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”Epigenetics of adipocyte commitment. Regulation of *Pparγ* and *Zfp423* expression”

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Epigenetics of adipocyte commitment.

Regulation of *Pparγ* and *Zfp423* expression”

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

Fiory F, Parrillo L, Raciti GA, Zatterale F, Nigro C, Mirra P, Falco R, Ulianich L, Di Jeso B, Formisano, Miele C, Beguinot F. *PED/PEA-15 inhibits hydrogen peroxide-induced apoptosis in Ins-1E pancreatic beta-cells via PLD-1*. PLoS One. 2014.

Luca Parrillo, Valerio Costa, Gregory Raciti, Michele Longo, Rosa Spinelli, Roberta Esposito, Cecilia Nigro, Viviana Vastolo, Antonella Desiderio, Federica Zatterale, Alfredo Ciccodicola, Pietro Formisano, Claudia Miele, and Francesco Beguinot. *Hoxa5 undergoes dynamic DNA methylation and transcriptional repression in the adipose tissue of mice exposed to high-fat diet*. International Journal of Obesity. 2015.

Longo M, Spinelli R, D'Esposito V, Zatterale F, Fiory F, Nigro C, Raciti GA, Miele C, Formisano P, Beguinot F, Di Jeso B. *Pathologic endoplasmic reticulum stress induced by glucotoxic insults inhibits adipocyte differentiation and induces an inflammatory phenotype*. Biochim Biophys Acta. 2016.

LIST OF ABBREVIATIONS

APC: Adenomatous polyposis coli

AZA: 5-Azacytidine

BMI: Body mass index

Bmp4: Bone morphogenetic protein 4

C/EBPα: CCAAT/ enhancer binding protein α

CAD: Coronary artery disease

CHD: Coronary heart disease

CK1: Casein kinase I

CS: Calf serum

CTRL: Control

DEX: Dexamethasone

DMEM: Dulbecco's modified Eagle's medium

Dnmt: DNA-methyltransferase

FBS: Fetal bovine serum

FFAs: Free fatty acids

FZ: Frizzled

Glut4: Glucose transporter 4

GSK3-β: Glycogen synthase kinase 3-beta

HDL: High-density lipoprotein

IBMX: 1-Methyl-3-Isobutylxanthine

IDF: International diabetes federation

LEF/TCF: Lymphoid enhancer factor/ T cell factor

LPL: Lipoprotein lipase

LRP5/6: Low density lipoprotein receptor-related proteins 5 and 6

MIX: Methyl isobutyl xanthine

MNase: Micrococcal nuclease

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NUC: Nucleosome

Pparγ: Peroxisome proliferator-activated receptor γ

TSA: Trichostatin A

TSS: Transcription start site

UCP-1: Uncoupling protein-1

VLDL: Very low density lipoprotein

WHO: World health organization

Wisp2: Wnt1 inducible signaling pathway protein 2

Zfp423: Zinc finger protein 423

ABSTRACT

Obesity is the most frequent metabolic disease worldwide and the major risk factor for metabolic disorders such as insulin resistance and type 2 diabetes. In response to overfeeding, excess lipids are stored in the adipocytes, leading to inappropriate adipose cell expansion (hypertrophic obesity), which is associated with local inflammation and dysregulated and insulin resistant adipose tissue. Hypertrophic obesity is also characterized by an inability to recruit and differentiate precursor cells into mature adipocytes. This is not due to lack of these precursor cells but instead is a consequence of an impaired ability to induce commitment and promote differentiation of available precursor cells, by inactivating inhibitory pathways, and/or activating pathways needed to commit and/or differentiate.

Very recently, the zinc finger protein *Zfp423* has been identified as a transcriptional regulator of preadipocyte determination. Zfp423 protein, indeed, directly binds the *peroxisome proliferator-activated receptor gamma* (*Pparγ*) promoter, thus promoting the gene transcription of the master regulator of adipocyte differentiation. Furthermore, Zfp423 protein is the point of convergence between the anti-adipogenic Wnt1-inducible-signaling pathway protein 2 (Wisp2) pathway and the pro-adipogenic bone morphogenetic protein 4 (Bmp4) pathway. In light of this, it is now clear that alterations impacting on Zfp423 and Pparγ protein and/or gene expressions are at least in part responsible for the restricted adipogenesis observed in hypertrophic obesity.

Increasing evidence sustains that even adipogenesis might be regulated by an epigenetically induced gene transcription reprogramming.

Thus, in light of all these observations, I aimed to establish which are the molecular mechanisms regulating the gene expression of *Zfp423* and of its downstream target *Pparγ* looking at epigenetic changes involvement.

Experiments were performed in two cell models: 3T3-L1 and NIH-3T3 cells, which are committed to and uncommitted to adipocyte lineage, respectively.

The mRNA expression of both *Zfp423* and *Pparγ* genes are increased in 3T3-L1 compared with NIH-3T3 cells. Furthermore, the expression differences among the two cellular models are not dependent on differences in the promoter DNA sequence of both genes, but rather to epigenetic mechanisms and chromatin remodeling. Indeed, performing methylation studies by bisulfite sequencing and nucleosomes positioning and occupancy analysis by micrococcal nuclease (MNase) digestion, I revealed that the DNA methylation status and the nucleosomes occupancy of *Zfp423* and *Pparγ* promoter regions are increased in NIH-3T3 compared with 3T3-L1 cells.

Finally, chemically-induced demethylation of the *Zfp423* and *Pparγ* promoters by DNA-methyltransferase (Dnmt) inhibitor 5-Azacyditine (AZA) treatment promotes adipocyte terminal differentiation in the uncommitted NIH-3T3 cell line.

In conclusion, I demonstrated that *Zfp423* and *Pparγ* genes, which are involved in adipocyte commitment and differentiation, are transcriptionally regulated by DNA methylation and dynamic chromatin remodeling, and that the modulation of the methylation status of the promoter region of these two genes is relevant to the regulation of adipocyte commitment and terminal differentiation *in vitro*.

1. INTRODUCTION

1.1 Obesity

Overweight and obesity result from a chronic imbalance between energy intake and energy expenditure, leading to an excessive accumulation of fat in adipose tissue. The current classification of overweight and obesity in adults is based on the body mass index (BMI). BMI, indeed, is an indirect measure of body fat, described as the person's weight in kilograms, divided by the square of height in meters (kg/m^2) (World Health Organization 2014).
Thus, according to World Health Organization (WHO) classification (World Health Organization 2014), overweight is defined as a BMI of 25 or more and includes pre-obesity (25-29.9) and obesity that is divided into three categories: Class I (30–34.9), Class II (35–39.9) and Class III (>40) (James *et al.* 2004) (Table 1).

Classification	BMI (Kg/m^2)
Underweight	<18.5
Normal range	18.5-24.9
Overweight	≥25
Pre-obese	25-29.9
Obese I	30-34.9
Obese II	35-39.9
Obese III	≥40

Table 1. Classification of adult underweight, overweight and obesity according to BMI. *According to WHO, overweight is defined as a BMI equal to or more than 25 whereas obesity as a BMI equal to or more than 30.*

The incidence of obesity and overweight is increasing dramatically in western society; it was estimated that the worldwide prevalence of obesity has doubled between 1980 and 2014. WHO (World Health Organization 2014) estimates that the 38% of males and 40% of women were overweight while the 11% of adult males and the 15% of adult females were obese in 2014 (Figure 1).

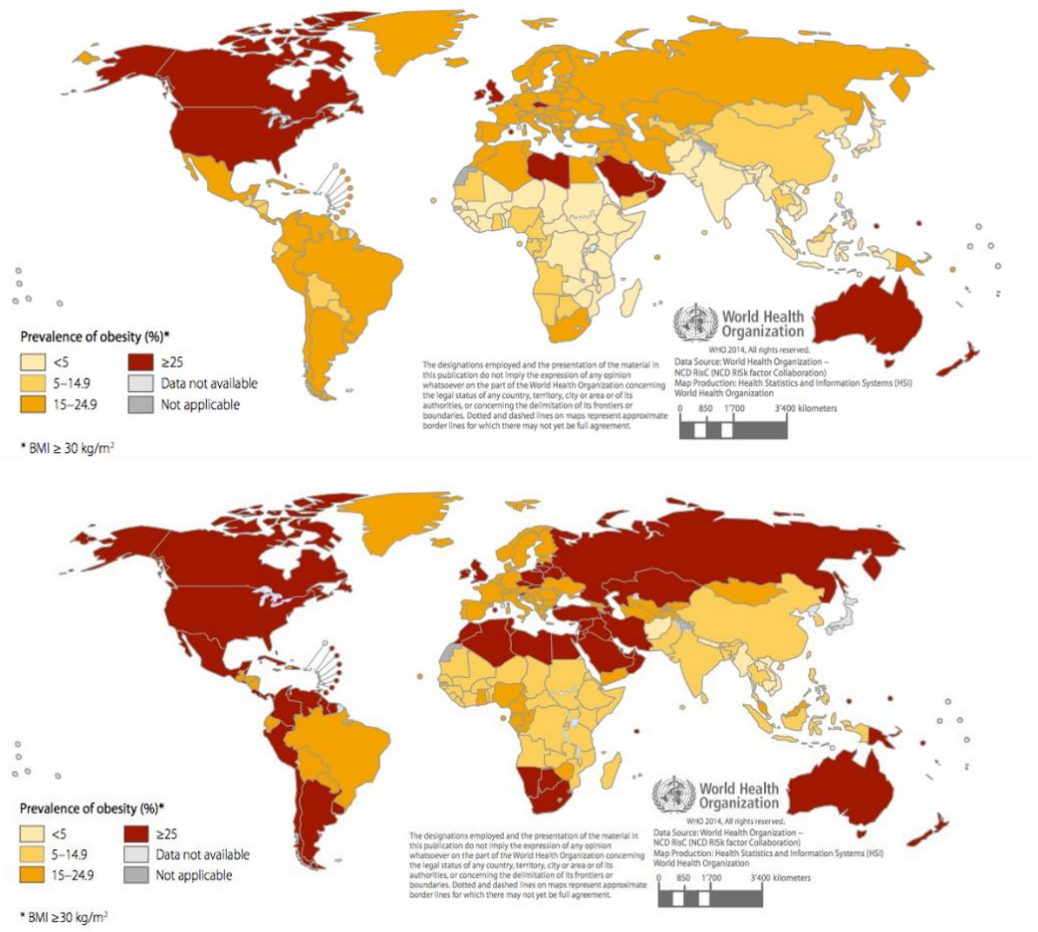

Figure 1. Prevalence of obesity in adulthood. *The maps indicate the adult prevalence of obesity respectively in males and females (World Health Organization 2014).*

Obesity is not a single disorder but a heterogeneous disease with multiple causes. Body weight, indeed, is regulated by a complex interaction between genetic, societal, environmental, and political influences (Frood et al. 2013).
On the genetic level, many evidence has shown that predisposing genes can contribute significantly to the development of obesity (Silventoinen et al. 2009; Silventoinen et al. 2010) by interacting with environmental factors such as physical inactivity and unhealthy diet (Choquet and Meyre 2011).
Twin studies suggest a heritability of fat mass of between 40% and 70% with a concordance rate of 0.7-0.9 between monozygotic twins compared to 0.35-0.45 among dizygotic twins (Stunkard et al. 1986). Furthermore, it has been also seen that if one's parent is obese, the own risk to develop obesity significantly increased compared with age and sex-matched individuals without familiarity of obesity, and if obesity is present during the early childhood, the individual chances to be obese throughout his/her own life are high compared with their lean counterparts.

In addition to genetics, also societal changes that modify individual behaviours, leading to a more sedentary life (Bauman et al. 2011), physical inactivity (Hansen et al. 2012), and poor dieting (Caraher et al. 2010; Perez-Escamilla et al. 2012), may also contribute to the increase of obesity incidence (Swinburn et al. 2011).
Many evidence suggests a strong correlation between BMI and the incidence of several chronic diseases, such as cancer, hypertension, coronary heart disease (CHD), respiratory complications and diabetes mellitus (Figure 2).
This relationship is approximately linear for a range of BMI less than 30, but the risk to develop the obesity-related comorbidities greatly increase for subjects with a BMI above 29, independently of gender (Willett et al. 1995; Willett et al. 1999). The development of these complications is strictly correlated with the increase of body fat that impairs the physiological functions. Indeed, an increased amount of fat results in alterations of total blood volume and cardiac function, whereas the distribution of fat around thoracic cage and abdomen impairs respiratory function by the reduction of respiratory compliance. Moreover, intra-abdominal visceral deposition of adipose tissue plays an important role in the development of hypertension, insulin resistance, hyperlipidemia and diabetes mellitus (Kopelman 2000).

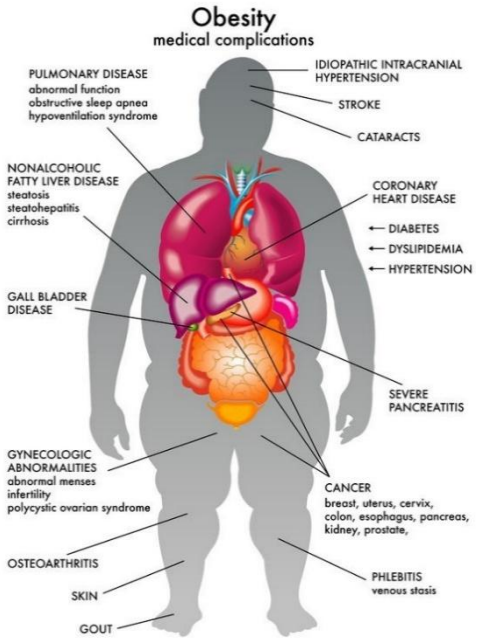

Figure 2. Representation of the most important comorbidities associated with obesity. *Obesity has a multitude of adverse metabolic health consequences such as diabetes mellitus, hypertension, dyslipidemia, and metabolic syndrome (Kopelman 2000).*

1.2 Diabetes

Diabetes mellitus is a group of chronic metabolic disorders characterized by elevated blood glucose levels (hyperglycemia) caused by defects in insulin secretion, insulin action or both (American Diabetes Association 2010).

The vast bulk of cases of diabetes falls into two main etiopathogenetic categories (American Diabetes Association 2010).

Type 1 diabetes occurs predominantly in young people and is characterized by an absolute deficiency of insulin, resulting from an autoimmune mediated destruction of β cells. Its frequency is low relative to type 2 diabetes, which accounts for over 90% of cases globally, and requires a daily injection of insulin.

Type 2 diabetes is the most common form of diabetes mellitus and is characterized by increased levels of blood glucose to which individuals are predisposed by an interaction between genetic, social and environmental risk factors (Khodabandehloo et al. 2016).

In type 2 diabetes, the hyperglycemia is initially due to a decreased insulin sensitivity, as a result of insulin resistance, which leads to hyperinsulinemia. Later on, when the pancreatic β cells are not capable of producing the amount of insulin needed to maintain normal glycemic status, chronic hyperglycemia and type 2 diabetes occur (Kahn S et al. 2006).

Primarily strategy for type 2 diabetes treatment consists of an individualized approach, consisting initially of dietary control and lifestyle modifications, followed by oral type 2 diabetes medications and insulin therapy (American Diabetes Association 2015).

The worldwide rapid increasing prevalence of type 2 diabetes is one of the major and serious public health burden (Tabish 2007). Type 2 diabetes reached an epidemic stage in line with the increasing obesity prevalence and has been particularly accelerated in the developing countries.

According to the International Diabetes Federation Atlas (International Diabetes Federation Atlas 2013), there are 382 million people in the world living with diabetes, defined as a fasting plasma glucose value ≥7.0 mmol/L (126 mg/dl) or 2-h plasma glucose ≥11.1 mmol/l (200 mg/dl) after a load of glucose (World Health Organization 2006), and it is expected to afflict around 592 million people by 2035 (International Diabetes Federation Atlas 2013) (Figure 3). This rise is largely driven by modifiable risk factors, such as physical inactivity, overweight and diet high in fat and salt (World Health Organization 2010).

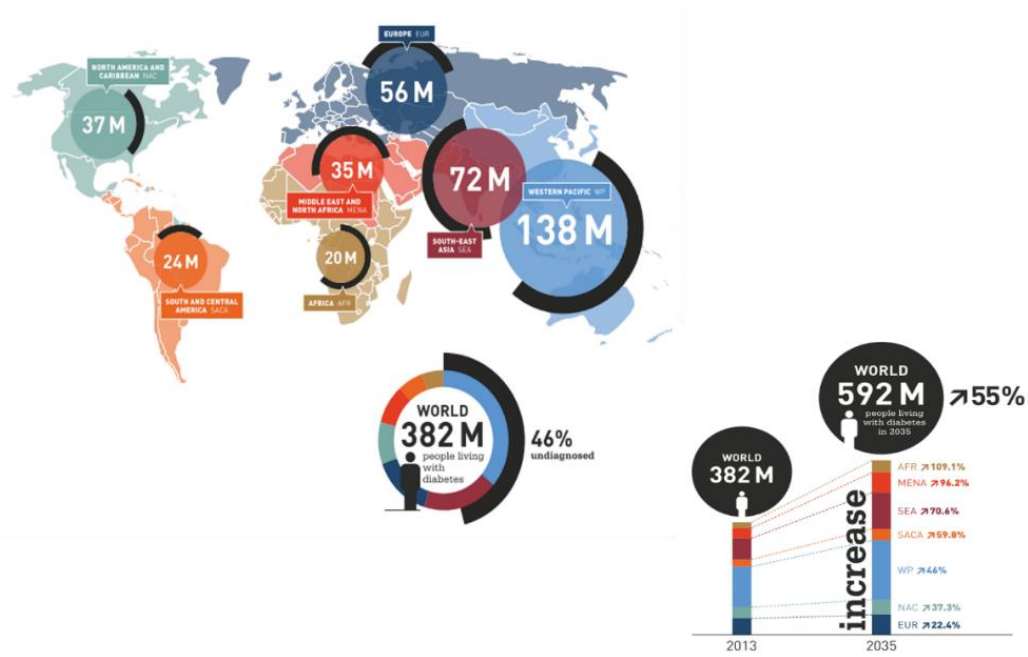

Figure 3. Prevalence of diabetes in the world. *The IDF Atlas (International Diabetes Federation Atlas 2013) figure provides a worrying indication of the future impact of diabetes on the global development.*

Diabetes and its complications are the major cause of morbidity and mortality around the world and result in higher health care costs.
Diabetes is the well-recognised cause of premature death and disability and is powerfully linked with both microvascular and macrovascular complications.
Microvascular complications affect small blood vessel and include damage to the nervous system (neuropathy), renal system (nephropathy) and eye (retinopathy) while macrovascular complications comprise coronary artery disease (CAD), stroke and myocardial infarction (World Health Organization 2010). Thus, looking at the rapid growth in the number of type 2 diabetics, the evolution and clinical implementation of effective psychosocial interventions are needed (Debono and Cachia 2007).

1.3 The relationship between obesity and type 2 diabetes

As previously mentioned, the worldwide increase in the prevalence of obesity largely explains the epidemic of type 2 diabetes over the past twenty years (Eckel et al. 2011). According to Ford and colleagues (Ford et al. 1997), the risk to develop diabetes increases between 4.5 and 9% for every kilogram of weight increase.
In 1995, the worldwide obese population was calculated to be around 200 million, while the subjects considered obese, in 2014, are 600 million.

Strikingly similar trends have been observed in type 2 diabetes statistics, and future predictions are alarming (King et al. 1998).
For this reason, Sims and colleagues (Sims et al. 1973), coined the term 'diabesity' to explain the close relationship existing between these two diseases. Obesity and type 2 diabetes often occur together and the risk to develop type 2 diabetes for a BMI over 35, compared to a BMI less than 23, increases by 50-80 fold (Figure 4) (Chan et al. 1994).

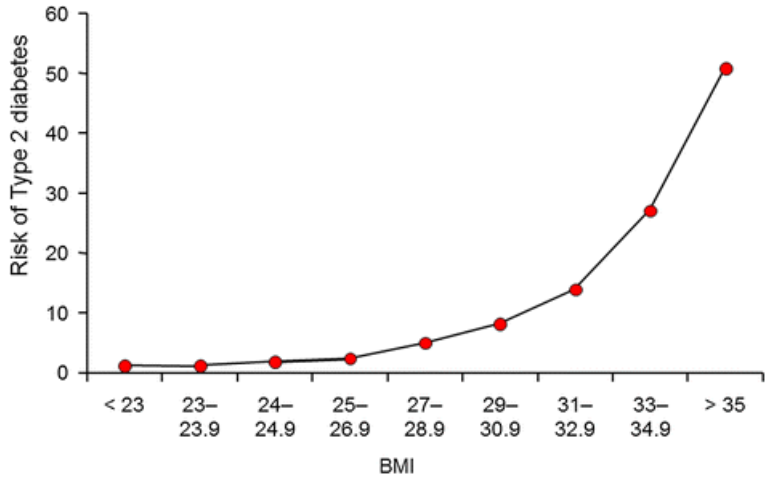

Figure 4. BMI and type 2 diabetes risk. *The relationship between BMI and risk to develop type 2 diabetes, derived from data extracted from Chang and colleagues (Chan et al. 1994).*

Therefore, excess weight is the primary risk factor for type 2 diabetes. Nevertheless, obesity is not a homogeneous condition. Indeed, approximately 10-30% of obese individuals do not show the associated metabolic complications and, for this reason, are defined as metabolically healthy individuals (Kloting et al. 2010). Nevertheless, a similar number of non-obese individuals show a dysmetabolic state and reduced insulin sensitivity (Scott et al. 2014). Thus, BMI *per se* is not a sufficiently sensitive marker of individual risk for obesity-related metabolic complications.
Conversely, increasing evidence suggests that adipose tissue distribution might be a more relevant marker for obesity-related metabolic complications. Indeed, abdominal distribution markedly increases both cardiovascular and diabetes risk (Fox et al. 2009; Despres and Lemieux 2006).
This finding has raised much interest in the possible role of regional differences in adipose tissue metabolism and, in particular, in the role of intra-abdominal and visceral fat in metabolic complications (Després and Lemieux 2006; Neeland et al. 2012).

1.4 Adipose tissue

Adipose tissue is a complex organ with heavy effects on physiology and pathophysiology. For many years, adipose tissue has been ignored because was considered just as a form of connective tissue, containing lipid droplets but without any link with the metabolism of the organisms (Rosen and Spiegelman 2014; Rosen and Spiegelman 2006). Nevertheless, this notion gradually began to shift with the realization that adipose tissue takes on a critical role in energy homeostasis and in the regulation of metabolic functions.

Adipose tissue is indeed a metabolically active tissue, consisting of lipid-filled cells, called adipocytes, that make up the 90% of the tissue volume (Lee et al. 2010). In addition, to mature adipocytes, fat tissue contains several metabolically active and inflammatory cells, including stromal vascular cells such as fibroblasts, leukocytes, macrophages, pericytes, endothelial cells, and preadipocytes, which take on a significant part in the integrity of tissue (Katz 2002).

The mammalian adipose tissue is divided into two functionally distinct forms of fat: white and brown. White adipose tissue is the primary site of energy storage (Ronti et al. 2006; Rosen and Spiegelman 2006) but can act also as a thermal insulator and protect other organs from mechanical damage (Trayhurn 2007). Brown adipose tissue, on the other hand, is highly specialized in the dissipation of stored chemical energy by the generation of heat by the actions of mitochondrial uncoupling protein-1 (UCP-1), a brown adipose tissue-specific protein located within the mitochondria (Rosen and Spiegelman 2014; Rosen and Spiegelman 2006). Nevertheless, the concept of two different forms of adipose tissue has been altered after the recognition that brown fat cells might be interspersed in the white adipose tissue to form “beige cells” (Boström et al. 2012). A recent study has shown that the fully stimulated brown and beige adipocytes contain comparable amounts of UCP-1, suggesting that they have a similar thermogenic capacity (Wu et al. 2012).

1.4.1 White adipose tissue as link between obesity and type 2 diabetes

As above mentioned, the main functions of white adipose tissue are: i) to store calories in the form of lipid droplets after food intake; ii) to release fat during the fasting state; iii) to cushion the body parts and to protect delicate organs from mechanical stress.

Furthermore, white adipose tissue plays a critical role in the energy balance and nutritional homeostasis (Rosen and Spiegelman 2014).

In humans, white adipose tissue constitutes the largest endocrine organ and is certainly so in overweight and obese individuals. Therefore, even minor metabolic changes in such a large secretory organ can impact on the whole-body metabolism (Trayhurn 2007).

White adipocytes in addition to store fatty acids, produce and release also a wide range of lipid, hormones and cytokines (adipokines) such as leptin, interleukin 6, adiponectin, monocyte chemoattractant protein 1 and tumour necrosis factor alpha, that are required for many biological functions as glucose and lipid metabolism, insulin sensitivity, inflammation, coagulation, blood pressure and feeding behaviour (Van Gaal et al. 2006; Müller et al. 2012; Wildman et al. 2008; Klein et al. 2004) (Figure 5).

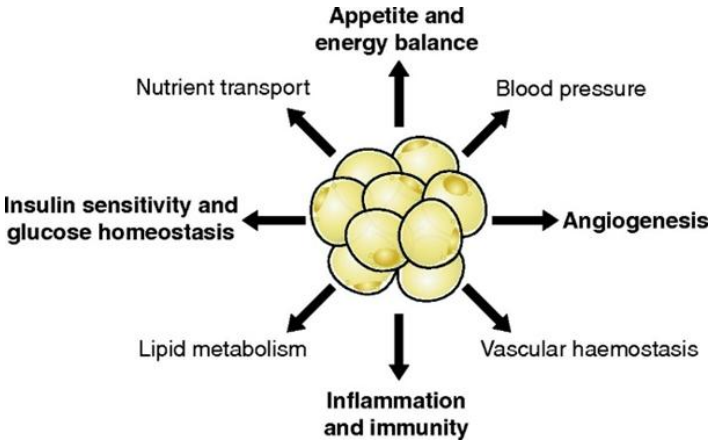

Figure 5. Adipose Tissue as an endocrine organ. *Adipose tissue is an endocrine organ that secretes various hormones and adipokines involved in the many physiological and metabolic processes (Trayhurn 2013).*

The white adipose organ includes multiple discrete locations (Lee et al. 2014; Shen et al. 2003). The most common classification distinguishes between subcutaneous and visceral fat (Figure 6) that are characterized by functional differences. Subcutaneous adipose tissues store more than 80% of total body fat in the body and is located beneath the skin; while visceral fat is around internal organs and comprises less that 10% of the total fat mass.

Being involved with its expansion in the development of obesity, white adipose tissue is now seen as the epicenter of the disorder and its associated comorbidities (Greenberg and Obin 2006).

Many studies have, indeed, shown that total adiposity is strongly associated with metabolism and cardiovascular risk. More, it has been also determined that the various fat depots contribute differently to this risk (Hassan et al. 2012). Indeed, the accumulation of visceral fat seems to correlate with an increased prevalence of insulin resistance, metabolic syndrome, and cardiovascular complications (Lemieux et al. 1996) whereas the fat accumulation in the peripheral subcutaneous adipose tissue does not seem to be associated with many of these obesity-related comorbidities (Porter et al. 2009).

A key role in the regional variation in fat tissue function is also played both by regional differences in preadipocytes characteristics (replication, differentiation, subtype abundance, susceptibility to apoptosis or senescence and gene expression) and by some aspects of adipocyte behavior (adipokine secretion, rates of lipolysis and triglyceride synthesis) (Tchkonia et al. 2010; Tchkonia et al. 2013).

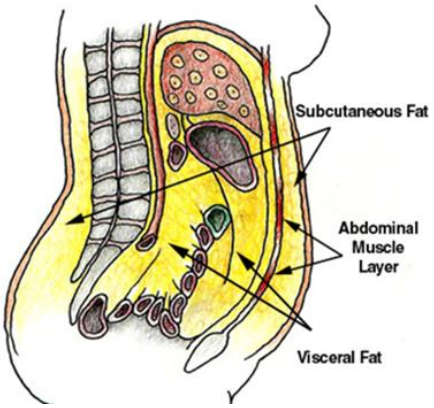

Figure 6. Representation of the anatomical localization of the main abdominal adipose tissue depots*. There are two major types of fatty tissue in the body: visceral and subcutaneous adipose tissue (Lee et al. 2014; Shen et al. 2003).*

1.4.2 White adipose tissue remodeling in obesity

White adipose tissue is characterized by an incredible ability to change its dimension (Rosen and MacDougald 2006; Rosen and Spiegelman 2014; Rosen and Spiegelman 2006), in response to nutrient excess or insufficiency (Martinez-Santibañez and Nien-Kai Lumeng 2014); this is essential for the maintenance of whole-body energy homeostasis.

White adipose tissue expansion is primarily achieved i) by increasing the size of individual adipocytes (hypertrophy), or ii) by recruiting new adipocytes from the resident pool of progenitors (hyperplasia) (Rosen and Spiegelman 2014).

In response to overfeeding, adipose depots expand first by hypertrophy, until a critical threshold (~0.7-0.8 µg/cell) is reached (Krotkiewski et al. 1983).

As shown by several studies, the balance between these two mechanisms depends on the location of the fat pad. However, in humans, the mechanisms of adipose tissue expansion during obesity are not fully delineated yet.

Anyway, many evidence suggests that overfeeding for several months induces an increase in cell size (Salans et al. 1971). A late version of this study suggests that acute excess of calories induces hypertrophy in the upper body subcutaneous fat and hyperplasia in the fat depots located below the waist (Tchoukalova et al. 2010).

More recently, Spalding and colleagues (Spalding et al. 2008), shown that "new adipocytes are generated constantly to replace lost adipocytes" and estimated that the half-life of the average adipocyte is in the order of 8.3 years (Sun et al. 2011). Moreover, it has been demonstrated that the number of adipocytes becomes already fixed in childhood and early adulthood (Sun et al. 2011; Spalding et al. 2008) and that significant weight loss is associated only with reduction of adipocyte volume and not of their number.
The last suggests that hypertrophy of adipocytes is the most significant mechanism by which white adipose tissue mainly undergoes alterations during adulthood (Spalding et al. 2008; Sun et al. 2011).
Nevertheless, as stated previously, the mechanisms by which adipose tissue expands in humans in obesity are not fully defined, and the patterns of fat deposition and expansion vary between people. This variance in adipose tissue distribution appears to determine the differences found in metabolic disease risk seen among individuals with a similar level of adiposity.
Many evidence suggests that in obese individuals, the metabolic dysfunction is related to large adipocytes, and is associated with the impaired ability to develop new cells (Hammarstedt et al. 2012; Isakson et al. 2009).
On the contrary, others indicate that an overabundance of small adipocytes is associated with a more unhealthy metabolic phenotype (McLaughlin et al. 2010; McLaughlin et al. 2007). In both instances, the final result is the same.
Indeed, the ability of the white adipose tissue to act as an efficient storage depot is impaired, inducing other peripheral tissues to compensate by accumulating inappropriately lipids.
Thus, the impairment in the ability of white adipose tissue to generate new cells through differentiation of preadipocytes is associated with the development of insulin resistance and diabetes mellitus (Karastergiou and Mohamed-Ali 2010) and the enlargement of adipocytes (hypertrophy) is correlated with death of adipocytes by necrosis, leading to local inflammation and recruitment of monocytes as a response (Strissel et al. 2007; Cinti et al. 2005).

1.4.2.1 Hypertrophic obesity: restricted adipogenesis and inflammatory response

As above mentioned, white adipocyte hypertrophy is characterized by the presence of large, lipid-laden adipocytes and may result from an intrinsically reduced capacity of *de novo* adipogenesis (Klöting and Blüher 2014).
In addition to the last observations, Smith and colleagues, analysing adipogenesis in stromal vascular cells from human abdominal subcutaneous depots (Gustafson and Smith 2012), have found poor differentiation in individuals with large subcutaneous adipose cells (hypertrophic obesity), whereas small adipose cells were associated with good adipogenesis,

suggesting thus a causal relationship between restricted adipogenesis and adipose cell volume (Figure 7).
Furthermore, these differences were not due to lack of precursor cells in hypertrophic obesity, but rather to the inappropriate signaling of pathways which promotes precursor cell differentiation and/or which enhances inhibitory signals promoting dedifferentiation (Talchai et al. 2012).
Unlike to hyperplastic obesity, many human studies have demonstrated that adipocyte hypertrophy is negatively correlated with the impairment of glucose and lipid metabolism and with the loss of insulin sensitivity, independently by the grade of obesity measured as BMI (Smith and Hammarstedt 2010; Blüher 2013; Klöting and Blüher 2014) and, plays a key function as an independent predictor of future type 2 diabetes onset (Lönn et al. 2010).

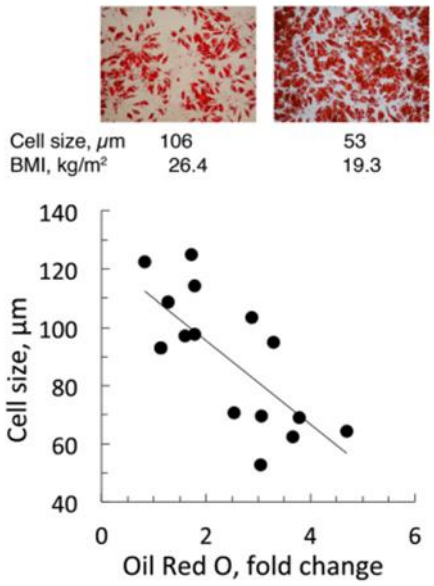

Figure 7. The distinction of human preadipocytes from the abdominal subcutaneous adipose tissue is concerned to the adipose cell size of the donors. *Purified stromal vascular cells were differentiated for 21 days in vitro and then stained with Oil Red O to show the lipid droplets (top).The negative correlation between the amount of lipids accumulated during differentiation and the adipose cell size of the donors is shown (bottom) (Gustafson and Smith 2012).*

Bluher and colleagues (Klöting and Blüher 2014), have furthermore demonstrated that adipocyte volume is associated not only with significantly impaired whole-body insulin sensitivity but also with increased circulating factors of inflammation, oxidative stress and an increased number of macrophage infiltration within adipose tissue (Klöting and Blüher 2014; Blüher 2013). Altogether these alterations cause local chronic low-grade inflammation (Sell et al. 2012; Bastard et al. 2006; Weisberg et al. 2003) (Figure 8).

The response of adipocytes, macrophages and other adipose tissue cells to these stress stimuli contributes to the deterioration of cellular functions and to the metabolic comorbidities associated with obesity (Rudich et al. 2007; Bashan et al. 2007). E.g., intracellular kinases involved in the transmission of the stress response, such as p38 and Jun N-terminal kinase, are upregulated and activated in adipose tissue of individuals with adipocyte hypertrophy (Bashan et al. 2007). Furthermore, a recent study suggests that in addition to adipocytes also macrophages in adipose tissue are exposed to the stress and may mediate the link between adipose tissue stress-response and the risk of obesity-induced metabolic diseases and inflammation. In addition, macrophages may report to other organs the stress and inflammatory status of the adipose tissue, leading to secondary organ failure.
In conclusion, hypertrophic obesity may start a sequence of pathogenic event that contributes to adipose tissue dysfunction and may subsequently have negative effects on other tissues (Blüher 2013).

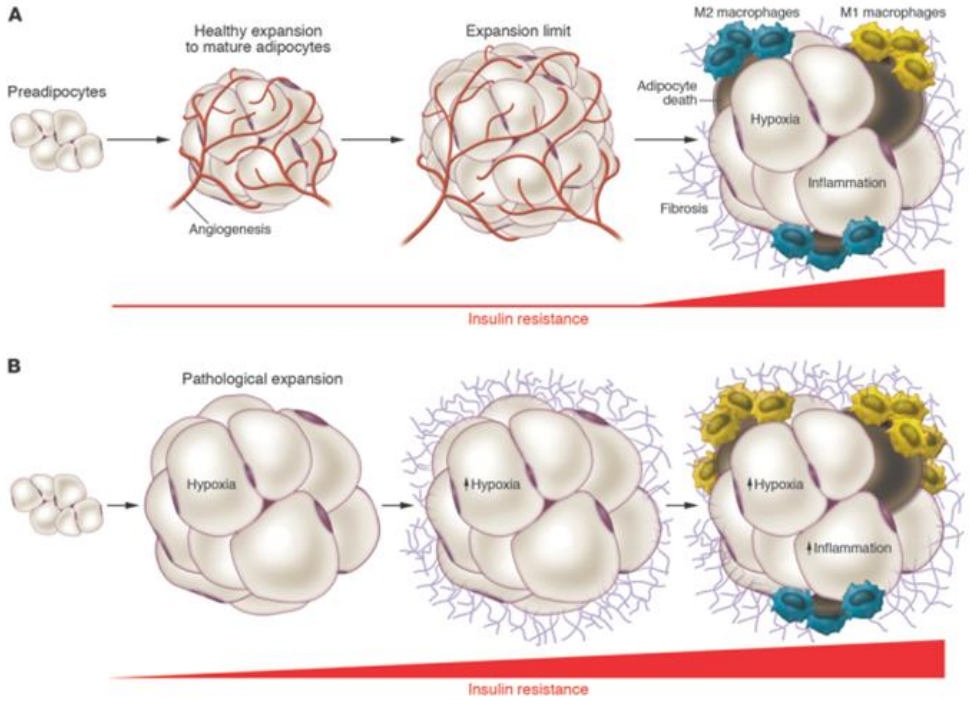

Figure 8. Adipose tissue expansion. *Healthy and unhealthy adipose tissue expansion during weight gain. (A) Healthy adipose tissue expansion comprises of an enlargement of adipose tissue through effective recruitment of adipogenic precursor cells to the adipogenic program. (B) In contrast, pathological adipose tissue expansion consists of an expansion of existing adipocytes, limited angiogenesis, and resulting hypoxia. Ultimately, the expansion of the adipose tissue leads to the recruitment of macrophages through various signals synthesized by adipocytes. These macrophages are found mainly around apoptotic adipocytes and M1-stage macrophages prevail, leading to an inflammatory phenotype that is powerfully linked with systemic insulin resistance (Sun et al. 2011).*

1.4.2.2 Hypertrophic obesity: adipose tissue expandability and ectopic fat accumulation

Adipose tissue expandability in response to positive energy balance has been considered an adaptive process. However, recent evidence suggests that adipose tissue expandability may play an important role in the development of the obesity-associated comorbidities (Slawik and Vidal-Puig 2006).
In particular, the subcutaneous adipose tissue has a higher capacity to expand its capillary network compared with visceral adipose tissue, but with the gain of fat mass (hypertrophy) this capacity decreases and, as a consequence, fat is stored in other metabolically more harmful ectopic lipid depots, such as intra-abdominal and visceral sites, liver, myocardium, and skeletal muscles (Gealekman et al. 2011; Van Harmelen et al. 2004; Bouloumie et al. 2002; Rupnick et al. 2002).
Many evidence suggests that both the restricted adipogenesis that the impaired tissue vascularization capacity play a critical role in the reduction of subcutaneous adipose tissue expandability and are responsible for the development of metabolic disorders (Gealekman et al. 2011; Van Harmelen et al. 2004); furthermore decrease in tissue expandability in humans positively correlates with whole body insulin resistance (Gealekman et al. 2011).
The inability of subcutaneous adipose tissue to expand proportionally to caloric excess represents an important junction in the development of adipose tissue dysfunction and ectopic fat accumulation (Blüher 2013; Van Harmelen et al. 2004; Bouloumie et al. 2002; Rupnick et al. 2002), defined as the accumulation of excess energy as fat in locations not classically associated with adipose tissue storage (Britton et al. 2011; Gustafson et al. 2007).
Ectopic fat depots can induce both systemic (as visceral adipose tissue and intrahepatic fat) that local effects (as pericardial, myocardial and perivascular fat) (Figure 9). Nevertheless, the mechanisms underlying the connection between obesity, body fat distribution, and metabolic derangements remain still incompletely understood (Cornier at al. 2011).

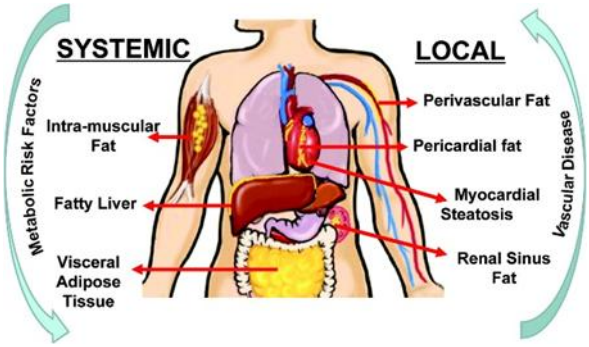

Figure 9. Classification of ectopic fat. *Ectopic fat depots in non-adipose tissues, promotes systemic and local effects, including dysfunction, insulin resistance, and inflammation in the liver, muscular tissue, pancreas and visceral fat (Britton et al. 2011).*

As stated previously, lipids can accumulate abnormally in hepatocytes, leading to an abnormal fat accumulation in the liver that is well known as a nonalcoholic fatty liver disease (NAFLD).
NAFLD is characterized by various hepatic abnormalities that ranging from hepatic steatosis to non-alcoholic steatohepatitis (NASH). Furthermore, the resulting accumulation of fat in hepatocytes can lead to hepatic insulin resistance, impairment in the insulin-mediated suppression of hepatic glucose production and hence fasting hyperglycemia (Lee et al. 2014). In addition, fatty acids impair the ability of hepatocytes to degrade insulin, leading to hyperinsulinemia, and provide substrates for triglyceride synthesis, contributing to hyperlipidemia by elevated hepatic very low-density lipoprotein (VLDL)-triglyceride secretion combined with elevated high-density lipoprotein (HDL)-cholesterol clearance (Choi and Ginsberg 2011).
On the other hand, ectopic fat accumulation surrounding the heart and blood vessels is assumed to have mostly local effects.
the epicardial adipose tissue under normal conditions is thought to serve a number of physiologic functions, including acting as an energy source to the myocardium by releasing fatty acids (Gustafson 2010; Gustafson et al. 2007; Talman et al. 2014).
Moreover, epicardial fat may directly affect the coronary arteries and myocardium through paracrine actions of locally secreted adipokines and other bioactive molecules (Ricote et al. 1998; Tannock et al. 2004), which promote macrophage infiltration into adipose tissue, and locally reduce insulin-induced vasodilatation, leading to vasoconstriction (Chen et al. 2001).
These alterations might have long-term effects on cardiovascular function and morphology and might be responsible for cardiovascular disorders (Ricote et al. 1998).

1.5 Adipocyte differentiation: pro- and anti-adipogenic pathways

The generation of adipocytes from precursor stem cells takes a complex and temporally regulated program of gene expression that is termed adipogenesis (Rosen and MacDougald 2006; Rosen and Spiegelman 2014; Rosen and Spiegelman 2006). At the cellular level, adipogenesis is divided into two phases: determination and terminal differentiation.
During determination, precursor cells became committed to adipocyte lineage and are identified as preadipocytes.
These cells cannot be distinguished morphologically from their precursors, but lost the potential to differentiate into other cell types In the second phase, known as terminal differentiation, preadipocyte acquires the machinery required for transport and synthesis of lipids, for responsiveness to insulin and for the secretion of adipocyte-specific proteins (Rosen and MacDougald 2006; Rosen and Spiegelman 2014).

The molecular regulation of terminal differentiation is more extensively characterized than the commitment, which has not been entirely understood yet (Rosen and Spiegelman 2014).
Several transcription factors, such as Pparγ and CCAAT/enhancer binding protein alpha (C/EBPα) (Wu et al. 1999) have been identified to be a regulator of adipocyte differentiation by activating adipocyte-specific genes (Niemelä et al. 2008) (Figure 10).
In details, Pparγ is a member of the nuclear receptor superfamily that plays an important role in the control of gene expression linked to a variety of physiological processes. Its most notable function is to regulate development of adipose tissue, which involves coordinating expression of many hundreds of genes responsible for the establishment of the mature adipocyte phenotype (Tontonoz et al. 1994; Evans et al. 2004; Farmer 2005).
In addition to Pparγ, Zfp423 was recently identified as a transcriptional regulator of preadipocyte commitment (Gupta et al. 2010; Cawthorn et al. 2012). Indeed, work from the Spiegelman lab showed that the Zfp423 transcription factor is enriched in adipogenic fibroblast cell lines, relative to non-adipogenic cells. Although *Zfp423* is not upregulated during adipogenesis, ectopic expression of *Zfp423* in non-adipogenic cells is sufficient to activate *Pparγ* expression and markedly increases the adipogenic potential of these cells (Gupta et al. 2010). Conversely, reduction of *Zfp423* expression in 3T3-L1 preadipocytes or mouse embryonic fibroblasts blunts preadipocyte *Pparγ* expression and diminishes the ability of these cells to differentiate.

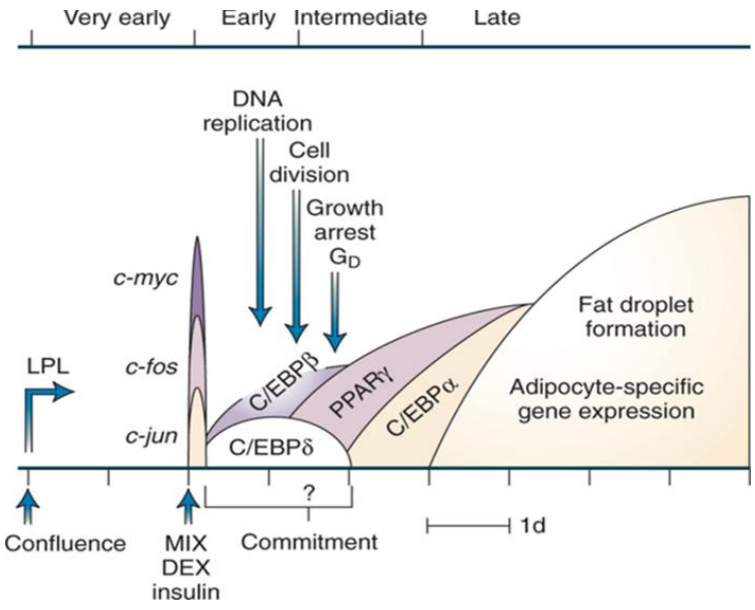

Figure 10. Adipocyte differentiation and gene expression. *The major identified events of preadipocyte differentiation are presented chronologically. Areas labeled with gene names represent periods of gene expression in the differentiation program. The discrete levels of differentiation (very early, early, intermediate and late) are likewise offered. LPL, lipoprotein lipase; MIX, methyl isobutyl xanthine; DEX, dexamethasone (Ntambi and Young-Cheul 2000).*

In addition to the previously mentioned proteins, other signaling pathways are involved in the regulation of commitment and adipogenesis such as the canonical WNT signaling pathway (Isakson et al. 2009; Logan and Nusse 2004).

WNT family members are secreted glycoproteins that regulate adult tissue homeostasis and remodeling by autocrine and paracrine mechanisms (Logan and Nusse 2004; Taipale and Beachy 2001), and exert their effects by signaling through multiple so-called 'canonical' and 'non-canonical' pathways.

Various reports have implicated WNT signaling molecules in the regulation of mesenchymal stem cell proliferation, cell fate determination and preadipocyte differentiation as well (Christodoulides et al. 2009) (Figure 11).

Specifically, the canonical WNT pathway activation in 3T3-L1 preadipocytes through the overexpression of WNT1 inhibits adipogenesis (Ross et al. 2000). Furthermore, other studies have demonstrated that the WNT signaling represses adipogenesis also by blocking the induction of *Pparγ* and *C/EBPα* (Christodoulides et al. 2009).

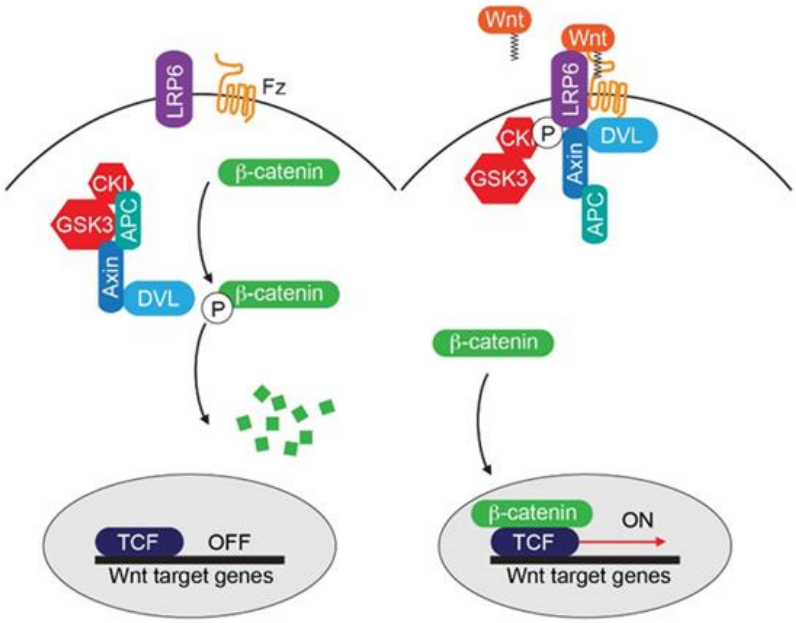

Figure 11. *The canonical WNT signaling cascade converges on the transcriptional regulator ß-catenin (Logan and Nusse 2004). In the absence of WNT signals, cytoplasmic ß-catenin is degraded by the action of the axin and adenomatous polyposis coli (APC) complex, which facilitates its phosphorylation by casein kinase I (CK1) and glycogen synthase kinase 3-beta (GSK3-ß), resulting in ß-catenin ubiquitination and proteasomal degradation (He et al. 2004). On the contrary, when a WNT ligand binds to the frizzled (FZ) receptors and the low-density-lipoprotein-receptor-related Protein-5 or -6 (LRP5/6) co-receptors, the ß-catenin degradation is inhibited and ß-catenin translocates to the nucleus, where forming complexes with the lymphoid enhancer binding factor/T cell specific transcription factor (LEF/TCF) family, activates WNT target gene expression.*

WNT signaling pathway thus plays a critical role in maintaining precursor cells uncommitted and undifferentiated (Isakson et al. 2009; Clevers and Nusse 2012) and its termination is a prerequisite to allowing induction of adipogenic differentiation. However, WNT inhibition is necessary, but not sufficient to induce commitment of the mesenchymal stem cells and of other adipogenic precursor cells. Indeed, additional signals are needed to be turned on or to be repressed in order to start the differentiation process.

E.g., the Bone Morphogenetic Proteins have been shown to play an important role in the induction of both white adipogenesis (Bmp2 and 4) and brown adipogenesis (Bmp7) (Bowers et al. 2006; Gustafson and Smith 2012; Tseng et al. 2008).

In particular, it has been recently demonstrated that Bmp4, which is a member of the transforming growth factor beta superfamily, induces the commitment and the subsequent adipogenesis of human subcutaneous adipose precursor cells (Gustafson and Smith 2012).

Interestingly, the pro-adipogenic effect of Bmp4 was likewise seen in adipose precursor cells from people with hypertrophic obesity supporting the idea that mesenchymal stem cells and other early precursor cells are present in the white adipose tissue and may be recruited following appropriate pro-adipogenic signals. In addition to the previously described findings, Smith and colleagues (Hammarstedt et al. 2013; Gustafson et al. 2013) have identified the Wisp2 protein as a novel adipokine involved in the crosstalk between WNT and Bmp4 signaling.

Their findings have indeed shown that Wisp22 protein, which is highly expressed in early adipogenic precursor cells, and, in particular, results to be upregulated in the subcutaneous adipose tissue of individuals with hypertrophic obesity, is able to inhibit adipogenesis by exerting a direct extracellular inhibitory signal on *Pparγ* activation and adipose cell differentiation as well as by preventing the effect of Bmp4 to induce precursor cell commitment.

Inside cells, the cytosolic Wisp2 protein forms a complex with the transcriptional regulator of preadipocyte determination Zfp423, preventing thus the Bmp4-induced Zfp423 translocation into the nucleus and the Zfp423-mediated regulation of *Pparγ* gene expression (Hammarstedt et al. 2013; Gustafson et al. 2013) (Figure 12).

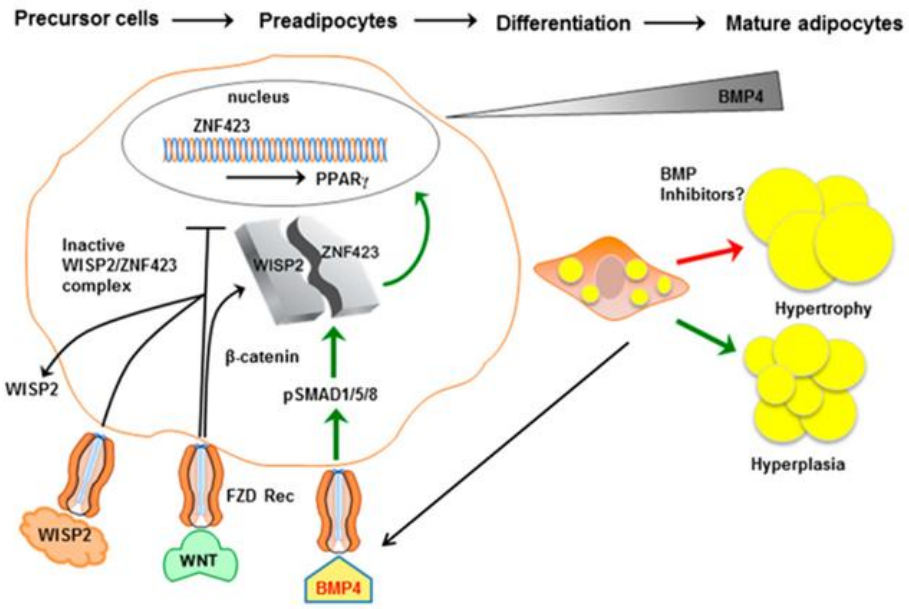

Figure 12. Regulation of precursor cell commitment by Bmp4 and subsequent differentiation of the preadipocytes*. Precursor cells are kept undifferentiated by WNT activation and the intracellular and secreted mediator Wisp2. Wisp2 preventing Pparγ activation by binding ZNF423/Zfp423 in the cytosol. Bmp4 induces Smad1/5/8 phosphorylation, which interacts with the Smad binding sites in ZNF423/Zfp423, dissociates the complex, allowing ZNF423/Zfp423 to enter in the nucleus and initiate Pparγ activation. This allows the cellular commitment, but overall differentiation is also regulated by extracellular Wnt/Wisp2, which prevents Pparγ activation through still unclear mechanisms (Hammarsted et al. 2013).*

1.6 Epigenetics and adipogenesis

As previously mentioned, obesity is a multifactorial disease and is caused by the combined action of genetic and environmental factors.
The presence of a hereditary component in obesity is fully supported by twin-based studies. The hereditability of BMI in twins indeed ranges from 40 to 70% (Stunkard et al. 1986). With regard to the genetic component, several obesity forms are recognized. Monogenic obesity is determined by mutations in the genes for *leptin*, *proopiomelanocortin*, or *melanocortin receptors 3 or 4* (Shapira et al. 2005). However, despite the strong familial clustering of obesity and the identification of several common variants, genome-wide association studies provide the limited capacity to predict risk genes for obesity.
Thus, the complexity of obesity cannot be entirely accounted by genetic causes. The observed familial clustering of obesity may thus be attributable to a significant epigenetic component.
With the term epigenetics are defined all the heritable and reversible changes in gene activity and expression that occur without alteration in DNA sequence (Herrera et al. 2011; Choi et al. 2013). Nowadays, epigenetics is recognized as a more than the plausible link between environment (wrong feeding behavior,

physical inactivity, smoking, etc.) and alterations in gene expression that might lead to disease phenotypes (El-Osta et al. 2008; Jirtle and Skinner 2007).
Thus, environmental exposures to nutritional, chemical and physical factors have the potential to alter gene expression and to modify adult disease susceptibility (obesity and type 2 diabetes) in various ways through changes in the epigenome (Jirtle and Skinner 2007), and the implications of different epigenetic processes in obesity onset are now being extensively investigated (Martínez et al. 2014).
Epigenetic alterations are often tissue specific and include DNA methylation and histone modifications which mediate several biological processes such as imprinting (Herrera et al. 2011). For instance, gestational metabolic programming by environmental cues is an important determinant of obesity predisposition.
Alterations in the DNA methylation pattern in intrauterine life are indeed capable of changing gene expression of several genes, and of contributing to the risk to develop obesity by increasing adipose tissue growth and expansion.
Even adipogenesis might be regulated by an epigenetically induced gene transcription reprogramming. E.g., Fujiki and colleagues (Fujiki et al. 2009), have demonstrated that the promoter of the *Pparγ*2 gene is hypermethylated in 3T3-L1 preadipocytes, but, following the induction of differentiation, it undergoes demethylation and the *Pparγ*2 mRNA expression increases as demethylation proceeds (Fujiki et al. 2009).
In addition, others have also shown that the promoter regions of late adipogenic genes, such as the insulin-responsive *Glut4* and *leptin* are demethylated during adipogenesis as well, and that this event is paralleled by a correspondent increased expression of these genes in mature adipocytes (Pinnick and Karpe 2011). Furthermore, Martinez and colleagues have recently provided high-resolution views of chromatin remodeling during cell differentiation and have allowed the designation of thousands of putative preadipocyte- and adipocyte-specific, cis-regulatory elements based on dynamic chromatin signatures (Martinez et al. 2014).
Therefore, based on the last examples there are at least two ways by which epigenetic modifications may influence the adipogenic programs.
The first one is by increasing or decreasing the levels of primary transcription factors, such as Pparγ, through the direct epigenetic regulation of their promoters. The second one is, instead, by assembling either activator or repressor multiprotein complexes to master transcription factor promoter regions. These proteins are localized downstream the promoter regions possessing specific recognition sequences, and often they operate including or excluding epigenetic modifying enzymes.

2. AIM OF THE STUDY

Hypertrophic obesity is typically associated with the development of insulin resistance and type 2 diabetes. It is due to the inability to recruit and differentiate available precursor cells in the subcutaneous adipose tissue, through the inactivation of canonical WNT signaling pathway, allowing Bmp4 to initiate their commitment (Hammarstedt et al. 2013; Gustafson et al. 2013). Emerging evidence suggests that the zinc-finger protein Zfp423 (a key mediator of Bmp4 pathway) controls preadipocyte determination directly regulating Pparγ gene expression (Gupta et al. 2010).

The elucidation of the molecular mechanisms regulating the expression of *Zfp423* and its downstream target *Pparγ* would be useful to understand the causes of the restricted adipogenesis observed in hypertrophic obesity.

Genetic studies performed so far have not identified genetic variants of *Zfp423* and *Pparγ* genes associating with hypertrophic obesity.

Furthermore, the complexity of obesity cannot be entirely accounted for genetic changes and the obesity-related comorbidities may be attributable to a significant epigenetic component.

For this reason, this work aims at investigating the contribution of epigenetic mechanisms to the transcriptional regulation of *Zfp423* and *Pparγ* genes.

Understanding of the molecular mechanisms regulating Zfp423 and Pparγ expression will help the identification of novel strategies aimed at preventing and treating the restricted adipogenesis typical of hypertrophic obesity.

3. MATERIALS AND METHODS

3.1 Materials

Media, sera, and antibiotics were purchased from Invitrogen (Paisley, UK). Rosiglitazone was purchased from Alexis (Milan, Italy). AZA, 3-isobutyl-1-methylxanthine and dexamethasone were from Sigma-Aldrich (St Louis, Missouri, USA). Insulin, TRIzol, and SuperScript III were from Invitrogen (Carlsbad, California, USA). pCpGfree-Lucia was from InvivoGen (St Diego, California, USA). SYBR Green Supermix was from Bio-Rad (Hercules, California, USA). EZ DNA Methylation Kit was from Zymo Research (Orange, California, USA). Micrococcal Nuclease, Dam^-/Dcm^- E.coli cells, HpyCH4IV enzyme and M.SssI, HhaI and HpaII methylases were purchased from New England Biolabs (Ipswich, Madison, USA). DNA Purification Kit and pGEM-T EASY Vector were from Promega (Madison, Wisconsin, USA). The QIAquick PCR Purification kit was from QIAGEN (Hilden, Germany). Big Dye Terminator v3.1 Cycle Sequencing Kit was from Applied Biosystems (Foster City, California, USA). PCR clean-up and gel extraction were from Macherey-Nagel (Neumann Neander, Duren, Germany).

3.2 Cell culture, treatment and adipocyte differentiation

3T3-L1 and NIH-3T3 mouse embryonic fibroblasts were obtained from the American Type Culture Collection (Manassas, Virginia, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% calf serum (CS), penicillin (200 IU/ml) and streptomycin (100 g/ml). Cells were maintained at 37° in a humidified CO_2 incubator.

For adipocyte differentiation assays, cells were grown to confluence in medium containing 10% CS. Two days after confluence, cells (day 0) were cultured in DMEM supplemented with a differentiation cocktail containing 5 μg/ml insulin, 0.5 mmol/l 3-isobutyl-1-methylxanthine (IBMX), 1 μmol/l dexamethasone (DEX), 1 μM rosiglitazone and 10% fetal bovine serum (FBS), for 2 days. Forty-eight hours after induction, cells were maintained in DMEM containing insulin (5 μg/ml) and 10% FBS until they were ready for collection.

The NIH-3T3 cells were cultured in the presence or absence AZA (5 μM) for 6h before the administration of differentiation cocktail.

Lipid accumulation of mature adipocytes was determined by Oil red O staining, as described by Isakson and colleagues (Isakson et al. 2009) and, quantified as an index of differentiation.

The cells were fixed in 4% formaldehyde and incubated for 20 min at room temperature. The formaldehyde was removed by washing the samples for 5 min in PBS.
Then, the cells were incubated for 60 min at RT in Oil Red O staining solution. Images were taken using an Olympus microscope system (Olympus, Center Valley, Pennsylvania, USA). For quantification, absorbance was measured at 510 nm using a spectrophotometer (Beckman, California, USA), after the addition of isopropanol and the incubation at room temperature for 10 minutes.

3.3 Rna isolation and real time RT-PCR

Total RNA was isolated by the TRIzol reagent, according to the manufacturer's protocol. Upon adding chloroform, the RNA remains in the aqueous phase, while proteins and high molecular weight DNA enter the organic phase and the interphase, respectively.
Total RNA is recovered by isopropanol precipitation. Reverse transcription of 1 μg of total RNA was performed using SuperScript III following the manufacturer's instructions. The cDNA obtained were used as a template for quantitative real time RT-PCR, performed in triplicate by using iQ SYBR Green Supermix on iCycler real time detection system (Bio-Rad). Relative quantification of gene expression was calculated by the $\Delta\Delta^{-Ct}$ method. Each *Ct* value was first normalized to the respective *Cyclophilin Ct* value of a sample to account for variability in the concentration of RNA and in the conversion efficiency of the reverse transcription reaction. The primers used are listed in the table below (Table 2).

C/EBPα	Forward 5'-GGCTCGCCATGCCGGGAGAA-3' Reverse 5'-CCGGGTCGATGTAGGCGCTG -3'
Pparγ2	Forward 5'-CAGTGGAGACCGCCCAGGCT- 3' Reverse 5'-TGGAGCAGGGGGTGAAGGCT- 3'
Fabp4/Ap2	Forward 5'-TCTCACCTGGAAGACAGCTCC- 3' Reverse 5'-GCTGATGATCATGTTGGGCTTGG- 3'
Glut4	Forward 5′-CAGAAGGTGATTGAACAGAG-3′ Reverse 5′-AATGATGCCAATGAGAAA-3
AdipoQ	Forward 5'-GTGACGACACCAAAAGGGCTC -3' Reverse 5' -TCCAACCTGCACAAGTTCCC- 3'
Cyclophilin	Forward 5'-GCAGACAAAGTTCCAAAGACAG- 3' Reverse 5'-CACCCTGGCACATGAATCC- 3'

Table 2. *Primer sequences used in quantitative real time RT-PCR analysis.*

3.4 MNase protection assay

Chromatin was analysed by an MNase that is an endo-exonuclease with the ability to induce double strand breaks in the linker region between individual nucleosomes, leading to the characteristic nucleosomal ladder. Because of this property, MNase can be used to determine the approximate positions of nucleosomes in a region of DNA, if the nucleosomes are consistently positioned (Richard-Foy and Hager 1987).

For each experiment, NIH-3T3 and 3T3-L1 cells were fixed for 10 min at 37° in growth medium by the addition of 37% (vol./vol.) formaldehyde to a final concentration of 1% (vol./vol.). The crosslinking reaction was stopped with glycine (from a 2.5 M stock) to a final concentration of 125 mM for 5 minutes at room temperature (quenches reaction). Nuclei were isolated from 5×10^5 cells, suspended in 1 ml of wash buffer (10 mmol/l Tris-HCl (pH 7.4), 15 mmol/l NaCl, 60 mmol/l KCl and 1mmol/l $CaCL_2$) and digested with 200 U of MNase (MNase) for 20 min at 37°. MNase digestion was stopped by adding 100 mmol/l EDTA and 10 mmol/l EGTA (PH 7.5).

RNA and proteins were degraded by adding of RNaseA (0,4 µg/µl), proteinase K (400 µg/ml) and NaCl (300mmol/l). Each sample was adjusted to 0.4% SDS and incubated overnight at 65° for deproteinization and crosslink reversal.

MNase digested DNA were electrophoretically separated on 1.5% agarose gel and mononucleosome-size (150 bp) bands were excised from the gel and purified by PCR clean-up and gel extraction (Macherey-Nagel) according to the manufacturer's instructions. The purified DNA was subsequently amplified by quantitative real time RT-PCR using primers designed as follows: NUC 1, NUC 2, NUC 3 and CTRL R primer set for *Pparγ* promoter and NUC1, NUC2 and CTRL R primer set for the *Zfp423* promoter region. The primers used are listed in the table below (Table 3).

NUC 1 Pparγ	Forward 5′- TAATATCCCCTTAAGGAAGAAGCTC -3′ Reverse 5′- TGCTCAGGAACCATCGGGA -3
NUC 2 Pparγ	Forward 5'- CAAGGGCTGCTCCCACGTTA - 3' Reverse 5'- TGCTTAAGGCCTTTGCCCTTTT- 3'
NUC 3 Pparγ	Forward 5'- TGTACAGTTCACGCCCCTCA - 3' Reverse 5'- TTGTCTCGCCAGTGACCCAC - 3'
CTRL R Pparγ	Forward 5'- AGCCTTTATTCTGTCAACTATTCCT - 3' Reverse 5'- GGTCCAAAATGTTACTGCTATCCA - 3'
NUC 1 Zfp423	Forward 5'- CCCGCACGGGCCTGTTA - 3' Reverse 5'- CTCTGACAGCACTGGGCA - 3'
NUC 2 Zfp423	Forward 5'-TGTGGCCGGACGCCTG - 3' Reverse 5'- CCTTCTCCTCCGCCCCTTG - 3'
CTRL R Zfp423	Forward 5'- GCCCGAGGGCAGGCA - 3' Reverse 5'- GCACGGGCATTGCTCAG - 3'

Table 3. *Primer sequences used in quantitative real time RT-PCR analysis.*

3.5 Determination of DNA methylation status

Treatment of denatured single-stranded genomic DNA with sodium bisulfite is used to convert unmethylated cytosine residues to uracil residues, whereas methylated cytosine bases are protected from conversion and remain unchanged. In particular, bisulfite conversion consists of three sequential chemical steps: sulfonation of cytosine to cytosine-6-sulfonate, deamination to uracil-6-sulfonate, and desulfonation to uracil (Figure 13).

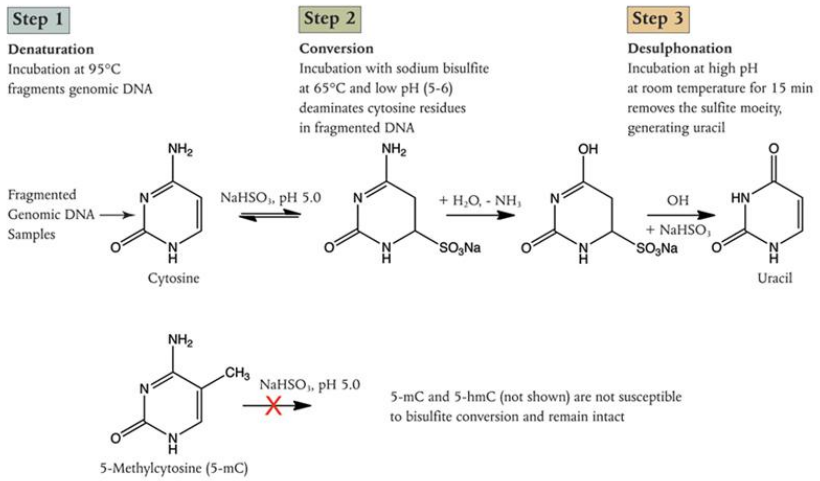

Figure 13. The principle of the bisulfite conversion and chemical background. *This procedure is based on the chemical modifications of cytosine residues in the presence of sodium bisulfite (HSO3–) at low pH and high temperatures. The chemistry of each reaction step is as follows: cytosine is sulfonated at the carbon-6 position and deaminated at the carbon-4 position to generate uracil sulfonate.A subsequent desulfonation under alkaline completes the conversion, generating uracil from cytosine.*

Genomic DNA from cultured cells was extracted by DNA Purification Kit (Promega). Bisulfite treatment of extracting genomic DNA was performed by EZ DNA Methylation Kit (Zymo Research), following the manufacturer's instructions. The *Pparγ* and *Zfp423* promoter region in the bisulfite converted genome was amplified by PCR using Bisulfite-specific primer sets. The Bisulfite Primer Seeker software (Zymo Research) was used to design the primers for CG-rich sequences on *Pparγ* and *Zfp423* promoter region. The primers used are listed in the table below (Table 4).

Pparγ	Forward 5'-GATGTGTGATTAGGAGTTTTAATTAAA-3' Reverse 5'-CAAACCTAAATTAACTAACACTATCCTAAC-3'
Zfp423	Forward 5'-GGTTTTATTATGTGTTTTTGTAGTGTA-3' Reverse 5'-ATATCCCTCAACTCAACCTACTTAA- 3'

Table 4. *Primer sequences used in quantitative real time RT-PCR analysis.*

Converted DNA was amplified under the following conditions: 95° for 10 min and 39 cycles of 96° for 1 min, 58° for 1 min, and 72° for 2 min. For bisulfite sequencing, each PCR product of *Pparγ* and *Zfp423* was subcloned using pGEM T-EASY Vector system (Promega). Then, competent E.coli cells were transformed and plated on X-GAL/IPTG LB-ampicillin Agar plates, where blue colonies represent an empty vector, and white colonies represent vectors inserted with target PCR product. Ten clones from each sample were selected and plasmids containing the target DNA are extracted by using the QIAprep Spin Miniprep Kit (QIAGEN) and subjected to standard sequencing analysis, using T7 universal primer and Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). All the procedures follow the manufacturer's protocol.

3.6 DNA sequencing by dideoxynucleotide chain termination method

DNA sequencing was carried out using the dideoxynucleotide chain termination method (Sanger et al. 1977) with dye terminator labeling of purified plasmids or PCR products. Specific forward and reverse primer for PCR products were used. The DNA samples were precipitated using ethanol and the pellets were washed twice with 70% ethanol.
The amplified DNA was purified using the QIAquick PCR Purification kit (QIAGEN). The purified templates were dissolved in DEPC treated water and about 50 to 100 ng of PCR fragments (10 ng per 100 bp) were used in one reaction. The sequencing was performed on an ABI 3500 Automatic Sequencer (Applied Biosystems) using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

3.7 Luciferase reporter assay and methylation assay *in vitro*

The 5'-flanking region of *Zfp423* gene (-1324 to -764) was amplified by PCR from genomic DNA prepared from NIH-3T3 cells, and the fragment was cloned into pCpGfree-Lucia (InvivoGen) luciferase reporter vector. Amplification of the reporter construct was carried out using Dam^-/Dcm^- E.coli cells (New England Biolabs). The luciferase reporter vector was incubated with 1 units/ug of M.SssI enzyme (methylates all CpG) and, with 1 units/ug of HhaI (modifies the cytosine residue of the sequence GCGC) and HpaII enzymes (methylates the cytosine residues in the sequence CCGG) in the presence of 160 μM S-adenosylmethionine (SAM) at 37° for 1h. Methylation was confirmed by digestion of HpyCH4IV, a methylation-sensitive restriction enzyme. The full methylated, unmethylated and partially methylated *Zfp423* reporter vectors were transfected in NIH-3T3 cells, using lipofectamine.
To normalize the luciferase activity, an amount of control plasmid encoding a Renilla luciferase gene was cotransfected into the cells.

After 48h, cells lysates were assayed for Firefly and Renilla luciferase activity using a single luciferase reporter assay kit, according to the manufacturer's instructions.

3.8 Statistical analysis

All data are presented as means ± SEM. The difference between groups was evaluated using the Student's t-test. A p value less than 0.05 was considered significant.

4. RESULTS

4.1 *Zfp423* and *Pparγ* mRNA expression in 3T3-L1 and NIH-3T3 cells

Based on the aims above described, all the experiments were performed in two cellular models with a different adipogenic capability; the 3T3-L1 cells, which are fibroblasts committed to adipocyte lineage and are widely used to study *in vitro* adipogenesis; and the NIH-3T3 cells, which are fibroblasts with a low rate of adipogenic capability and are generally used in many studies as negative controls for *in vitro* adipogenesis.
Firstly, the mRNA expression of both *Zfp423* and *Pparγ* genes have been evaluated in both NIH-3T3 and 3T3-L1 cells by quantitative real time RT-PCR. As shown in Figure 14, a 174.1-fold increase in *Zfp423* mRNA and a 394.7-fold up-regulation in *Pparγ* mRNA are detected in 3T3-L1 compared with NIH-3T3 cells.

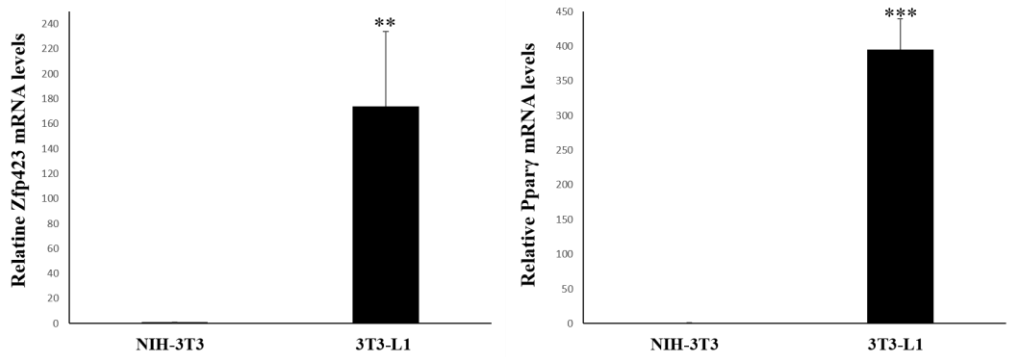

Figure 14. Zfp423 and Pparγ mRNA expression in NIH-3T3 and 3T3-L1 cells*. Total RNA was isolated from cells and the mRNA levels of Zfp423 and Pparγ genes were assessed by quantitative real time RT-PCR in untreated 3T3-L1 and NIH-3T3 cells. Experimental normalization has been performed using the housekeeping Cyclophilin gene as internal control. Results are means ± SD from three independent experiments. **p<0.01, and ***p<0.001 vs NIH-3T3 in a 2-tailed Student's t-test.*

Then, to exclude that the observed divergences in *Zfp423* and *Pparγ* gene expression were associated with differences in the DNA sequence of these genes in the two cellular lines, *Zfp423* and *Pparγ* promoters have been sequenced.
The sequencing analysis of about 2000 bp of the promoter of each gene has revealed that the *Zfp423* and *Pparγ* genes are genetically identical in 3T3-L1 and NIH-3T3 cells (data not shown).
These data, thus, indicate that the differences in the expression levels of *Zfp23* and *Pparγ* genes among NIH-3T3 and 3T3-L1 cells are not ascribable to the DNA sequences of their promoters, but are to be found.

4.2 DNA Methylation status of *Zfp423* and *Pparγ* promoters in NIH-3T3 and 3T3-L1

Then, to understand the reasons at the basis of the increased *Zfp423* and *Pparγ* expression observed in 3T3-L1 cells, the contribution of DNA methylation in the transcriptional regulation of these two genes was examined and the specific methylation status of the two gene promoters was determined *in vitro* with appropriate experimental procedures in both NIH-3T3 and 3T3-L1 cells.

Firstly, by using the bioinformatic tool EMBOSS Cpgplot, I have identified CpG-rich sequences of *Zfp423* and *Pparγ* promoters that may be sensitive to methylation. In details, in the *Zfp423* promoter region a CpG-rich sequence containing 62 CpG sites located within 560 bp upstream the transcription start site (TSS) has been identified; whereas in the *Pparγ* promoter 4 CpGs, which methylation status has been already negatively correlated with *Pparγ* expression (Fujiki et al. 2009), have been found.

Successively, I have established the methylation status of these CpG-rich sequences by sodium bisulfite sequencing in both NIH-3T3 and 3T3-L1 cells. The DNA methylation status of *Zfp423* promoter is higher in NIH-3T3 compared with 3T3-L1 cells [CpG methylation %: 90.2% (NIH-3T3) Vs 15.3% (3T3-L1)] (Figure 15A). The DNA methylation status of *Pparγ* promoter results to be more methylated in NIH-3T3 compared with 3T3-L1 cells, as well [CpG methylation %: 75.0% (NIH-3T3) Vs 30.0% (3T3-L1)] (Figure 15B).

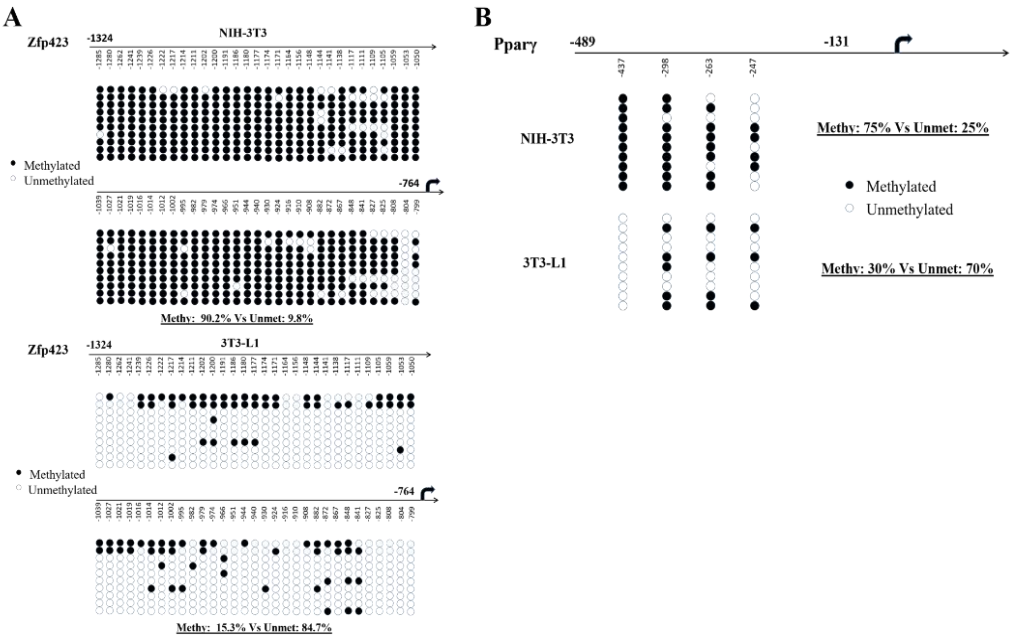

Figure 15. DNA methylation of the Zfp423 and Pparγ promoters in NIH-3T3 and 3T3-L1 cells. *Bisulfite sequencing analysis of DNA methylation status and percentages of each methylated CpG sites in (A) Zfp423 and (B) Pparγ promoters in NIH-3T3 and 3T3-L1 cells. Each PCR product was sub-cloned and ten clones were analyzed by sodium bisulfite sequencing. The methylation profile of each CpG site in Zfp423 and Pparγ promoter, either methylated (black circles) or unmethylated (white circles), is aligned corresponding to their sequence order (as shown on the top of the figure for NIH-3T3 cells and 3T3-L1).*

Then, I have evaluated *in vitro* the causal relationship between the methylation status of the *Zfp423* promoter and its mRNA expression.
To this aim, I have cloned the *Zfp423* promoter region into a luciferase reporter (pCpGfree-Lucia) vector, which was or completely methylated by M.SssI enzyme, or partially methylated by HhaI and HpaII enzymes, or not methylated.
Methylation status of these vectors was confirmed by digestion with HpyCH4IV, a methylation-sensitive restriction enzyme (data not shown).
I have, then, performed luciferase reporter assay in NIH-3T3 cells transiently transfected with the *in vitro* methylated, partially methylated, or unmethylated vectors. The luciferase activity of the fully methylated and of the partially methylated *Zfp423* promoter region are reduced by about 80% and 40%, respectively, compared with the activity of the unmethylated region (Figure 16). These results demonstrate that CpG methylation of *Zfp423* promoter regulates *Zfp423* gene transcription *in vitro*.

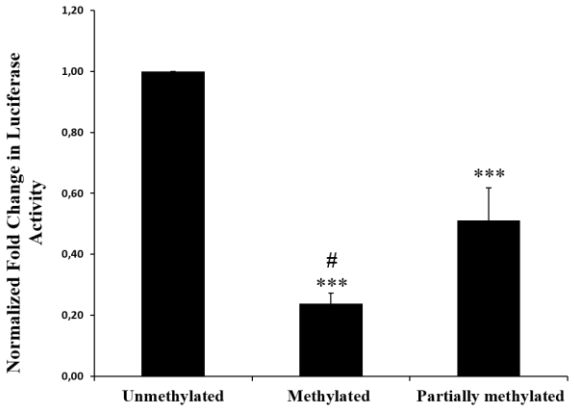

Figure 16. DNA methylation effect of Zfp423 promoter activity. *Luciferase activity of the in vitro methylated, partially methylated, or unmethylated Zfp423 promoter reporter constructs in NIH-3T3 cells. Relative luciferase activity normalized to the activity of a cotransfected internal vector is shown in the graph. Results are means ± SD from three independent experiments. ***p<0.001 vs unmethylated vector, and #p<0.05 vs partially methylated vector in a 2-tailed Student's t-test.*

4.3 Nucleosome occupancy of *Zfp423* and *Pparγ* promoters in 3T3-L1 and NIH-3T3 cells

Next, I have wondered whether the differences in gene expression of *Zfp423* and *Pparγ* in the two cellular systems may be linked to different nucleosome occupancy of the gene promoters.

To predict nucleosome positioning on *Zfp423* and *Pparγ* promoter regions, I have analysed the genomic sequence of these two genes using the bioinformatic tool NuPoP. As indicated in Figure 17, both the promoter regions of *Zfp423* and *Pparγ* contain several potential regions for positioning of nucleosomes (Figure 17).

Then, to validate the highest scores predicted nucleosomes and evaluate nucleosome occupancy on *Zfp423* and *Pparγ* promoter regions among NIH-3T3 and 3T3-L1 cells, I have firstly performed an MNase protection assay, which was followed by quantitative real time RT-PCR for the potential nucleosome sites. In detail, chromatin and genomic DNA purified from NIH-3T3 and 3T3-L1 cells were digested with MNase (200 U) to obtain mono-nucleosomal DNA fragments averaging 150 bp in size (Figure 17).

Then, the potential positioning of nucleosome was checked in the mono-nucleosomal DNA isolated from the agarose gel by quantitative real time RT-PCR, using specific sets of primers for *Pparγ* promoter (NUC 1, NUC 2, NUC 3 and CTRL R) and for *Zfp423* promoter (NUC 1, NUC 2 and CTRL R).

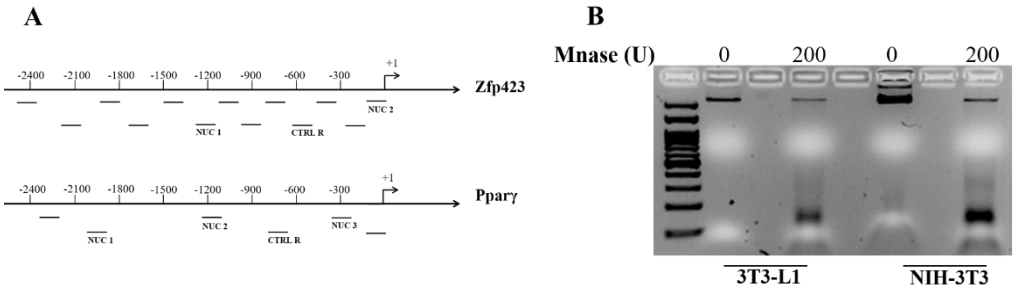

Figure 17. A schematic representation of Pparγ and Zfp423 promoter regions. *(A) Here are indicated the regions, relatively to the transcription start site, which are potentially occupied by nucleosomes on the Zfp423 promoter (NUC 1, NUC 2 and CTRL R) and on the Pparγ promoter (NUC 1, NUC 2, NUC 3 and CTRL R); (B) Genomic DNA obtained by nuclei lysis of 3T3-L1 and NIH-3T3 cells digested with 200 U of MNase for 20 min at 37°. Mono-nucleosomal DNA fragments averaging 150 bp in size have been obtained by MNase digestion.*

As shown in Figure 18, the percentage of nucleosome occupancy of three selected regions on the *Pparγ* promoter is higher in NIH-3T3 compared with 3T3-L1 cells [NUC1 % of occupancy: 21.2% (3T3-L1) vs 62.0% (NIH-3T3); NUC2 % of occupancy: 9.2% (3T3-L1) vs 45.1% (NIH-3T3); NUC3 % of occupancy: 10.9% (3T3-L1) vs 53.1% (NIH-3T3)] (Figure 18).
Similarly, the nucleosomes occupancy of *Zfp423* promoter regions is also increased in NIH-3T3 compared with 3T3-L1 cells [NUC1 % of occupancy: 51.5% (3T3-L1) vs 72.2% (NIH-3T3); NUC2 % of occupancy: 46.4% (3T3-L1) vs 94.6% (NIH-3T3)] (Figure 18).
As expected, no significant quantitative differences were found among the two cells in regions, which no nucleosomal positioning was predicted by the bioinformatic software.
All these data indicate that the decreased nucleosome occupancy of the 3T3-L1 inversely correlates with the increased expression levels of these two genes and thus suggest that both *Pparγ* and *Zfp423* genes may be transcriptionally regulated by dynamic chromatin remodeling.

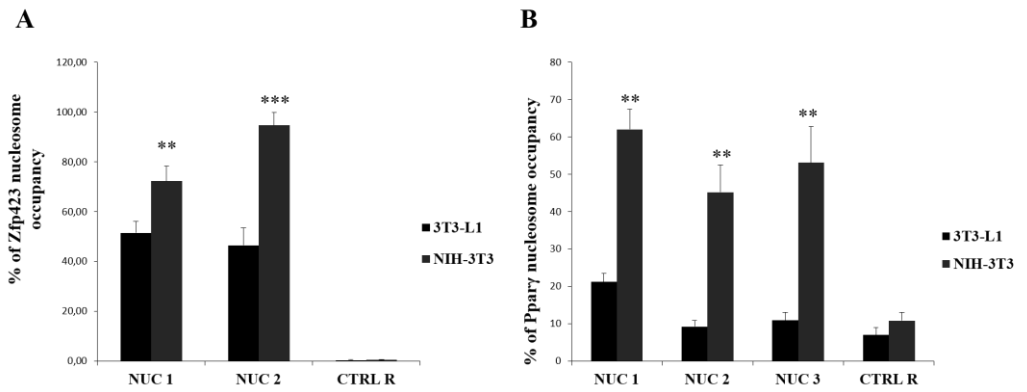

***Figure 18**. **Nucleosome occupancy percentage of Zfp423 and Pparγ promoter regions in 3T3-L1 and NIH-3T3 cells**. The percentage of nucleosome occupancy was analysed by quantitative real time RT-PCR; A) in two regions of the Zfp423 promoter and B) in three regions of the Pparγ promoter. Results are means ± SD from three independent experiments. **p<0.01, and ***p<0.001 vs 3T3-L1 in a 2-tailed Student's t-test.*

4.4 Contribution of DNA methylation to *Zfp423* and *Pparγ* expression

To evaluate how DNA methylation and/or nucleosome occupancy contribute to *Zfp423* and *Pparγ* expression, I have tested the ability of the class II histone deacetylase inhibitor Trichostatin A (TSA) and of the DNA methyltransferase inhibitor AZA to remove the transcriptional block of *Zfp423* and *Pparγ* expression in NIH-3T3 cells. For this purpose, NIH-3T3 cells were exposed to TSA (330 nm) and to AZA (5 μM) for 24h and the *Zfp423* and *Pparγ* gene expression was then determined by quantitative real time RT-PCR.
As shown in Figure 19, the expression of *Zfp423* and *Pparγ* mRNA is induced by AZA treatment compared with untreated cells. By contrast, treatment of cells with TSA does not affect both *Zfp423* and *Pparγ* mRNA expression (Figure 19).
NIH-3T3 cells were also treated for 6h with AZA, showing a greater increase in the expression of *Zfp423* and *Pparγ* compared to expression levels observed after the 24h treatment (data not shown).
Taken together these results demonstrate that DNA methylation rather than histone acetylation is the main epigenetic mechanism involved in the regulation of *Zfp423* and *Pparγ* gene expression.

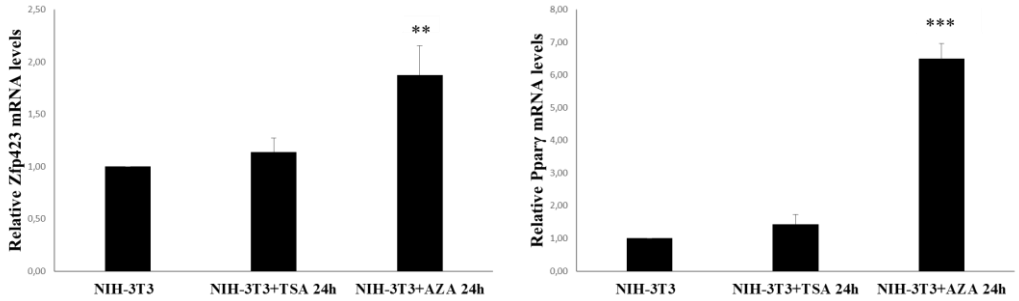

Figure 19. Zfp423 and Pparγ gene expression upon TSA and AZA treatment in NIH-3T3 cells. *Cells were cultured in growth medium containing 330 nM TSA or 5 μM AZA for 24h. Expression of Zfp423 and Pparγ mRNA were then measured by quantitative real time RT-PCR. Experimental normalization has been performed using the housekeeping Cyclophilin gene as internal control. Results are means ± SD from three independent experiments. **p<0.01, and ***p<0.001 vs NIH-3T3 in a 2-tailed Student's t-test.*

To further support the previous experiments, we performed an MNase protection assay in NIH-3T3 cells treated with AZA.

As reported in Figure 20, the percentage of nucleosome occupancy of the previous investigated regions on the *Pparγ* promoter was lowered in AZA-treated NIH-3T3 cells compared with NIH-3T3 cells [NUC1 % of occupancy: 38.9% (NIH-3T3+AZA) vs 62.0% (NIH-3T3); NUC2% of occupancy: 37.4% (NIH-3T3+AZA) vs 45.1% (NIH-3T3); NUC3% of occupancy: 22.8% (NIH-3T3+AZA) vs 53.1% (NIH-3T3)] (Figure 20).

Similarly, the nucleosomes occupancy of *Zfp423* promoter regions is also lowered in AZA-treated NIH-3T3 compared with NIH-3T3 cells [NUC1 % of occupancy: 65.1% (NIH-3T3+AZA) vs 72.2% (NIH-3T3); NUC2 % of occupancy: 46.9% (NIH-3T3+AZA) vs 94.6% (NIH-3T3)] (Figure 20).

These results suggest that the inhibition of DNA methyltransferases restores the expression of these two genes by inducing chromatin remodeling on *Zfp423* and *Pparγ* promoters.

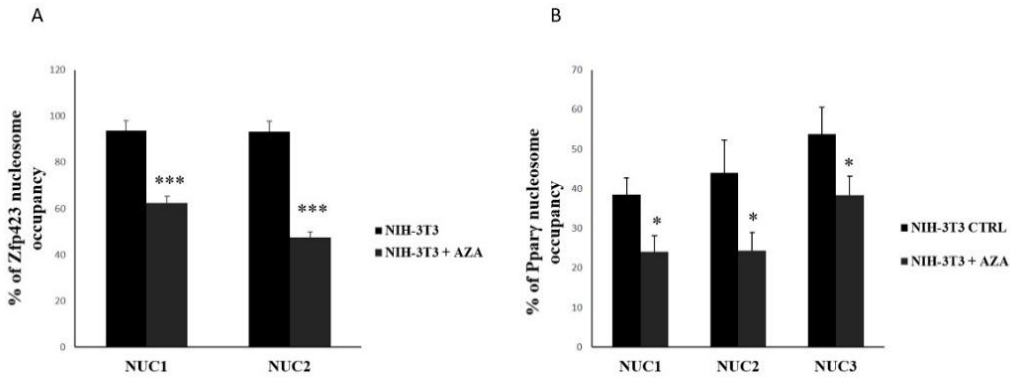

Figure 20. Nucleosome occupancy percentage of Zfp423 and Pparγ promoters in NIH-3T3 cells treated with AZA*. The percentage of nucleosome occupancy was analysed by quantitative real time RT-PCR in two regions of Zfp423 promoter and in three regions of the Pparγ promoter and. Results are means ± SD from three independent experiments. *p<0.05, and ***p<0.001 vs NIH-3T3 in a 2-tailed Student's t-test.*

4.5 DNA methyltransferase inhibitor AZA treatment effect on adipogenesis in NIH-3T3 cells

To examine the functional impact of these epigenetic changes, I have evaluated adipocyte differentiation in NIH-3T3 cells in the presence of AZA. In details, adipogenesis was induced using a differentiation cocktail, according to the Materials and Methods section, in presence or absence of the DNA methyltransferase inhibitor AZA.

Adipocyte differentiation and the lipid content of NIH-3T3 cells were respectively assessed by evaluating the expression of the adipogenesis-related markers genes *Pparγ*, *adipocyte lipid-binding protein* (*Ap2/Fabp4*), *adiponectin* (*AdipoQ*), and *Glut4* on days 0 and 7 of differentiation by quantitative real time RT-PCR, and by Oil Red O staining on day 7 of differentiation. In NIH-3T3, which, as previously said, are cells with a limited adipogenic capability, when adipogenic differentiation was induced there is only a slight increase in both the mRNA expression of the adipogenesis-related markers genes *Pparγ*, *Ap2/Fabp4*, *AdipoQ* and *Glut4* and in the lipid accumulation upon Oil Red O staining (Figure 21).

On the other hand, when AZA was administrated to cells in the post-confluent stage, AZA treatment robustly improves adipogenic differentiation as shown by the increased expression of *Pparγ*, *Ap2/Fabp4*, *AdipoQ*, and *Glut4* expression, as well as, by the increased lipid accumulation compared with the untreated control cells.

These results suggest that the methylation of CpG sites in the promoter regions of *Zfp423* and *Pparγ* genes promotes the adipocyte differentiation of non-adipogenic NIH-3T3 cell line.

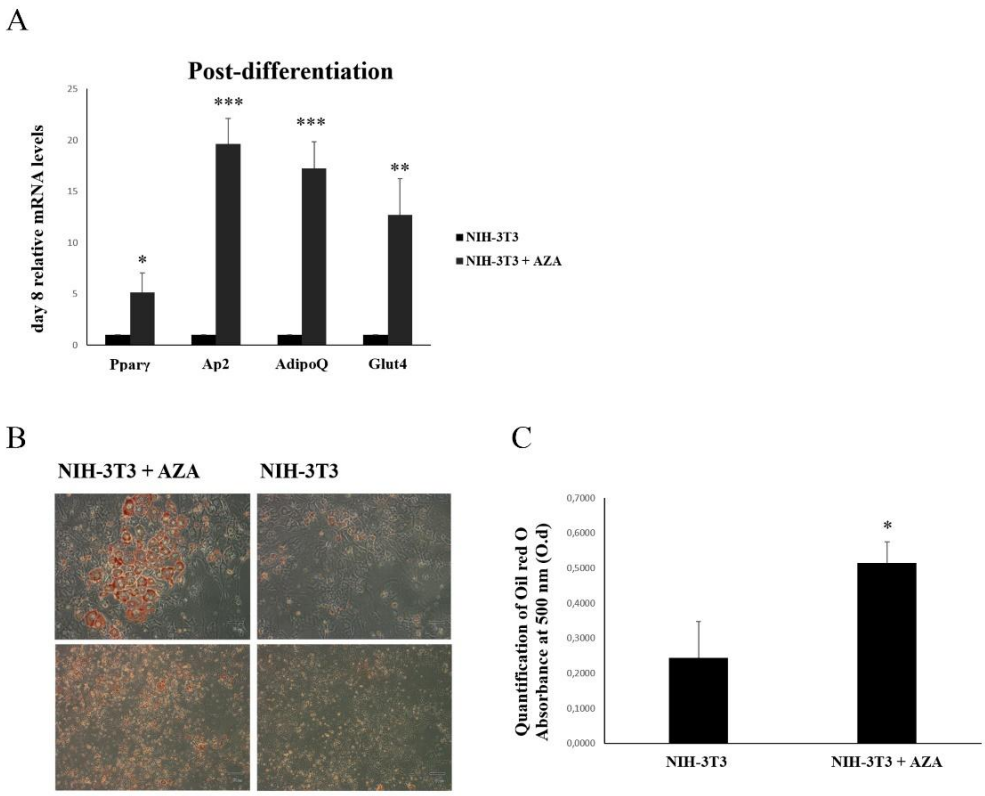

Figure 21. Effect of AZA on the differentiation of NIH-3T3 cells. *Cells were cultured in the presence or absence of AZA during the period from days -2 to day 0 (post-confluent). Seven days upon adipocyte differentiation induction, gene expression, and lipid accumulation were assayed in cells treated or not with AZA. A) The relative mRNA levels of Pparγ, Ap2/Fabp4, AdipoQ and Glut4 were determined on days 0 and 7 of differentiation by quantitative real time RT-PCR. Experimental normalization has been performed using the housekeeping Cyclophilin gene as internal control. Results are means ± SD from three independent experiments. ***p<0.001 vs NIH-3T3 Ctrl in a 2-tailed Student's t-test. B) On days 0 and 7, cells were fixed and stained with Oil red O. Representative images of Oil red O staining are shown. C) Quantification of adipocyte differentiation by Oil Red O staining. Quantities of Oil Red O are denoted as an Increased percentage of the untreated differentiated cells values. Results are means ± SD from three independent experiments. *p<0.05 vs NIH-3T3 in a 2-tailed Student's t-test.*

5. DISCUSSION

Obesity, often defined as a condition of abnormal or excessive fat accumulation in the adipose tissue, is now the most common metabolic disorder in developed countries (Westley and May 2013; Caterson and Gill 2002; Formiguera and Canton 2004; WHO 2004) and responsible, in the absence of immediate actions, of very serious health problems in the coming years. Indeed, obesity is associated with premature death and is universally recognized as major risk factor for metabolic disorders, such as insulin resistance, diabetes and cardiovascular disease (Fiel et al. 2001; Visscher and Seidell 2001).

Accumulation of excess lipids in adipose tissue is due to the increase in adipocyte size (hypertrophy) and/or the recruitment of the new adipocytes from the resident pool of progenitor cells (hyperplasia) (Rosen and Spiegelman 2014). Unlike to hyperplastic obesity, adipocyte hypertrophy is associated with impairment of glucose and lipid metabolism and loss of insulin sensitivity (Blüher 2013; Klöting and Blüher 2014). In addition, hypertrophic obesity is associated with adipose tissue dysfunction, inflammation, and local and systemic insulin resistance. Moreover, insulin sensitivity is inversely correlated with adipose cell size as well as restricted adipogenesis (Gustafson 2010; Gustafson et al. 2013).

Hypertrophic obesity, indeed, is associated with an inability to recruit and differentiate new subcutaneous adipose cells. This is not due to lack of precursor cells but to the inability of these cells to be committed and/or to differentiate. Failures of inactivation of the canonical WNT signaling pathway, which inhibits adipocyte lineage commitment, and/or of Bmp4 signaling which instead promotes the commitment, are directly responsible for precursor cell restricted adipogenesis. However, the mechanisms involved are not fully understood yet.

Thus, it is clear that understanding the underlying mechanisms responsible for the restricted adipogenesis characterizing hypertrophic obesity, may prevent the metabolic complications associated with obesity.

In the last years, it has been identified Wisp2, as a novel adipokine, which has provided new insight into the crosstalk between WNT and Bmp4 signaling (Hammarstedt et al. 2013). Wisp2 expression is considerably higher in the subcutaneous adipose tissue than in visceral adipose tissue, and its expression is positively associated with hypertrophic obesity, markers of ectopic fat accumulation, and degree of insulin resistance (Hammarstedt et al. 2013). Wisp2 is highly expressed in early adipogenic precursor cells and prevents adipogenesis through, at least, two mechanisms: one of these is mediated by the secreted Wisp2, which inhibits adipogenesis with a still not completely known mechanism. The second one is mediated by retaining in the cytosol the transcriptional activator of *Pparγ*, Zfp423 (Hammarstedt et al. 2013).

Zfp423 is a zinc finger protein previously shown to regulate brain development (Warming et al. 2006; Cheng et al. 2007).
It is highly expressed in preadipose rather than non-preadipose fibroblasts and very recently, it has been demonstrated that plays a critical role in adipogenesis. Ectopic expression of *Zfp423* in non-adipogenic fibroblasts NIH-3T3 activates *Pparγ* expression in undifferentiated cells, promoting adipocyte differentiation. In addition, *in vivo* analysis of *Zfp423* in embryos obtained from *Zfp423*-deficient mouse showed impaired brown and white adipocyte differentiation, supporting the notion that *Zfp423* is a critical regulator of preadipose cell determination of both brown and white fat depots (Gupta et al. 2010). Furthermore, Gustafson recently demonstrated that Wisp2/Zfp423 complex can be dissociated by Bmp4, thereby allowing Zfp423 to enter the nucleus, thus activating *Pparγ* expression, and committing these cells to the adipose lineage. In light of this, it is clear that identify the molecular mechanisms regulating *Pparγ* and *Zfp423* gene expression could, at least partially, help to recognize the causes of the restricted adipogenesis observed in hypertrophic obesity. Indeed, what drives the restricted adipogenesis is currently unclear. This insufficiency could be ascribed to epigenetic modifications more than genetic polymorphism.
In the present study, thus, I have investigated the contribution of epigenetic alterations in the development of restricted adipogenesis observed in hypertrophic obesity, focusing my attention on the role of *Zfp423* and *Pparγ* gene itself.
To this end, I have performed experiments in 3T3-L1 and NIH-3T3 cells. These cell lines have been extensively used in adipogenesis studies. Indeed, 3T3-L1 cells are committed to adipocyte lineage whereas NIH-3T3 cells are non-adipogenic fibroblasts and negative control of adipogenesis (Fujiki et al. 2009; Shao et al. 2013).
In this study, comparing *Zfp423* and *Pparγ* expression levels in 3T3-L1 and NIH-3T3 cells, I have demonstrated that the expression of both *Zfp423* and *Pparγ* genes are higher in 3T3-L1 compared to NIH-3T3 cells. These data are in accordance with Gupta and colleagues, which have previously shown that the expression levels of *Zfp423* are higher in several preadipose cell lines when compared to non-adipogenic fibroblasts. Additionally, they also demonstrate that in these preadipocyte cell lines, the expression of *Zfp423* is not significantly modified during adipocyte differentiation (Gupta et al. 2010), indicating that is the basal expression of *Zfp423* in the undifferentiated state that reflects the ability of these preadipose cell lines to differentiate.
3T3-L1 and NIH-3T3 are a continuous substrain of 3T3-Swiss Albino cells developed through clonal isolation.
Subsequently, I have demonstrated that the differences found in the expression levels of *Zfp423* and *Pparγ* were not due to the presence of genetic mutations accumulated during the clonal isolation.
Moreover, I have shown that methylation status of *Zfp423* and *Pparγ* promoters is reduced in preadipose 3T3-L1 cells compared to non-adipogenic

fibroblasts NIH-3T3 and that the methylation level of each promoter inversely correlates with the expression level of its gene. These findings are in agreement with those previously reported by Fujiki and colleagues (Fujiki et al. 2009), who showed that the expression of *Pparγ* is repressed by DNA methylation in visceral adipose tissue of mouse models of diabetes.

In addition to DNA methylation status, performing MNase digestion, a method which allows the determination of nucleosome occupancy and positioning, I have demonstrated that the reduced expression levels of *Zfp423* and *Pparγ* genes are also regulated by chromatin remodeling. Indeed, genome regulation also occurs at the level of chromatin. Nucleosome packaging restricts protein binding and interferes with DNA-templated reactions. Local modulation of DNA accessibility thereby provides an opportunity to influence the fundamental processes of transcription, replication and repair. Some studies have uncovered cell-type-specific chromatin signature that suggests a dynamic interplay between tissue-specific regulation by transcription factors and chromatin structure and also can regulate adipocyte determination (Bell et al. 2011).

Subsequently, a MNase protection assay performed in NIH-3T3 cells treated with AZA clearly shown that the inhibition of DNMTs is able to restore the *Zfp423* and *Pparγ* gene expression reducing not only the methylation levels but also the degree of chromatin compaction, promoting the recruitment and activation of the transcriptional machinery to the promoters of the investigated genes. These data clearly highlight that DNA methylation acts upstream of chromatin remodeling with a coordinated and synergistic action of DNMTs and histone modifying enzymes, confirming that DNA methylation may serve as a template for the establishment of certain histone modifications (Jin et al. 2011).

Once I have determined that both *Zfp423* and *Pparγ* are transcriptionally regulated by epigenetic modifications, I have investigated the effects of these epigenetic mechanisms on cell commitment. It is known that the exposure of proliferating stem cells derived from mouse embryos to DNA methylation inhibitor results in a subpopulation of cells with the ability to undergo commitment to new cell phenotypes (Taylor and Jones 1979). In light of this, adipocyte differentiation was induced in NIH-3T3 cells treated with AZA for 6h before the administration of differentiation cocktail. AZA treatment has revealed a significant improvement in adipogenic differentiation, suggesting that AZA treatment may induce *Zfp423* and *Pparγ* gene expression, promoting adipocyte commitment and differentiation of NIH-3T3 cells.

In accordance with my data, several studies support the hypothesis that the block of DNA methylation induces the stable expression of one or few genes responsible for lineage commitment (Pinney and Emerson 1989; Taylor and Jones 1979). Indeed, Lane and colleagues have previously shown that the exposure of proliferating 10T1/2 stem cells (derived from mouse embryos) to AZA gave rise to a subpopulation of cells with the capacity to differentiate into adipocytes upon treatment with terminal differentiation inducers, and have also

provided convincing evidence for the participation of Bmp4 in adipocyte lineage determination (Bowers et al. 2006).
Although this study highlights the importance of *Bmp4*, no alterations in *Bmp4* methylation are identified. Although this study highlights the importance of *Bmp4*, my data provide supporting evidence that methylation of *Zfp423* and *Pparγ* controls preadipocyte determination in NIH-3T3 cells.

6. CONCLUSIONS

In this study, I have demonstrated that: i) *Zfp423* and *Pparγ*, main genes involved in the commitment and differentiation are differentially expressed in 3T3-L1 and NIH-3T3 cells; ii) the expression differences are not due to the presence of genetic mutations in the promoter sequence of *Zfp423* and *Pparγ* genes; iii) Zfp423 and Pparγ genes are transcriptionally regulated by DNA methylation and chromatin remodelling, iiii) the inhibition of DNA-methyltransferase restores the expression of *Zfp423* and *Pparγ*, induces chromatin remodeling of their promoters, and improve the adipocyte commitment and differentiation of the NIH-3T3 cell line. Taken together, these results suggest that epigenetic mechanisms could impact on the expression of these two key modulators of adipocyte differentiation, resulting in the modulation of the adipogenic process.

My observations are the starting point for a better understanding of these pathophysiological alterations. Indeed, a complete understanding of molecular mechanisms regulating the expression of *Zfp423* and *Pparγ* genes may provide novel therapeutic strategies for the treatment of obesity-related comorbidities. Meanwhile, identify the environmental factors that lead to epigenetics changes in these two genes, could play a critical role in the prevention of hypertrophic obesity and its related metabolic complications.

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