vinculin (loading control).

Taken together, these results indicate that Ankrd26 protein is an early target of the anorexigenic insulin and leptin stimuli. Indeed, both insulin and leptin directly modulated the hypothalamic Ankrd26 protein, up-regulating, as early as 5 minutes from the stimuli, and long-term sustaining its expression (up to 1 hour).

4.2.3 The anti-obesity drug Exendin-4 up-regulates the Ankrd26 protein expression in the mHypoE-N46 cells

Successively, I wondered whether anti-obesity agents with anorexigenic effect on the hypothalamus could modulate Ankrd26 protein levels. To investigate this issue, hypothalamic mHypoE-N46 cells were treated up to 1 hour with Exendin-4 (100 nmol/l), a GLP1R agonist, approved for the treatment of obesity/T2D in humans [91].

Interestingly, Exendin-4 treatment up-regulates the Ankrd26 protein expression already after 5 minutes. Increased levels of Ankrd26 protein were also maintained at 15, 30, and 60 minutes upon Exendin-4 stimulation (Figure 17). In this case, the phosphorylation levels of AMPK and ERK1/2 proteins were evaluated as controls of the GLP1 pathway activation. As shown in Figure 17, Exendin-4 activates both AMPK and ERK1/2 protein, while stimulatory effects were observed on Akt activation (negative control) (Figure 17). These results indicate that the hypothalamic Ankrd26 protein is also an early target of the anorexigenic drug Exendin-4. The last data, together with the previous ones with insulin and leptin treatments, points out the possibility that Ankrd26 is a protein

directly involved in the downstream regulatory effects of anorexigenic signals in hypothalamic cells.

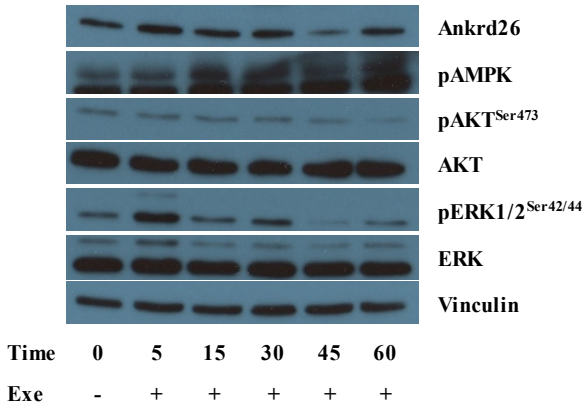

Figure 17. Exendin-4-mediated Ankrd26 protein expression in hypothalamic mHypoE-N46 cells. The mHypoE-N46 cells were starved over-night and then treated with Exendin-4 (100 nmol/l) for 5, 15, 30, 45 and 60 minutes. The representative western blots show the protein levels of Ankrd26, p-AMPK, p-Akt (Ser^{473}), Total Akt, p-ERK1/2 ($Ser^{42/44}$), Total ERK1/2, and Vinculin (loading control).

4.2.4 Over-expression of *Ankrd26* modulates neuropeptide expression in the hypothalamic mHypoE-N46 cells

Finally, to test the hypothesis that the up-regulation of the Ankrd26 protein might be somehow involved in the downstream effects of the anorexigenic stimuli, hypothalamic cells over-expressing Ankrd26 were generated by transiently transfecting mHypoE-N46 cells with the *pCMV6-Ankrd26* expression vector. As show in Figure 18, the *Ankrd26* mRNA levels were highly increased (more than 1000-fold) in mHypoE-N46-*Ankrd26* cells upon 24 and 48 hours from transfection compared to not transfected control cells. Concurrently to the increased mRNA levels, the Ankrd26 protein levels were highly increased in transfected cells compared to the control cells, as well (Figure 18).

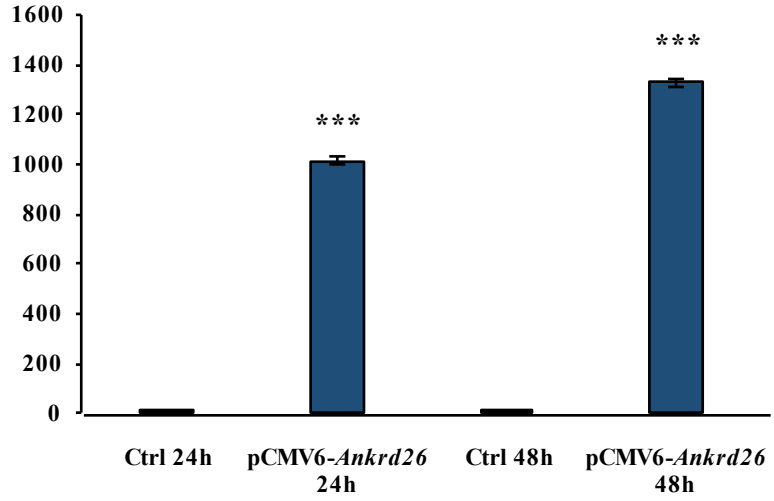

Figure 18. Ankrd26 mRNA expression in hypothalamic mHypoE-N46 cells transfected with *pCMV6-Ankrd26*. *Ankrd26* mRNA levels were evaluated by quantitative real time PCR. *Vinculin* was used as housekeeping gene upon 24 and 48 hours from transfection. Values are mean ± SD of 3 independent experiments and are expressed as Relative Expression Unit (REU). ***$p < 0.001$ vs Ctrl.

Once the Ankrd26 protein over-expression was accomplished, the basal activation of the anorexigenic signalling mediators, Akt and ERK1/2, and the mRNA expression of the hypothalamic anorexigenic (*POMC* and *CART*) and orexigenic (*NPY* and *AgRP*) neuropeptides, were investigated.

Interestingly, the over-expression of Ankrd26 slightly reduced AKT basal activation, while increased basal phosphorylation of pERK1/2 levels in the mHypoE-N46-*Ankrd26* cells compared to not transfected control cells (Figure 19). These data suggest that the over-expression of *Ankrd26* may favor the activation of the MAPK pathway in spite of the PI3K/Akt pathway in basal condition.

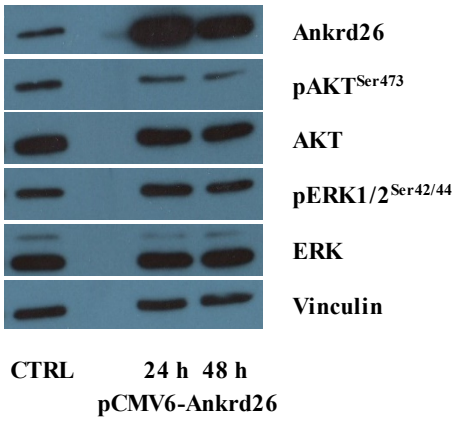

Figure 19. Ankrd26, Akt and ERK 1/2 expression in hypothalamic mHypoE-N46 cells transfected with *pCMV6-Ankrd26*. The representative western blots show the protein levels of Ankrd26, p-Akt (Ser^{473}), Total Akt, p-ERK1/2 ($Ser^{42/44}$), Total ERK1/2, and Vinculin (loading control) upon 24 and 48 hours from transfection.

Also, the over-expression of Ankrd26 in hypothalamic mHypoE-N46 cells increased of about 5- and 3-folds the mRNA expression of the anorexigenic *POMC* and *CART* neuropeptides, respectively (Figure 20a and 20b), and reduced by 50 % the mRNA expression of the orexigenic *AgRP* (Figure 20d). No differences on *NPY* mRNA were instead found between mHypoE-N46-*Ankrd26* cells and mHypoE-N46 not transfected control cells (Figure 20c). These data indicated that Ankrd26 protein is directly involved in neuropeptide gene expression and suggest that its up-regulation mediates anorexigenic signals. Indeed, its protein over-expression up-regulates the mRNA levels of the anorexigenic in spite of orexigenic neuropeptides.

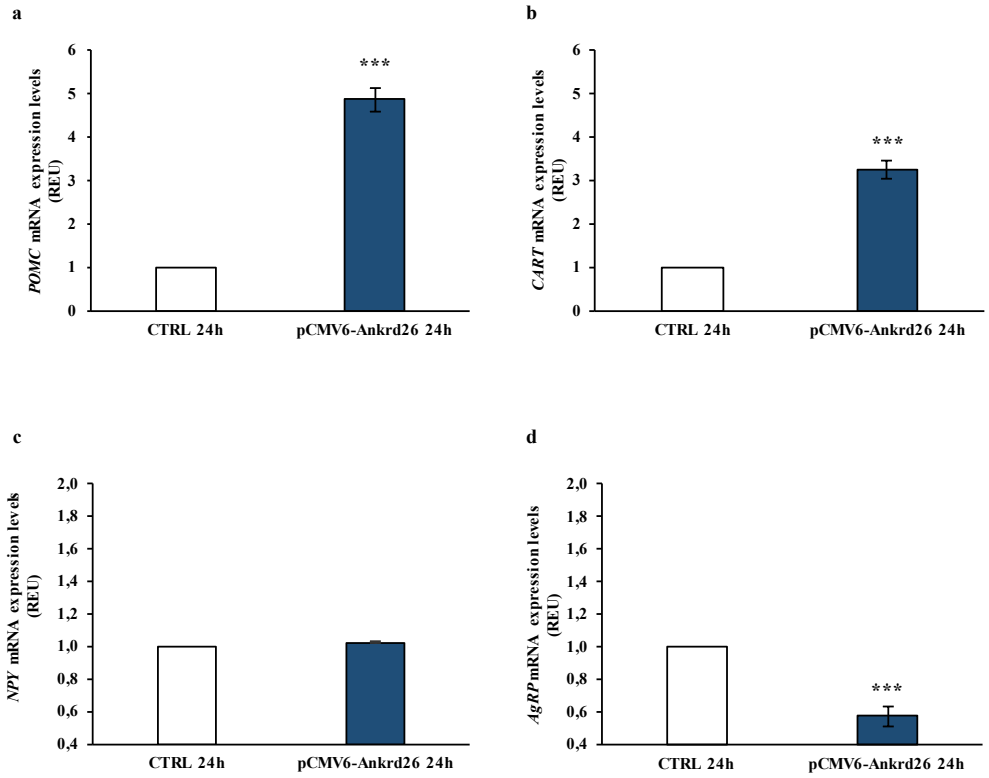

Figure 20. Neuropeptide mRNA expression in hypothalamic mHypoE-N46 cells transfected with *pCMV6-Ankrd26.* *POMC* (a), *CART* (b), *NPY* (c) and *AgRP* (d) mRNA levels were evaluated by quantitative real time PCR in mHypoE-N46-pCMV6-*Ankrd26* cells upon 24 hours of transfection. *Vinculin* was used as housekeeping gene. Values are mean ± SD of 3 independent experiments and are expressed as Relative Expression Unit (REU). ***$p < 0.001$ *vs* Ctrl.

5. Conclusion and future perspectives

At the interface between the genome and the environment, the epigenome undergoes continuous changes through the individual lifetime, which may have major implications for disease susceptibility. Consistently, mounting evidence indicates an important role of the epigenetic modifications in determining the risk of obesity. *E.g.*, obesity has been indeed associated with DNA methylation changes at genes involved in the regulation of adipogenesis, energy homeostasis, appetite and satiety. Some of these changes might even have an important role in the mediation of down-stream adverse responses to increased BMI.

In my PhD thesis, I dealt with the issue of epigenetics in the context of obesity, particularly focusing my research work on the study of DNA methylation changes at the *ANKRD26* gene and on the relevance of these modifications in relation to obesity-related unhealthy outcomes in humans. Also, I dealt with the issue of the functional consequences of these modifications on the regulation of appetite and satiety signals *in vivo* and *in vitro*, investigating methylation changes at *Ankrd26* gene in murine hypothalami from obese mice and in hypothalamic cells over-expressing Ankrd26 protein.

From the first section of my experiments (studies in humans), I can conclude that the promoter hyper-methylation at specific CpGs within the *ANKRD26* gene represents a common abnormality in obese patients metabolically unhealthy and causes silencing of the gene expression in these subjects. Further, changes in *ANKRD26* DNA methylation and gene expression correlate to increased pro-inflammatory cytokines and chemokines and cardio-metabolic risk factors, suggesting that they mark the adverse health outcome occurring in humans, especially if obese. However, some limitations exist in my current study, starting from the limited sample size of the recruited individuals. Our findings should be therefore replicated in other large and independent population. Furthermore, whether the epigenetic gene silencing of the *ANKRD26* gene precedes or is subsequent to obesity has not been addressed here and deserves to be investigated.

In the second part of my experiments, from the molecular studies *in vivo*, I can conclude that obesity leads to the epigenetic silencing of the *Ankrd26* gene in murine hypothalami, suggesting that changes in DNA methylation within the *ANKRD26* gene are events, which concurrently occurred in tissues, including WAT and hypothalamus, directly involved in the regulation of energy

homeostasis. In addition, from the molecular studies *in vitro* in hypothalamic cells, I can also conclude that Ankrd26 protein *i.* is object of up-regulation by hormones, including insulin and leptin, and drugs (Exendin-4) and *ii.* directly modulated MAPK signaling and neuropeptide gene expression, significantly increasing the mRNA of the anorexigenic *POMC* and *CART* and decreasing the mRNA of the orexigenic *AgRP*. These data, thus, suggest that Ankrd26 protein is a down-stream effector of anorexigenic signals in neuronal cells. Therefore, the epigenetic silencing of the *Ankrd26* gene might directly participate to the impairment of appetite and satiety regulation typical of obesity. However, this last aspect needs to be better addressed. Gain and loss of function experiments will help to clarify this point further.

In conclusion, the results showed in my PhD thesis have reinforced our hypothesis that *i.* the ANKRD26 might play a pivotal role in the regulation of energy homeostasis, centrally in the hypothalamic nuclei and peripherally in the WAT depots, and *ii.* the impairment of its expression by epigenetic methylation, which occurs in obesity (humans and mice), might be relevant to gain major insight into disease pathogenesis and biomarker discovery and as a novel pharmacological target.

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