EPIGENETIC REGULATION OF ADIPOCYTES PHENOTYPE: IMPLICATION FOR PERIVASCULAR ADIPOSE TISSUE CONTRIBUTION TO CARDIOMETABOLIC DISEASES

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Abstract

Perivascular adipose tissue (PVAT) has emerged in the past decades as an integral part of the vasculature (*tunica adiposa*), contributing by the release of adipokines, to important physiological process such as vascular tone regulation. The metabolic syndrome is associated with an increase deposition of PVAT and the development of atherosclerosis. Whereas healthy PVAT protect the vasculature from inflammation, lipid accumulation and hypothermia, the dysfunctional PVAT observed in the metabolic syndrome favour many features of atherosclerosis such as endothelial dysfunction, vascular smooth muscle proliferation and inflammation. The down- and up-regulation of adiponectin, leptin and nerve growth factor, respectively in the dysfunctional PVAT perfectly illustrates this change. Phenotypically, healthy PVAT resembles the thermogenic brown and beige adipocytes, however, recent data suggest a loss of PVAT thermogenic phenotype during the metabolic syndrome in a process called “PVAT whitening”. This review will provide a current perspective on the factors susceptible to influence PVAT epigenetics and the consequences of PVAT dysfunction on cardiometabolic diseases.

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Key words: *tunica adiposa*, WAT, BAT, cardiometabolic diseases, fetal programming, leptin, adiponectin, nerve growth factor, AMP-activated kinase

Abbreviations used

ADCF: Adipose derived constricting factor; ADRF: Adipose derived relaxing factor; AMPK-5': Adenosine monophosphate-activated protein kinase; BAT: Brown adipose tissue; CMD: Cardiometabolic diseases; CpG: 5'-C-phosphate-G-3'; DNA: Deoxyribonucleic acid; DNMT: DNA methyltransferase; EHMT1: Euchromatic histone lysine n-methyltransferase 1; FNDC5: Fibronectin type III domain-containing protein 5; H₂S: Hydrogen sulphide; HDAC: Histones deacetylase; HFD: High fat diet; IL-6: Interleukin-6; iNOS: inducible nitric oxide synthase; JAK: Janus kinase; KDM1: Lysine (K)-specific demethylase 1; KMT1: Lysine (K)-specific methyltransferases 1; LIF: Leukaemia Inhibitory factor; LSD1: Lysine-specific histone demethylase 1; MCP-1: Monocyte chemoattractant protein-1; Myf5: Myogenic factor 5; Myh11: Myosin heavy chain 11; NGF: Nerve growth factor; NO: Nitric oxide; p75^{NTR}: p75 (pan) neurotrophin receptor; Pax7: Paired box homeotic gene 7; PDGFR α : Platelet derived growth factor receptor alpha; PGC-1 α : Peroxisome proliferator-activated receptor γ coactivator-1 α ; PPAR γ : Peroxisome proliferator activated receptor gamma; PRDM16: PR domain containing 16; PVAT: Perivascular adipose tissue; SAT: Subcutaneous adipose tissue; SM22 α : Smooth muscle protein 22-alpha; STAT3: Signal transducer and activator of transcription 3; TDG: Thymine DNA glycosylase; TET: Ten-eleven translocation; TNF α : Tumour necrosis factor alpha; TrkA: Tropomyosin-related kinase receptor A; UCP-1: Uncoupling protein-1; VAT: Visceral adipose tissue; WAT: White adipose tissue; WHO: World Health Organization

Prologue

In our post-industrial society, the combination of the abundance of processed food, sedentary life style, stress, and exposure to endocrine disruptor favours the explosion of obesity. According to the World Health Organization (WHO) obesity has doubled since 1980, and by 2030 some studies suggest that almost half (42%) of adult in the United States could be affected (Finkelstein *et al.* 2012). During the same period the burden of cardiometabolic diseases (CMD) has also grown making them for the first time in human history the leading cause of premature mortality ahead of infectious diseases (WHO report 2008). About 260 years ago the Italian physician Morgagni described the association between hyperuricemia, visceral obesity, hypertension, obstructive sleep apnoea and atherosclerosis (Crepaldi *et al.* 2006). It was not before 1998 that the term metabolic syndrome was defined by the WHO as a syndrome comprising type 2 diabetes or impaired glucose tolerance associated with at least two out of the four other factors, namely high blood pressure, high blood fat, obesity and microalbuminuria (Grundy *et al.* 2004, Crepaldi

et al. 2006). Since then the scientific community has deployed constant effort to understand the interconnections between the different components of the metabolic syndrome leading to atherosclerosis development.

Introduction

Adipose tissue is widely distributed throughout the human body and can be subdivided according to its anatomical location. Subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) are the main pools of adipose tissue and account for 82-97% and 10-15% of total body fat respectively (Gastaldelli *et al.* 2010, Lee *et al.* 2013). However, small adipose tissue depots are also found in the bone marrow, skeletal muscles and mammary glands (Rosen *et al.* 2014). In the past decades perivascular adipose tissue (PVAT) has emerged as an integral part (*tunica adiposa*) of human arteries (Chaldakov *et al.* 2009, 2012, 2014; Ayala-Lopez and Watts 2016) and veins of large, medium and small sizes with the notable exception of cerebral vessels (Houben *et al.* 2012, Szasz *et al.* 2013, Brown *et al.* 2014), and represent an average of 3% of total fat in human (Gastaldelli *et al.* 2010; Lee *et al.* 2013). In the small vessels of some organs, however, PVAT may be constituted by the adipose depots embedding this organ as it is the case for the pericoronary/epicardial PVAT (Britton *et al.* 2011). It is now accepted that PVAT is not only a connective tissue, providing the vessel with mechanical protection, but also a regulator of vascular functions through the secretion of many adipokines with vasoactive, pro- and anti-inflammatory, and neuro-metabotropic properties (Chaldakov *et al.* 2014; Kostopoulos *et al.* 2014; Iacobellis 2016). Although adipocytes are the main component of PVAT it also consists of nerves and resident immune cells (e.g. macrophages, mast cells) whose interactions ensure vascular homeostasis (Chaldakov *et al.* 2014, Ayala-Lopez and Watts 2016). Whereas SAT and VAT are the main contributors to the systemic adipokines level, the proximity of PVAT with the *vasa vasorum* may allow PVAT-derived adipokines to reach the inner vascular cells (i.e. vascular smooth muscle and endothelial cells) at high concentration and thereby strongly impact vascular function. Thus, it has been shown that PVAT exert anticontractile functions through the release of adipose derived relaxing factors (ADRF) such as adiponectin, hydrogen sulphide (H₂S), NO and prostacyclin (Wojcicka *et al.* 2011, Nava *et al.* 2016). On the other hand, PVAT can mediate nerve dependant vasoconstriction by releasing adipose derived constricting factors (ADCF) such as superoxide anion, angiotensin II and endothelin-1 (Gao *et al.* 2007).

Obesity is associated with an increase in PVAT deposition, which is strongly related to atherosclerotic plaque development (Mahabadi *et al.* 2010, Maurovich-Horvat *et al.* 2011). Moreover,

in a swine model of obesity the increases in PVAT deposition negatively correlate with coronary vasodilatation (Owen *et al.* 2013). Accumulating data suggest also a phenotypic switch of PVAT during metabolic syndrome development which can be summarized under the term “PVAT dysfunction”. The dysfunctional PVAT is characterized by a loss of its anticontractile property, and an increase in the release of proinflammatory and proangiogenic adipokines (Chang *et al.* 2013; Kostopoulos *et al.* 2014). These adipokines, by promoting endothelial dysfunction, macrophage chemotaxis, together with the proliferation of vascular SMC and fibroblasts, contribute to the vascular remodeling and the progression of atherosclerotic plaque. On the other hand, when compared to other fat depots such as SAT and VAT, mouse PVAT was found to be resistant to a high fat diet (HFD) induced inflammation (Fitzgibbons *et al.* 2011). The protective role of PVAT could be linked to its thermogenic potential that regulates intravascular temperature, increases plasma triglyceride clearance and confer resistance to inflammation. Therefore, it is important to identify the molecular (i.e. Mediators, signaling pathway, epigenetic factor) determinants of PVAT phenotype in health and disease. Several studies suggest that *in utero* and long term exposure to the metabolic syndrome could lead to a loss of PVAT thermogenic ability by modifying its epigenetic memory. In the past decades epigenetics has emerged as a new level of gene regulation involving modification of DNA accessibility by post-translational modification of histones (e.g. phosphorylation, acetylation, and methylation) and DNA methylation. Recent studies in humans (Mamun *et al.* 2009, Oken 2009, Fraser *et al.* 2010) and rodents (Samuelsson *et al.* 2008, Shankar *et al.* 2008) models have shown the links between maternal obesity or weight gain during pregnancy and metabolic disorders such as obesity, glucose intolerance and CMD in offspring. In this review, we are presenting the recent evidences of epigenetic regulation of adipocytes phenotype relevant to PVAT biology in health and disease.

PVAT phenotype in the spectrum of adipocytes phenotype

In addition to differences in location, there are also substantial functional differences between adipose tissue depots. Two main types of adipocytes are distinguished in human and mouse. Most adipocytes of SAT and VAT constitute the white adipose tissue (WAT). The white adipocytes constituting WAT are characterized by a unilocular large lipid droplets, small peripheral nucleus, few mitochondria's and are mainly responsible for energy storage in the form of lipids (Rosen *et al.* 2014). On the other hand thermogenic or brown adipose tissue (BAT) in humans is found in cervical, supraclavicular, paravertebral, mediastinal,

and peri-renal regions (Sacks *et al.* 2013). The brown adipocytes constituting BAT are smaller, present multilocular lipid droplets, centralized nucleus, and higher numbers of mitochondria. The main physiological function of brown adipocyte is to maintain body temperature and avoid hypothermia. The thermogenic capacity of brown adipocytes relies on the expression of the uncoupling protein-1 (UCP-1) within the mitochondria (Rosen *et al.* 2014). This protein by catalysing a proton leak across the inner mitochondrial membrane disconnect fatty acid oxidation from ATP production, thus producing heat (Rosen *et al.* 2014). Recently a third category of adipocyte called beige or brite (for brown in white adipocytes) has been characterized (Chechi *et al.* 2013). These cells are mainly induced in SAT by stimuli such as cold exposure and β -adrenergic stimulation. Similar to brown adipocytes, beige adipocytes express high levels of UCP-1 whose expression is required for their thermogenic function (Lee *et al.* 2013). In human most studies on PVAT has been made on the pericoronary/epicardial and periaortic adipose tissue and conclude that human PVAT phenotype resembles beige/brown adipocytes and is characterized by a small size, multilocular lipid droplets, abundant mitochondria, as well as high expression of UCP-1 (Sacks *et al.* 2013, Brown *et al.* 2014). In agreement with the findings in human, studies in mice showed that aortic PVAT present a large similarity to BAT and is also resistant to diet-induced inflammation (Fitzgibbons *et al.* 2011). Brown adipocytes in rodents has been shown to improve several characteristics of the metabolic syndrome, such as hyperglycaemia, insulin resistance and the excess of free fatty acid characterizing dyslipidaemia (Bartelt *et al.* 2011, Shimizu *et al.* 2015, Villarroja 2016). In accordance with a similar protective effect of PVAT, Chang *et al.* showed that PVAT free mice had impaired ability to regulate triglyceride levels and intravascular temperature (Chang *et al.* 2012). In conclusion the thermogenic potential of most PVAT depots (with the exception of mesenteric PVAT) is associated with vasculoprotective functions. Therefore, it is crucial to understand the mechanisms regulating PVAT thermogenic phenotype, and PVAT dysfunction in order to prevent, and manage the vascular diseases associated with the metabolic syndrome.

Role of epigenetics in adipocytes differentiation and PVAT phenotypic diversity

From a developmental perspective, brown and white adipocytes arise from two different precursors (Fig. 1A). Whereas brown adipocytes derived from a Pax7⁺/Myf5⁺ stem cells involved in skeletal muscle development, white and beige adipocytes are derived from a common PAX7⁺/Myf5⁺/PDGFR- α ⁺ precursor (Brown *et al.* 2014). In a very interesting article Chang *et al.* demonstrated that specific deletion of PPAR γ in SM22a α -

Figure 1. A. Representation of adipocytes foetal programming induced by *in utero* exposure to a high fat diet (HFD). Epigenetics plays an important role in the development of most tissues including adipose tissue and allow the generation of brown (BAT), brown in white (BRITE), white (WAT) and perivascular (PVAT) adipose tissue depots from mesenchymal/mesodermal stem cells (Siersbaek *et al.* 2012). *In utero* exposure to a HFD, by generating inflammation and the loss of metabolic homeostasis induces a reduction of thermogenic adipocytes while inducing WAT hypertrophy and PVAT “whitening”. Whether this alteration also affects the generation of the intermediate progenitors remains unknown. Adapted from (Brown *et al.* 2014). **B.** Hypothetical model of adipose tissue phenotype diversity based on location. According to the results of Satoor *et al.* (2011) adipose tissues location define their exposure to local parameters such as glucose concentration ([glucose]), leading to epigenetic regulation of their phenotype. Beyond [glucose] other mediators derived from nearby resident cells, such as, cytokines, growth factors may also regulate adipocytes phenotype through epigenetic regulation resulting in specific phenotypes and functions. * Not applicable to mesenteric PVAT.

pressing cells (i.e. Vascular smooth muscle cells) impairs aortic and mesenteric PVAT development without any effect on BAT and WAT development (Chang *et al.* 2012). This strongly suggests that mouse PVAT derived from a precursor of the vascular smooth muscle cell lineage. Consequently, compared to others fat depots such as SAT, PVAT present a reduced adipogenic potential and a heightened pro-inflammatory phenotype (Chatterjee *et al.* 2009, Gil-Ortega *et al.* 2015). Despite these differences in origin, both brown and beige adipocytes express the transcription factor PRDM16 which play a critical role in the thermogenic differentiation of adipocytes. Interestingly, it has been shown that PRDM16 promotes the thermogenic differentiation of adipocytes through its association with the euchromatic histone-lysine N-methyltransferase 1 (EHMT1). Through

this interaction PRDM16 repress the genetic program leading to skeletal muscle differentiation (Ohno *et al.* 2013). Similar to PVAT development, it has been shown recently that a subset of beige adipocytes induced by cold, is derived from a vascular smooth muscle cell (SMC) precursor expressing myosin heavy chain 11 (Myh11⁺) (Long *et al.* 2014, Berry *et al.* 2016). Interestingly the expression of PRDM16 in vascular smooth muscle cells is able to turn them into beige adipocytes (Long *et al.* 2014). This suggests that PRDM16 through interactions with EHMT1 or another epigenetic regulator could repress vascular smooth muscle cell differentiation in the SM22 α ⁺ precursor and promote PVAT differentiation. This hypothesis is also supported by the fact that human epicardial adipose tissue (peri-coronary adipose tissue) display great similarities with beige adipocytes

(Sacks *et al.* 2013). Despite a common developmental path PVAT phenotype varies according to location in human and mouse. Thus, in mice the phenotype of PVAT in the thoracic aorta resembles BAT, whereas mesenteric PVAT resembles WAT. Finally, aortic and renal adipocytes resembles beige adipocytes (Brown *et al.* 2014). These observations support a high plasticity of PVAT adipocytes and the critical role of environmental and cellular interactions in adipocytes phenotype. In a remarkable work, Satoor *et al.* showed that transfer of VAT in the subcutaneous space was able to modify the phenotype of the engrafted VAT by modifying its epigenetic memory (Satoor *et al.* 2011). Indeed, they showed that the high glucose concentration in the mesenteric circulation was able to epigenetically repress VAT expression of adipokines (i.e. adiponectin, leptin, resistin and visfatin) by recruiting the histone demethylase LSD1 and KMT1 (Satoor *et al.* 2011). This could explain the fact that mesenteric PVAT unlike others present a phenotype of white adipocytes. Interestingly, diabetes, which is characterized by hyperglycaemia is associated with PVAT inflammation that may result from a “PVAT whitening” (Lastra *et al.* 2015). Beyond glucose concentration other physiological parameters varying with location, such as temperature, exposure to myokines, inflammatory and neuromediators have been shown to influence the terminal differentiation of adipocytes (Wang *et al.* 2016). This suggests that the phenotype of each fat depot results from the sensing and integration of many environmental factors and that, epigenetics may be a critical mechanism controlling adipose tissue diversity and adaptation (Fig. 1B). By screening a collection of epigenetic compounds recently identified the histone demethylase LSD1/KDM1 as a key regulator of brown adipogenesis (Chen *et al.* 2016). Using RNA interference and knock-out mice, they further demonstrated that LSD1/KDM1 promotes brown adipogenesis through Wnt pathway inhibition and stimulation of UCP-1 expression (Chen *et al.* 2016). In another study KDM1A has been shown to promote adipogenic differentiation and repress the expression of inflammatory mediators (i.e. IL-6) in the 3T3-L1 pre-adipocyte cell line (Hanzu *et al.* 2013). Although the role of LSD1/KDM1 in PVAT has not been studied yet it is interesting to notice that LSD1/KDM1 deficient mice (i.e. LSD1/KDM1^{+/-}) are more sensitive than their WT counterpart to salt induced hypertension due to alterations in NO-cGMP induced vascular relaxation (Pojoga *et al.* 2011). Whether this phenotype is due to a “PVAT whitening” remain to be demonstrated. This illustrates the potential role of epigenetic factors controlling the thermogenic adipocyte program in vascular biology. In a recent article Inagaki *et al.* provided a complete review of the transcriptional and epigenetic regulators controlling brown and beige adipocytes differentiation that could be used to further inves-

tigate the path of PVAT development and function (Inagaki *et al.* 2016).

In an effort to uncover the mechanisms behind adipocytes phenotypic diversity Gehrke *et al.* (2003) studied the DNA methylation profile of different fat depots (i.e. abdominal and gluteal) in humans. They showed that DNA methylation profile of genes involved in fatty acid and triglyceride metabolism correlates with their levels of expression in these depots resulting in differential fatty acid and triglycerides saturation. These results clearly demonstrate that epigenetic regulation is an important aspect of adipocytes functional diversity. But recent data's also suggest a role for epigenetic regulation in dysfunctional adipocytes. Indeed, increases in the DNA methylation profile of genes relevant to adiposity in SAT of humans correlate with body mass index (BMI), android adipose mass, android:gynoid adipose ratio and trunk:limb adipose ratio (Agha *et al.* 2015). Moreover, differential methylation of genes related to obesity has also been reported in the SAT of obese woman (Arner *et al.* 2015) and post-obese woman whose obesity is characterized by adipocytes hyperplasia (Dahlman *et al.* 2015). Thereby, epigenetic modifications such as histones and DNA methylation are not only involved in adipocytes differentiation, but also in the maintenance of depots specific phenotype and the acquisition of a pathologic phenotype in patients with obesity.

PVAT dysfunction caused by fetal programming

Further evidences of the epigenetic regulation of PVAT phenotype in health and disease has been brought by experimental models of foetal programming (Fig. 1A). In these models the foetus undergoing epigenetic regulation of its fat development is exposed to a pathologic maternal milieu induced by HFD (Laker 2013). The interference of this pathological maternal milieu (i.e. hyperglycaemia, dyslipidaemia and inflammation) with the ongoing epigenetic regulation of adipogenesis results in obesity, glucose intolerance and CMD in the offspring that are transmitted to the next generations (Laker 2013). Thus, Zaborska *et al.* (2016) recently showed that the offspring of obese rats fed an HFD during pregnancy and lactation lost the anticontractile function of their PVAT despite being fed a normal diet for 12 or 24 weeks after weaning. Furthermore this loss of anticontractile function resulted in hypertension at 24 weeks old of age. From a mechanistic perspective, the study showed that the loss of anticontractile effect of PVAT was due to the release of ADF and a decrease in nitric oxide (NO) bioavailability (Zaborska *et al.* 2016). This study suggests that *in utero* exposure to metabolic and adipokines imbalance, could imprint a pathological memory in adipocytes predisposing the offspring to hypertension. Beyond the loss of anti-contractile properties, *in utero* exposure

to a HFD has also been shown to favour atherosclerosis development and a pro-inflammatory phenotype of PVAT especially by increasing the expression of the chemokines MCP-1 (Wakana *et al.* 2015). The contribution of PVAT dysfunction to atherosclerosis development has been further demonstrated by transplantation experiments. Indeed transplantation of PVAT from 8 weeks old mice exposed *in utero* to a HFD was associated with a twofold increased in atherosclerotic plaque development in the nearby artery when compared to transplantation of PVAT from mice exposed *in utero* to a normal diet (Wakana *et al.* 2015). A recent report also showed that coronary atherosclerosis induced by a HFD is accompanied by a transition of the epicardial fat phenotype from brown to white (Wang *et al.* 2015). This transition was characterized by a decrease in the number of mitochondria as well as in the expression of UCP-1 and PPAR γ (Wang *et al.* 2015). Consistently it has been shown that the offspring of mice fed a HFD during lactation present a reduction in BAT depots and a reduced thermogenic capacity of their adipocytes (Liang *et al.* 2016). One of the major regulator of PPAR γ function and UCP-1 expression during the development of thermogenic adipocytes is the peroxisome proliferator-activated receptor γ coactivator-1 α (Pgc-1 α). Interestingly a HFD has been shown to repress PGC-1 α expression in skeletal muscle through methylation of its promoter (Laker *et al.* 2014). Thus the epigenetic repression of PGC-1 α in PVAT could explain the impaired UCP-1 and PPAR γ expression observed in epicardial fat of rats exposed to a HFD. UCP-1 expression in adipocytes has been shown to be regulated through both DNA methylation and histones modification in response to cold exposure (Shore *et al.* 2010). The detrimental effect of a possible “PVAT whitening” on atherosclerosis development is provided by transplant experiments showing that VAT transplant is more proatherogenic than SAT which include numbers of thermogenic (i.e. beige adipocytes) adipocytes (Ohman *et al.* 2011). It is therefore important to find new targets allowing the maintenance of PVAT thermogenic phenotype and the prevention of its “whitening”.

Effect of exercise training on PVAT function and adipocytes thermogenic program

Whereas *in utero* exposure to a HFD is associated with PVAT “whitening” in offspring, studies on the effect of exercise training suggest a possible “re-browning” of PVAT. Indeed, in a rat model of metabolic syndrome (i.e. obese Zucker rats) associated with PVAT dysfunction (i.e. loss of anti-contractile function, decreased UCP-1 expression, increased inflammatory mediator expression) exercise training have been shown to improve UCP-1 expression and to inhibit the release of IL-6 and TNF α by PVAT (DeVallance *et al.* 2016). Recent reports showed that exercise

training was able to improve the dysfunction of adipocytes induced by a HFD in rats through induction of beige adipocytes in their WAT. The authors observed an exercise dependant increase in fibronectin type III domain-containing protein 5 (FNDC5) the precursor of the myokine irisin (Rocha-Rodrigues *et al.* 2016, Rocha-Rodrigues *et al.* 2016; for exercise-adiponectin link see Öztürk *et al.* in this volume of *Adipobiology*). Previously irisin has been reported to induce white fat browning through stimulation of UCP-1 expression (Bostrom *et al.* 2012). Interestingly a recent article showed that irisin restores the anticontractile function of mouse PVAT in a model of obesity induced by a HFD by increasing its expression of heme oxygenase and adiponectin while reducing that of superoxide and TNF α (Hou *et al.* 2016). However, human data's failed to show a substantial impact of training exercise on circulating irisin and SAT browning (Norheim *et al.* 2014). Although the authors did not investigate the contribution of epigenetics to the “re-browning” of PVAT in their models, studies in humans showed that a 6 months exercise training was able to change the DNA methylation profile of 7663 genes in adipocytes (Ronn *et al.* 2013). Interestingly, one of the genes found to be hyper methylated and therefore inhibited in adipocytes after the 6 months exercise training was the histone deacetylase 4 (HDAC4). Further investigations using the pre-adipocyte cell line 3T3-L1 showed that HDAC4 silencing was associated with increase basal and insulin-induced lipogenesis (Ronn *et al.* 2013). Moreover the exercise training has been shown to prevent epigenetic repression of PGC-1 α in the skeletal muscle of mice exposed *in utero* to a HFD (Laker *et al.* 2014). Such an effect in PVAT could explain the beneficial effect of exercise training on mouse PVAT dysfunction induced by *foetal* programming. Further experiments are needed to determine whether the improvement in PVAT function associated with exercise training could be due a myokines dependant “re-browning” induced through epigenetic regulation.

Epigenetic regulation of adiponectin expression and effect on cardiometabolic diseases

Adiponectin is the main adipokine secreted by adipose tissue and represent in an average 0.01% of plasma protein. Adiponectin play important metabolic functions by promoting fatty acid oxidation, glucose uptake and preventing glucose induced ROS synthesis (Shimomura *et al.* 2006; Van de Voorde *et al.* 2013). Animal studies in models of CMD showed that adiponectin has cardioprotective, anti-inflammatory, -oxidant, -atherogenic, and -apoptotic properties (Chow *et al.* 2007, Goncalves *et al.* 2015). Moreover down regulation of its receptor in obese patients is related to insulin resistance and the intramuscular deposition of lipids (Yamauchi *et al.* 2001). The protective role of adiponectin

in CMD is further supported by the inverse correlation existing between its plasma concentration, BMI and the risk of hypertension (Cnop *et al.* 2003). Consistently, adiponectin has been shown to contribute to mouse PVAT anticontractile function (Lynch *et al.* 2013). Therefore the downregulation of adiponectin is a hallmark of adipose tissue dysfunction associated with the metabolic syndrome and thereby has been studied intensively. A recent study of adiponectin gene methylation status in adipose tissue of obese patients showed a positive correlation with BMI suggesting that the downregulation of adiponectin expression associated with adipose tissue dysfunction is due to epigenetic repression (Houde *et al.* 2015). In a model of *in utero* exposure to a HFD researchers confirmed the role of adiponectin promoter methylation as the probable cause of decreased circulating adiponectin associated with the metabolic syndrome. In another research article using the same model, it has been shown that epigenetic regulation of adiponectin also involves histone modifications. In this study adiponectin promoter repression in WAT was associated to a decreased acetylation and an increase methylation of histone H3 at lysine 9 (Masuyama *et al.* 2012). The decrease in histone H3 acetylation could be due to the action of the Histone Deacetylase 9 (HDAC9), since its inhibition in a model of chronic consumption of a HFD in mice was associated with an increase adiponectin expression and enhanced energy expenditure by promoting Beige adipogenesis (Chatterjee *et al.* 2014). Further studies showed that the epigenetic changes induced by *in utero* exposure to a HFD in the adiponectin promoter are transmitted up to 3 generations (Masuyama *et al.* 2015). Although such epigenetic modifications in adiponectin gene has not been directly studied in PVAT several studies have shown decreases in adiponectin expression at the mRNA and protein level in the PVAT of human diseased vessels (Ozen *et al.* 2015). Moreover the detrimental effect of adiponectin repression in PVAT on atherosclerosis development has recently been shown. Indeed engraftment of PVAT from adiponectin deficient mice in a model of collar induced carotid atherosclerosis resulted in the accelerated atherosclerotic plaque formation (Li *et al.* 2015). The protective effect of adiponectin in this model was due to its ability to promote macrophages autophagy (Li *et al.* 2015). Antonopoulos *et al.* also recently highlighted the role of PVAT derived adiponectin as an important anti-oxidant mediator in the internal mammary artery of patients suffering with type II diabetes (Antonopoulos *et al.* 2015). However a recent analysis of adiponectin expression in the PVAT of patients undergoing carotid endarterectomy showed a 2 fold increase of its expression in symptomatic plaques, suggesting a detrimental role of adiponectin in the stability of atherosclerotic plaques (Sharma *et al.* 2015). In conclusion adiponectin expression in PVAT strongly

influence vascular biology and is regulated in adipocytes partly through epigenetic mechanisms. Further experiments are required to fully understand the mechanisms involved in PVAT adiponectin repression.

Epigenetic regulation of leptin expression and effects on cardiometabolic diseases

Unlike adiponectin, the plasma concentration of leptin, another important adipokine mainly secreted by adipocytes, is positively correlated to obesity (31.3 ± 24.1 ng/ml vs 7.5 ± 9.3 ng/ml in non-obese subjects) (Considine *et al.* 1996). However, these patients also present leptin resistance due to decreased expression of the leptin receptor. Leptin at first has been involved in energy metabolism and appetite regulation and was perceived as a protective adipokine with anti-obesogenic and anti-diabetic properties (Oral *et al.* 2002). However, from a cardiovascular perspective, leptin has been shown to promote several processes involved in CMD such as platelet aggregation, oxidative stress, vascular smooth muscle cell proliferation, and inflammation (Beltowski 2006). The role of epigenetics and particularly DNA methylation in leptin expression associated with obesity has recently been studied in SAT, VAT and blood cells of severely obese patients. The data showed an inverse correlation between leptin promoter methylation in blood cells and BMI, suggesting a role for leptin promoter demethylation in the hyperleptinemia associated with obesity (Houde *et al.* 2015). The contribution of epigenetics to the dysfunctional leptin system associated with the metabolic syndrome has also been studied using offspring of mice fed with a HFD during pregnancy. In addition, to increase in body mass, dyslipidaemia and glucose intolerance the offspring also present hyperleptinemia (Khalyfa *et al.* 2013). The authors showed that the hyperleptinemia induced by a HFD was due to the hypomethylation of CpG sites in leptin promoter. Conversely the expression of the leptin receptor in visceral fat was downregulated due to hypermethylation of its promoter (Khalyfa *et al.* 2013). In another study using the same model it has been shown that a HFD increases leptin expression in adipocytes through hypermethylation of the lysine 20 of histone H4 at its promoter (Masuyama *et al.* 2012). Similar to the modifications in adiponectin promoter the complete loss of the epigenetic marks induced by *in utero* exposure to a HFD in leptin promoter occurs after 3 generations of return to a normal diet (Masuyama *et al.* 2015). The increased expression of leptin in PVAT of patients with CMD has been observed at the mRNA and protein level (Ozen *et al.* 2015), however the role of epigenetics in this increase remains to be established. Further studies on the epigenetic regulation of leptin and adiponectin expression in PVAT could lead in the future to the identification of targets, allowing

the improvement of PVAT function in individuals affected by the metabolic syndrome.

Nerve growth factor a potential marker of adipose tissue inflammation in cardiometabolic diseases

Nerve growth factor (NGF) is a prototype member of the protein family of neurotrophins (that induce the survival and development of neurons) discovered by Rita Levi-Montalcini in the early 1950's. Since then NGF has been involved in a wide range of human diseases, including, neurodegenerative and psychiatric disorders and CMD (Aloe *et al.* 2013; Yanev *et al.* 2013). Nerve growth factor exerts its biological effects on cells by binding to a high affinity receptor called tropomyosin-related kinase receptor A (TrkA) and a low affinity receptor named p75 (pan) neurotrophin receptor (p75^{NTR}). Whereas TrkA activation mediates protective functions, especially in neurodegenerative disorders, p75^{NTR} on the other hand, has been shown to mediate proNGF-induced apoptosis (Aloe *et al.* 2013). Therefore the relative expression of both receptors deeply influences NGF pathophysiology.

From a physiological perspective NGF expression in PVAT and other adipose depots may be related to the recruitment and maintenance of innervation as recently demonstrated for the perivascular innervation of mouse neovessels (Goda *et al.* 2016). In a recent review Ayala-Lopez and Watt (2016) highlighted the potential role of the sympathetic innervation and the adrenergic system of PVAT in obesity-associated hypertension. In addition to hypertension, altered interactions between nerves and adipocytes in PVAT may contribute to atherosclerosis development. Indeed, advanced coronary atherosclerosis in human is associated with a reduced amount of NGF and an increase in mast cells and *vasa vasorum* number and p75^{NTR} expression in the subepicardial adipose tissue (Chaldakov *et al.* 2004). Both WAT and BAT has been shown to express NGF and brain-derived neurotrophic factor (BDNF) in mouse (Sornelli *et al.* 2009) and in human (Nisoli *et al.* 1996, Peeraully *et al.* 2004). And obesity was associated with an increase of NGF circulating level in women (Bullo *et al.* 2007) and its expression in SAT (Atanassova *et al.* 2014). Moreover, in a model of type 1 diabetes induced by streptozotocin injection in rat, NGF expression has been shown to increase in both WAT and BAT (Sornelli *et al.* 2009). Nevertheless, several studies showed that NGF expression in fat depots of obese patients was primarily due to non-adipocyte cells, including stromal vascular cells (Fain 2010). In fact, analysis of NGF expression during human adipogenesis *in vitro* revealed a downregulation in differentiated adipocytes (Wang *et al.* 2005). Consistent with this result treatment of adipocytes with the PPAR γ agonists, rosiglitazone (Ryan *et al.*

2008) and 15-deoxy- $\Delta^{12,14}$ -PGJ2 (Bullo *et al.* 2005) results in reduced NGF secretion. On the other hand, NGF expression *in vitro* is consistently increase in adipocytes by several atherosclerosis related mediators such as TNF α (Peeraully *et al.* 2004, Ryan *et al.* 2008), LPS (Ryan *et al.* 2008) and prostaglandins (Bullo *et al.* 2005). This suggests that the increase expression of NGF in PVAT could be due to the recruitment of NGF expressing immune cells and a "PVAT whitening" induced by inflammatory mediators. This hypothesis is supported by results showing that stimuli promoting brown adipogenesis such as cold and norepinephrine were able to decrease the NGF expression in BAT (Nisoli *et al.* 1996). In contrast with these results other has shown increased expression of NGF in BAT of rats and hamsters exposed to cold (Nechad *et al.* 1994).

Whereas the role of epigenetics in NGF expression by adipocytes has not been studied yet, results obtained in other cell types support this possibility. Thus, in a model of chronic neuropathic pain induced by chronic constriction injury of the rat sciatic nerve, Tao *et al.* showed that infusion of the brainstem nucleus raphe magnus with an HDAC inhibitor was associated with an increase in histone H4 acetylation at the NGF promoter resulting in increased NGF expression (Tao *et al.* 2016). Such an association between histone H4 acetylation and NGF expression has also been observed in the central amygdala in a model of addiction induced by morphine stimulation in rat (Bie *et al.* 2012). In addition to histones acetylation, epigenetic regulation of NGF also involves methylation of its promoter. Indeed, during alcohol withdrawal in human the decrease in circulating NGF correlates with the increased methylation of CpG islands in the NGF promoter of blood cells (Heberlein *et al.* 2013). Finally, it has been shown in human colorectal cancer cells that the expression of the NGF receptor p75^{NTR} was also frequently repressed by methylation of its promoter (Yang *et al.* 2015).

In conclusion NGF is consistently induced in adipocytes *in vitro* by inflammatory mediators and *in vivo* by CMD with an important inflammatory component. Further studies are required to define the sources, modalities of regulation and the outcomes of NGF increases in healthy and dysfunctional PVAT associated with CMD with particular emphasize on inflammation and epigenetics.

Role of inflammation in the epigenetic regulation of PVAT dysfunction

Inflammation is an important player of the metabolic syndrome linking obesity to insulin resistance and has been involved in the initiation as well as the rupture of atherosclerotic plaques (Bentzon *et al.* 2014). Therefore PVAT in patients affected by the metabolic syndrome is exposed to the influence of systemic

and local inflammatory mediators secreted respectively by dysfunctional SAT/VAT and damaged vascular cells and inflammatory cells (e.g. Macrophages) (Verhagen *et al.* 2011, Fernandez-Alfonso *et al.* 2013, Castellon *et al.* 2016). In a recent article Kim *et al.* (2015) investigated the regulation of adiponectin expression in adipose tissue of obese patients and identified the DNA methyl transferase 1 (DNMT1) as the responsible for the epigenetic silencing of adiponectin in these cells. Whereas adiponectin repression, has been reported in response to endoplasmic reticulum stress, mitochondrial dysfunction, hypoxia or inflammation, they found that only proinflammatory cytokines such as IL-1 β and TNF α was able to induce DNMT1 expression in adipocytes (Kim *et al.* 2015). Interestingly, in a mouse model of PVAT inflammation induced by endovascular injury the associated inhibition of adiponectin expression in PVAT was absent in TNF α deficient mice and rescued by adjunction of TNF α to the perivascular area of the vessel (Takaoka *et al.* 2010). Moreover, in a mouse model of type 1 diabetes associated with repression of adiponectin expression in PVAT, Infliximab (TNF α blocking antibody) therapy restored adiponectin expression in mouse PVAT and improves endothelial nitric oxide-mediated vasodilation (Nacci *et al.* 2016). Both results strongly support a role for TNF α /DNMT1 in the epigenetic repression of adiponectin observed in dysfunctional PVAT. Since IL-1 β is also an inducer of DNMT1 it could be involved in the repression of adiponectin expression and PVAT thermogenic program. Indeed IL-1 β stimulation has been shown to inhibit the expression of UCP-1 induced by cold *in vivo* and β -adrenergic stimulation of adipocytes *in vitro* (Goto *et al.* 2016).

The role of inflammation in the demethylation of the leptin gene promoter on the other hand has not been reported yet. Active demethylation can be achieved by the action of the ten-eleven translocation (TET) and the thymine DNA Glycosylase (TDG) family enzymes (Kohli *et al.* 2013). To date, no report showed a positive regulation of these enzymes by inflammatory mediators in adipocytes. However a recent work aimed at investigating the role of epicardial fat in a rabbit model of coronary atherosclerosis showed that IL-6 stimulation of peri-coronary fat was able to increase leptin expression through activation of the JAK-STAT3 pathway (Wang *et al.* 2015). Interestingly, LIF, a cytokine belonging to the IL-6 family of cytokine, has been shown to maintain embryonic stem cell pluripotency through the regulation of several DNA demethylases (e.g. DNMT1, 3a/b and 3I) and histones deacetylases (HDACs) in a JAK-STAT3 pathway dependant fashion (Tang *et al.* 2013). These results suggest that IL-6 could upregulate leptin expression in PVAT by decreasing the methylation of its promoter and thereby aggravate atherosclerosis development. The promotion of PVAT dysfunction is

not restricted to IL-6 and TNF α , since in a model of inflammation induced by a HFD it has been shown that Oncostatin M expression was increased and responsible for the reduced BAT development and SAT browning observed in this model (Sanchez-Infantes *et al.* 2016). Further studies are required to better understand the contribution of epigenetics to the PVAT dysfunction induced by inflammatory mediators.

Role of the AMP-activated protein kinase in the epigenetic regulation of PVAT phenotype

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase playing a central role in intracellular energy sensing and whole body metabolism (Almabrouk *et al.* 2014). Therefore AMPK has been shown to play important role in the regulation of adipocytes metabolism as well as in endothelial and vascular smooth muscle cell function (Almabrouk *et al.* 2014).

Inhibition of AMPK in response to a HFD has been reported in many rat tissues as an early event preceding hyperglycaemia or systemic inflammation (Lindholm *et al.* 2013). Similar to what observed in the offspring of mice fed a HFD, AMPK-1 deficient mice also present a loss of the anti-contractile potential of their PVAT (Almabrouk *et al.* 2016). The authors further showed that this loss of anti-contractile function was due to impaired adiponectin secretion (Almabrouk *et al.* 2016). Knowing the role of epigenetics in PVAT adiponectin repression induced by a HFD, one may think that AMPK-1 could be involved in the epigenetic regulation of BAT and PVAT. Indeed, it has been shown that an AMPK-1 deficiency in mouse is associated with impaired BAT development due to an epigenetic repression of PRDM16 expression (Yang *et al.* 2016). In fact the authors showed that AMPK-1 regulates the isocitrate dehydrogenase 2, an enzyme involved in the production of α -ketoglutarate a metabolite require for TET mediated DNA demethylation. Thereby in AMPK-1 deficient mice the lack of α -ketoglutarate prevent PRDM16 promoter demethylation and mRNA expression leading to the inhibition of the development of their thermogenic fat (Yang *et al.* 2016).

The involvement of AMPK in epigenetic regulation of thermogenic adipogenesis may also explain the resistance of PVAT and BAT to the inflammation induced by a HFD. Indeed a recent report showed that the protective cardiovascular effects, of a plant derived steroid sapogenin named diogenin was mediated through AMPK activation (Chen *et al.* 2016). In a rat model of PVAT inflammation and endothelial dysfunction induced by a HFD the authors showed that diogenin treatment increases the basal AMPK expression and prevent the decrease induced by a HFD. The increase AMPK activity, then inhibits the PVAT expression of TNF α , IL-6, MCP-1 and iNOS induced by a HFD

while promoting adiponectin, PPAR γ and arginase-1 expression (Chen *et al.* 2016). Moreover, using an *in vitro* model of PVAT/endothelium interaction induced by exposure of PVAT to fatty acids (i.e. palmitic acid) it has been shown that, activation of AMPK in PVAT was able to prevent its release of inflammatory mediators and thereby its inhibition of the endothelial dependant vasodilatation (Sun *et al.* 2014). These latter authors found that the beneficial effects of AMPK activation was lost in the presence of nicotinamide, an inhibitor of the deacetylase and the key epigenetic regulator sirtuin 1 (SIRT1). However the exact interaction linking AMPK and SIRT1 in PVAT resistance to a HFD remains to be fully elucidated. In addition to its potential epigenetic regulation of inflammatory genes SIRT1 could also improve PVAT function by inducing mitochondrial biogenesis. Indeed Mice overexpressing SIRT1 under the control of its own promoter, has been shown to be resistant to the metabolic damages (i.e. adipose mass gain, glucose intolerance, hepatic steatosis, inflammation) induced by a HFD in particular by activating the PGC-1 α leading to increased mitochondrial biogenesis and higher energy expenditure (Pfluger *et al.* 2008). AMPK has been involved in endothelial, vascular smooth muscle cells and adipose tissue dysfunction associated with the metabolic syndrome (Almabrouk *et al.* 2014). A better understanding of its beneficial

effect on PVAT function through epigenetic regulation of key adipokines such as leptin and adiponectin could help find new targets for managing cardiovascular diseases.

Coda

There is growing evidence that PVAT dysfunction is a key aspect in the pathogenesis of CMD induced by the metabolic syndrome. However PVAT research still represents a small percentage of research effort in adipobiology. Although PVAT resembles brown/beige adipocytes, it arises from a distinct vascular smooth muscle progenitor (a cell lineage yet to be confirmed independently by other investigators) showing its singularity among adipocytes and its definitive belonging to the vascular structure. Therefore, it is difficult to determine to what extent findings in brown and beige adipocytes can be applied to PVAT. The data presented in this review suggest that epigenetics may play a central role in adipogenesis, the maintenance of adipocytes phenotype in adulthood (Fig. 1) and their dysfunction during metabolic syndrome (Fig. 2). The improvements in PVAT dysfunction induced by exercise training potentially through epigenetic regulation support the promises of targeting PVAT epigenetic program in preventing and managing CMD associated with the metabolic syndrome.

Figure 2. Hypothetical model of perivascular adipose tissue (PVAT) phenotype regulation in health and during the metabolic syndrome. In this model hyperglycaemia, dyslipidaemia and inflammation induce a "whitening" of the PVAT by epigenetic repression of their thermogenic programme. The resulting dysfunctional PVAT then favour vascular hypertension, inflammation and remodelling through the release of adipose-derived constricting factor (ADCF), nerve growth factor (NGF), cytokines and other growth factors. However the beneficial effect of physical activity (exercise training) on PVAT dysfunction suggest that other known inducers of BAT such as cold and β -adrenergic stimulation may also induce a PVAT "re-browning" and the restoration of a protective vascular function. * Not applicable to mesenteric PVAT.

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