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MicroRNAs and downstream Adipokines regulated by Adiponectin in vivo are novel targets for controlling adipose tissue inflammation

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Table of contents

List of Abbreviations

ABCA1	Cholesterol transporter ATP-binding cassette A1
ADAM	Disintegrin and metalloprotease
AdipoR	Adiponectin Receptor
Ago	Argonaute protein
AMPK	AMP-activated kinase
aP2	Adipocyte protein 2
AP-2β	Activating enhancer-binding protein
APPL1	Leucine zipper motif
ARE	AU-rich element
AT	Adipose tissue
ATF6	Activating transcription factor-6
BDNF	Brain-derived neurotrophic factor
BMI	Body Mass Index
BMP	Bone morphogenetic protein
CBP	CREB-binding protein
C/EBP	CCAAT Enhancer Binding Protein
CAM	Cell Adhesion Molecules
cAMP	Cyclic adenosine monophosphate
DsbA-L	Disulfide-bond A oxidoreductase-like protein
CORS-26	Collagenous repeat containing sequence of 26 kDa protein
CREB	c-AMP-Response-Element-Binding protein
CRP	C reactive protein
CTRP-3	C1q/TNF-related protein-3
eIF2α	Eukaryotic Initiation Factor 2 alpha
eNOS	Endothelial Nitric Oxide synthases
ECM	Extracellular matrix
ER	Endoplasmic Reticulum
ERK	Extra-cellular signal Regulated Kinase
Ero 1-Lα	ER oxidoreductase 1-Lα
FATP-1	Fatty acid transporter-1
FGF	Fibroblast growth factors

FFA	Free fatty acids
FOXO1	Forkhead box protein O1
GCSF	Granulocyte colony-stimulating factors
GMCSF	Granulocyte-macrophage colony-stimulating factors
GLUT	Glucose transporter
GPCR	G-protein coupled protein
hASCs	Human adipose-derived stem cells
HbA1C	hemoglobin A1c
HDL	High density lipoprotein
HIF-1α	Hypoxia Inducible Factor 1-α
HMGA	Cyclin-dependent kinase 4 inhibitor A
HMW	High Molecular Weight
HO-1	Heme oxygenase-1
ICAM	Intercellular Adhesion Molecule
IGF	Insulin-like growth factor
IGFBP3	IGF-binding protein 3
iNOS	Inducible Nitric Oxide synthases
IKK	IκB kinase
IL-1Rα	Interleukin-1 receptor alpha
IRE-1α	Inositol-Requiring Enzyme-1 alpha
JAKs	Janus kinases
JNK	JunN-terminal Kinase
LMW	Low Molecular Weight
LPL	Lipoproteinlipase
LPS	Lipopolysaccharide
LRH-RE	Liver Receptor Homolog-1
MAPK	Mitogen-activated protein kinase
MCP	Monocyte Chemoattractant Protein
MIF	Macrophage migration inhibitory factor
MMPs	Matrix metalloproteinases
MMW	Medium Molecular Weight
MS	Metabolic Syndrome

MSCs	multipotent mesenchymal stem cells
NEFA	Non-esterified free fatty acids
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear Factor of Activated T-cells
PAI-1	Plasminogen activator inhibitor-1
PERK	PKR-like Eukaryotic initiation factor 2α Kinase
PI3K	Phosphatidylinositol 3-kinases
PIGF	Placental growth factor
PPAR	Peroxisome Proliferator-Activated Receptors
PPRE	PPAR-Responsive Element
RANTES	Regulated upon Activation Normal T-cell Expressed and Secreted
RISC	RNA-induced silencing complex
ROS	Reactive Oxygen Species
SRE	Sterol-Regulatory Element
SREBP	Sterol-Regulatory Element Binding Protein
SVC	Stromal Vascular Cells
Th cells	T helper cells
TG	Triglycerides
TGF-β	Transforming growth factor beta
TIMP	Tissue Inhibitor of Matrix Metalloproteinase
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-alpha
TPO	Thrombopoeitin
Treg cells	Regulatory T cells
TZD	Thiazolidinediones
UCP	Uncoupling protein
UPR	Unfoulded-Protein Response
UTR	Untranslated region
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular endothelial growth factor
Wnt	wingless-related MMTV integration site

Chapter 1: Introduction

I. Obesity, inflammation and metabolic syndrome

1. Obesity

The incidence of obesity defined as body mass index (BMI) ≥ 30 kg/ m^2 has increased drastically worldwide during recent decades. According to the World Health Organization, more than 1 billion adults (~15% of the world population) are overweight (BMI >25) and over 300 million rank as truly obese (BMI ≥ 30), and these numbers are expected to increase by more than half again by the year 2025 (1). Obesity is a major cause of morbidity and mortality, associated with many health problems, including increased risk of insulin resistance, type 2 diabetes, fatty liver disease, atherosclerosis, and certain forms of cancer (Figure 1). Consequently, obesity and associated disorders now constitute a serious threat to global human health and welfare.

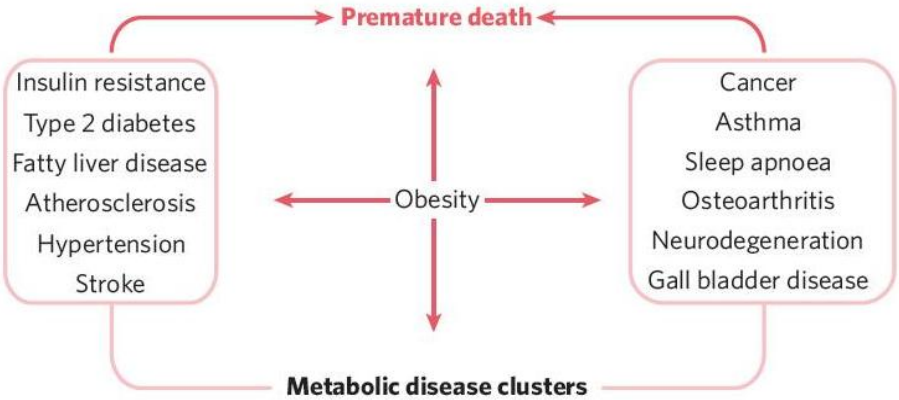

Figure 1: Clustering of metabolic diseases. Obesity is considered to be a central feature that increases the risk for a vast array of diseases, with significant morbidity and mortality. In general, the mechanistic basis of the link between obesity and the diseases listed on the right is poorly understood compared with that of those listed on the left (2).

Obesity is characterized by excessive expansion of adipose tissue (AT). White AT is mainly composed of adipocytes, the other part corresponds to the stromal-vascular fraction, which contains preadipocytes, endothelial cells, fibroblasts, macrophages, monocytes, and lymphocytes. Although AT's principal function deals with energy storage, it serves as an active secretory organ as well. A number of bioactive peptides or proteins, collectively named "adipokines", are produced and secreted by fat and/or non-fat cells of white AT (3; 4). They act in an autocrine/paracrine manner to regulate local AT function, and also act in an endocrine manner to influence the functions of distant tissues such as liver, skeletal muscle, cardiovascular and central nervous systems (4).

White AT is distributed throughout the body in a variety of locations including subcutaneous depots and visceral depots (5). It has been well recognized that fat distribution rather than total body fat is an important risk factor for obesity-related disorders. Individuals with excess of intra-abdominal visceral fat (central obesity) are at a greater risk of developing type 2 diabetes, dyslipidaemia, hypertension and cardiovascular disease (CVD) than those with excess of subcutaneous fat (peripheral obesity). Heterogeneity of function and responsiveness of the adipocytes from visceral and subcutaneous depots may contribute to this higher risk (6). First, visceral adipocytes are more insulin resistant than subcutaneous fat cells. This may be explained by various functional differences in these cells at the level of the insulin receptor and the post-receptor insulin signaling cascade. Second, visceral AT has higher lipolytic rate than subcutaneous AT due to higher β-adrenoceptor responsiveness and lower α2-adrenoceptor responsiveness, which results in excessive release of free fatty acids (FFA) into circulation. Elevation in circulating FFA and ectopic accumulation of FFA are highly associated with increased cardiovascular risk and systemic insulin resistance. Finally, adipocytes and resident macrophages of the visceral depot produce more pro-inflammatory cytokines like TNF-α, IL-6 and less protective adipokines like ApN (6). This depot-specific release of adipokines may also have a substantial impact on systemic glucose homeostasis, insulin resistance and vascular disorders (6). The most accurate way to measure central obesity is by magnetic resonance imaging or computer-assisted tomography scanning, but this approach is too expensive for routine use. Simple waist circumference measurements can be used. In Europe, waist circumference values greater than 94 cm in men and 80 cm in women are recomended as cutoff points for abdominal obesity. In Asia, values greater than 90 cm for men and 80 cm for women are recomended as optimal cutoffs (7).

2. Metabolic syndrome

The metabolic syndrome (MS) refers to the clustering of cardiovascular risk factors that include central obesity, hyperglycaemia, dyslipidaemia and hypertension. The ultimate importance of this cluster is to identify individuals at high risk of both type 2 diabetes and CVD. In 2005, the International Diabetes Federation (IDF) proposed a definition based on clinical criteria and designed for global application in clinical practice. This definition places emphasis on visceral obesity (measured by waist circumference) as the core feature of the syndrome (8). However in 2009, the IDF and American Heart Association/National Heart, Lung, and Blood Institute modified the definition. Both scientific associations agreed that

abdominal obesity should not be a prerequisite for diagnosis but one of the criteria. The presence of any 3 of the 5 risk factors constitutes a diagnosis of MS (Table 1) (7). The IDF consensus group had also recommended additional criteria that should be part of further research into MS, including elevated circulating inflammatory and/or thrombotic markers [C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and plasminogen activator inhibitor type 1 (PAI-1)] or reduced levels of anti-inflammatory molecules like ApN (8; 9). This syndrome has become one of the major public-health challenges. In the San Antonio Heart Study performed in ~2.600 individuals (age range 25–64 years), 25–40% participants have the MS. In a European Study, 30% of an Italian cohort (n = 3000; age range ~55 years) also have the MS (4).

Table 1: Metabolic syndrome definition (7)

Measure	Categorical Cut Points
Elevated waist circumference*	Population- and country-specific definitions
Elevated triglycerides (drug treatment for elevated triglycerides is an alternate indicator†)	≥150 mg/dL (1.7 mmol/L)
Reduced HDL-C (drug treatment for reduced HDL-C is an alternate indicator†)	<40 mg/dL (1.0 mmol/L) in males; <50 mg/dL (1.3 mmol/L) in females
Elevated blood pressure (antihypertensive drug treatment in a patient with a history of hypertension is an alternate indicator)	Systolic ≥130 and/or diastolic ≥85 mm Hg
Elevated fasting glucose‡ (drug treatment of elevated glucose is an alternate indicator)	≥100 mg/dL

HDL-c indicates high-density lipoprotein cholesterol

3. Obesity-induced inflammation as a mechanism linking obesity to metabolic syndrome

During the past decade, it became clear that inflammation is a key feature of obesity (2). The inflammatory response triggered by obesity involves many components of the classical inflammatory response to pathogens and includes increases in circulating inflammatory cytokines and acute phase proteins (e.g. C-reactive protein), recruitment of leukocytes to

inflamed tissues, activation of tissue leukocytes, and generation of reparative tissue responses (e.g. fibrosis). However, the nature of obesity-induced inflammation, referred to as "metainflammation" (metabolically triggered inflammation), is unique compared with other inflammatory paradigms (e.g. infection and autoimmune disease) in several key aspects. First, the chronic nature of obesity produces a low-grade activation of the innate immune system that affects metabolic homeostasis over time. Second, childhood obesity may place individuals at risk for lifelong inflammation, since inflammatory markers are elevated in obese children as young as 3 years old. Third, this chronic inflammation is composed of recurrent acute episodes of nutrition-related immune activation induced by nutrient availability (fasting or high-fat meals). This fluctuation may be associated with the induction of pro- or anti-inflammatory mediators. Finally, obesity-induced chronic inflammation is unique by its multi-organ involvement (10).

Growing evidence implicates obesity-induced inflammation as an important mechanism linking obesity to the MS in metabolically active organs. Assessment of gene expression networks in obese AT has identified a robust pattern of overexpressed inflammatory genes associated with metabolic disease (11; 12). Multiple inflammatory mediators abnormally secreted by AT and the crosstalk between immune and metabolic cells can impair insulin signaling and induce oxidative stress and endothelial dysfunction, leading to systemic insulin resistance and cardiovascular disease (4). Deregulated macrophage-myocyte and macrophage-hepatocyte signaling can impair insulin sensitivity as well (13; 14). Hypothalamic inflammation, which is induced very rapidly by a high-fat diet (15) may provoke hyperphagia and has been documented to impair insulin release from β cells, peripheral insulin action, and potentiate hypertension (16-18). Thus, chronic excess of nutrients, such as lipids and glucose may simultaneously trigger inflammatory responses, which further disrupt metabolic function, enhancing stress and inflammation. Such a vicious cycle is identified as a mechanism leading to further metabolic deterioration. Therefore, breaking this vicious circle by resetting the immunological balance in obesity is a crucial approach for the management of MS.

4. Insights into anti-inflammatory therapies

Several drugs in current clinical practice have been shown to have anti-inflammatory properties.

One of these is the agonists of the peroxisome proliferator-activated receptor (PPAR) family. A growing number of studies strongly support the anti-inflammatory properties of PPARs including PPARα and PPARγ. They are ligand-activated transcription factors which regulate gene expression by binding with their heterodimeric partner retinoid X receptor (RXR) to specific PPAR-response elements (PPREs). PPARα reduces hepatic fat accumulation mainly by stimulating fatty acid oxidation. It can also downregulate inflammatory gene expression in the liver by interacting several proinflammatory transcription factors including signal transducer and activator of transcription (STAT), activator protein-1(AP-1), and NF-κB. The synthetic PPARα agonists Fibrates are widely used for the treatment of dyslipidemia. They can also effectively reduce circulating levels of TNF-α, IL-6, fibrinogen and CRP, as demonstrated by several clinical trials in type 2 diabetic or hyperlipidemic patients (19; 20). PPARγ is indispensable for adipogenesis and lipogenesis in AT, PPARγ is also involved in governing the AT inflammatory response through two different molecular mechanisms: (a) interacting with proinflammatory transcription factors including STAT, NF-κB, and AP-1, and (b) preventing removal of co-repressor complexes from gene promoter regions (20; 21). The PPARγ agonist thiazolidinedione (TZD) class reduces markers of inflammation besides their primary insulin-sensitizing action. The mechanisms for the anti-inflammatory effects of TZD include PPARγ-independent activation of the glucocorticoid receptor (22) and PPARγ-dependent transrepression of inflammatory genes (21; 23).

Members of HMG CoA reductase inhibitors (commonly referred to as statins) have also been recognized for their ability to reduce CRP (24). However, some recent trials revealed an increased risk of new-onset type 2 diabetes for the use of statins (25).

Recent studies also suggest that the nonacetylated salicylates, a class of non-steroidal anti-inflammatory drugs may have promising glucose-lowering effects due to NF-κB inhibition (26). The clinical trial TINSAL-T2D (Targeting Inflammation Using Salsalate in Type 2 Diabetes) further confirmed that salicylates are an effective and relatively safe treatment for insulin resistance in humans (27). New trials with more patients and longer exposure times are now under way.

Finally, some other potential therapeutic approaches have also been investigated to control obesity-induced inflammation. Although there have been some encouraging results by targeting the cytokines, chemokines or their receptors (e.g., the anti-TNF or anti-CCR2 (chemokine (C–C motif) receptor 2) therapies), it is likely that the benefits of targeting a

single cytokine or signaling receptor are limited (2). For example, short-term TNF-α blockade with etanercept had a significant beneficial effect on systemic inflammatory markers in obese subjects, but did not improve vascular or metabolic insulin sensitivity (28; 29).

Because several inflammatory mediators associated with metabolic disorders are secreted by AT, reducing chronic inflammation of AT could be a primary goal of pharmacological intervention. In the next section, I shall therefore focus on the pathogenesis of AT inflammation.

II. Adipose tissue inflammation

1. Adipokine dysregulation in obesity

Numerous adipokines including pro-inflammatory cytokines, chemokines, growth factors, acute-phase proteins and complement-like factors secreted by adipocytes or nonadipocytes point to a fundamental role of AT in the pathogenesis of the MS (4) (Figure 2). Virtually all known adipokines are deregulated via multiple mechanisms triggered by obesity, type 2 diabetes or MS. Many adipokines implicated in the pathogenesis of inflammation and insulin resistance are overproduced with increasing adiposity, thereby promoting metabolic complications. On the contrary, some adipokines with anti-inflammatory or insulin-sensitizing properties, such as adiponectin, are decreased. Such a deregulation ultimately shifts the immune balance in AT toward a pro-inflammatory phenotype and further exacerbate metabolic function (4). Herein, I shall introduce the representative adipokines implicated in the pathogenesis of the MS. These adipokines are classified into "deleterious" or "beneficial" ones depending on whether their major net effect is to promote or slow down the development of obesity related complications.

It is important to note that the actions of most adipokines are well established in rodents while their roles in humans are not so directly proven and may be distinct due to differences between species. For example, recombinant leptin increases energy expenditure in *ob/ob* (lacking leptin) mice, but has no such an effect in leptin-deficient humans (30). Resistin was originally described as an adipocyte-secreted peptide that induced insulin resistance in rodents. However, human resistin is predominantly expressed in bone marrow and it serves as a pro-inflammatory mediator rather than an insulin resistance inducer in human (31).

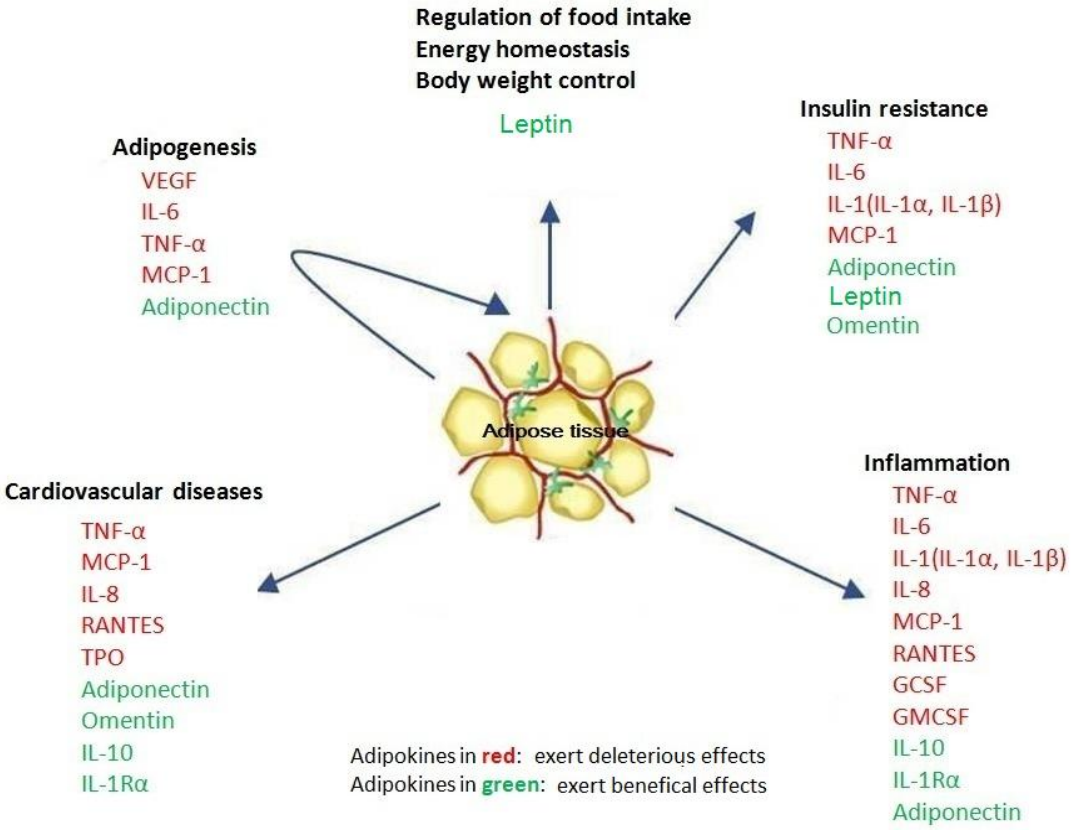

Figure 2: Adipose tissue is a highly active and regulated secretory organ. White adipose tissue (AT) produces a large variety of adipokines regulating metabolism and inflammation and contributing to the pathogenesis of obesity-related metabolic and vascular complications.

1.1 Deleterious adipokines

1.1.1 Pro-inflammatory cytokines

Tumor necrosis factor-α (TNF-α) Circulating levels of TNF-α are elevated in obese subjects and fall after weight loss (32). TNF-α gene is also overexpressed in AT of obese mice and humans (33; 34). Increased TNF-α in obese AT has provided the first clear link between obesity, chronic inflammation and insulin resistance. Mice lacking TNF-α or TNF-α receptors are resistant to the development of diabetes, while neutralization of TNF in rodents reduces the severity of insulin resistance (35). The molecular mechanisms underlying TNF-α induced insulin resistance involve two transcription factor-signaling pathways: the nuclear factor-κB (NF-κB) pathway and the c-Jun NH2-terminal kinase (JNK) pathway. NF-κB activation induces transcription of various pro-inflammatory markers and mediators that can cause insulin resistance. JNK activation promotes phosphorylation of insulin receptor substrate 1 (IRS-1) at serine sites that inhibit normal tyrosine phosphorylation of IRS-1 and downstream insulin signaling (36; 37). The other effects of TNF-α on adipocytes include increased lipolysis, increased leptin secretion (3), decreased adiponectin secretion (38) and antagonism

of TZDs/PPAR-γ (39). TNF-α also contributes to cardiovascular disease by inducing an increased expression of adhesion molecules in vascular wall, increasing the scavenger receptor class A expression and oxidized LDL uptake in macrophages and stimulating their infiltration in vascular wall (40).

Interleukin-6 (IL-6) It has been estimated that white AT contributes to ~25 % of the circulating IL-6 in humans (41). During LPS-induced systemic inflammation in mice, adipose tissue is the major source of systemic IL-6 (42). As described for TNF-α, circulating levels and AT production of IL-6 are increased in obesity (43). IL-6 is a major stimulator of acute-phase protein response in liver and AT during inflammation (44). Increased production of IL-6 by AT induces hepatic insulin resistance (36). This hepatic insulin resistance could be mediated, in part, by the increased expression of suppressor of cytokine signaling (SOCS)-3, a protein that binds and inhibits the insulin receptor and also targets IRS proteins for proteosomal degradation and negatively regulates the IRS-1 transcription (36). Finally, IL-6 also induces fatty acid oxidation and lipolysis (45).

Interleukin-1 (IL-1α, IL-1β) IL-1α and IL-1β are pro-inflammatory cytokines that exert similar but not redundant functions. They induce acute-phase proteins, proliferation of different cell types (fibroblasts, smooth muscle and mesangial cells) and secretion of other pro-inflammatory cytokines. IL-1α-knockout mice have lower fasting glucose and insulin levels and improved insulin sensitivity when compared with wild-type controls (46). Expression and secretion of IL-1β are increased in obese insulin-resistant mice and humans (47). A combined elevation of IL-1β and IL-6 levels has been reported to predict the risk for type 2 diabetes better than each cytokine alone (48). IL-1 (IL-1α, IL-1β) also have pro-angiogenic actions and direct effects on adipocytes, where they increase lipolysis and impair adipocyte differentiation through the inhibition of PPARγ (49).

1.1.2 Chemokines

Monocyte chemoattractant protein-1 (MCP-1) MCP-1, also known as chemokine (C-C motif) ligand 2 (CCL-2), is potent chemoattractant which plays a crucial role in recruiting monocytes/macrophages into the AT (4; 50). Circulating and AT levels of MCP-1 are elevated in obesity (51). MCP-1 and its receptor (CCR2) are required for macrophage infiltration into AT. When compared to wild-type mice, MCP-1-or CCR2-deficient (knockout, KO) mice fed a high-fat diet exhibited fewer macrophages and a lower inflammatory gene profile in AT together with reduced insulin resistance and hepatic steatosis (52; 53).

Treatment of 3T3-L1 differentiated adipocytes with MCP-1 decreased insulin-stimulated glucose uptake and the expression of several adipogenic genes (50). Eventually, in a large cohort of Caucasians, a polymorphism in the MCP-1 gene, which may impair MCP-1 expression at the transcriptional level, was negatively correlated with plasma MCP-1 levels and the prevalence of insulin resistance and type 2 diabetes (54). MCP-1 is also an inflammatory factor linking obesity to the development of CVD. Recruitment of monocytes/macrophages into the arterial vessel wall by MCP-1 is a major event leading to atherosclerotic lesions. Circulating MCP-1 levels were elevated in patients with coronary artery disease (55). Conversely, mice prone to develop atherosclerosis crossed with MCP-1-KO mice had a reduction in atherosclerotic lesions (56).

Regulated on activation normal T-cell expressed and secreted (RANTES) RANTES is a chemokine implicated in in cardiovascular inflammatory disorders after organ transplantation or arterial injury (57). In patients with end-stage renal disease and type 2 diabetes, RANTES (or CCL5) gene polymorphisms have also been associated with increased mortality due to cardiac events (58). Veillard *et al*. showed that antagonism of RANTES receptor reduced atherosclerotic plaque formation in a hypercholesterolemic mouse model (57). Furthermore, systemic concentrations of RANTES have been reported to be higher in individuals with impaired glucose tolerance or type 2 diabetes than in control subjects. It has therefore been suggested that this chemokine could be involved in the development of type 2 diabetes, independently of other risk factors that are classically related to the metabolic syndrome (59).

Interleukin-8 (IL-8) IL-8 (or CXCL-8) is another chemokine involved in atherogenesis. Adipocytes secrete significant amounts of IL-8, whose circulating levels are increased in human obesity (60). IL-8 is known to recruit and activate monocytes and induce the migration of different cell types including neutrophils, T-cells, basophils and smooth muscle cells in inflammatory conditions (61). Mice lacking the IL-8 receptor CXCR2, showed a significant reduction in atherosclerotic lesions and leukocyte accumulation (62).

1.1.3 Growth factors

Adipose tissue secretes growth factors, some of which have angiogenic properties such as vascular endothelial growth factor (VEGF), Granulocyte colony-stimulating factor (GCSF), Granulocyte-macrophage colony-stimulating factor (GMCSF), placenta growth factor (PlGF) and thrombopoietin (TPO), …(63; 64). They may control adipose vasculogenesis and adipocyte development. They can control immunity, blood pressure and atherosclerosis as

well (65). Localization studies have shown that omental adipocytes are the primary source of VEGF, which accounts for most of the angiogenic activity of AT (66). Inactivation of a VEGF family member (PlGF) has been shown to impair AT development by reducing angiogenesis in diet-induced obese mice (67). VEGF also acts as a pro-inflammatory cytokine by increasing the permeability of endothelial cells, inducing the expression of endothelial adhesion molecules and by acting as chemoattractant (68). GCSF and GM-CSF are potent determinants for inflammatory cell recruitment in AT (69; 70). In addition, Kim *et al.* reported GM-CSF knockout mice submitted to a high-fat diet exhibited reduced insulin resistance, as a result of decreased production of pro-inflammatory cytokines in AT (IL-1β, TNF-α, MIP-1α) (70). TPO has been reported to be oversecreted by omental AT from obese subjects (64). It stimulates the proliferation and differentiation of megakaryocytes, increasing both platelet count and size. High systemic TPO levels could participate to the pathogenesis of the acute coronary syndrome (71).

1.2 Beneficial adipokines

1.2.1 Anti-inflammatory cytokines

IL-10 IL-10 is produced by immune cells of AT and acts on adipocytes to improve insulin signaling, decreasing further macrophage recruitment. A significant positive association has been reported between circulating IL-10 levels and whole-body insulin sensitivity in young healthy individuals (72). This report is in agreement with other studies linking low IL-10 production with type 2 diabetes in an elderly population and low IL-10 levels with the metabolic syndrome and obesity (69). IL-10 treatment of 3T3-L1 adipocytes has been shown to inhibit MCP-1 secretion via signal transducer and activator of transcription-3 (STAT-3) and to reverse TNF-α-induced decreases in GLUT4 and IRS-1 tyrosine phosphorylation (73).

IL-1Ra (**Interleukin-1 receptor antagonist)** IL-1Ra is a naturally occurring cytokine limiting inflammation by competing with IL-1β for binding to its receptor. IL-1Ra is largely produced in AT in response to stress and by M2 macrophages to create an anti-inflammatory AT milieu in physiological conditions (74).

1.2.2 C1q/TNF-related protein family members

Proteins with a gC1q domain similar to that of TNF, C1q and adiponectin have been classified as members of the newly described C1q/TNF molecular superfamily (75; 76). Some of the

secreted C1q/TNF family proteins, such as adiponectin, C1q and CORS-26 (collagenous repeat containing sequence of 26 kDa protein, also known as CTRP-3), are highly expressed and secreted by AT. CORS-26 may be regarded as a potent anti-inflammatory adipokine, which can reduce LPS-induced IL-6 and TNF-α secretion from monocytes by suppressing NF-κB signaling (77). Moreover, recombinant CORS-26 stimulates ApN secretion from mature adipocytes (78). Adiponectin will further be described in Section III.

1.2.3 Omentin

Omentin is highly produced by visceral AT compared to subcutaneous AT, with a main contribution of SVC (79). Both Omentin expression in AT and circulating levels are decreased in obesity (80) and its circulating levels are negatively correlated with insulin resistance (80; 81). Omentin exerts an insulin-like action in vitro: it enhances insulin-stimulated glucose uptake in both subcutaneous and omental human adipocytes through the involvement of Akt pathway (79). Omentin has also been reported to play beneficial role in CVD by causing vasodilatation in isolated blood vessels (82) and decreasing migration and angiogenesis in human endothelial cells induced *in vitro* by VEGF and CRP (83).

1.2.4 Leptin

Leptin is secreted almost exclusively by adipocytes and its circulating levels reflect fat mass. The implication of leptin in energy homeostasis has been well described at the central level. Leptin binding to its receptors in the hypothalamus suppresses the production of "orexigenic peptides" (e.g., neuropeptide Y and agouti-related protein) while increasing those of "anorexigenic peptides" (e.g., proopiomelanocortin and cocaine-and amphetamine-regulated transcript), thereby inducing satiety (4; 84). The metabolic actions of leptin on peripheral tissues include enhanced glucose uptake and fatty acid oxidation and subsequent prevention of lipid accumulation in AT and non-adipose tissues as well. These metabolic effects of leptin are in part centrally mediated by activation of the hypothalamic–sympathetic nervous system axis (85; 86). Leptin replacement therapy in subjects with congenital complete leptin deficiency or in subjects with partial leptin deficiency (e.g., lipoatrophy, hypothalamic amenorrhea) reverses the metabolic and endocrine abnormalities associated with these conditions. In contrast, leptin's effects are largely absent in the obese hyperleptinemic state, probably due to leptin resistance (87). In addition to its role in energy homeostasis, leptin has an important immunomodulatory role. Leptin is involved in development, proliferation and activation of immune cells. High levels of leptin induce inflammation by activating pro-inflammatory cells, promoting T-helper 1 (Th1) responses and mediating the production of

other pro-inflammatory cytokines in mice (88). On the contrary, leptin deficiency may impair immune response, leading to increased susceptibility to infections. Immune deficiencies have been observed in both *ob/ob* (lacking leptin) and *db/db* (lacking leptin receptor) mice, as well in patients with leptin mutations and in malnourished children, which have low plasma leptin (87; 88).

2. Adipokine receptors and signal transduction

AT not only secretes multiple adipokines, but also responds to immunological signals by expressing a wide variety of receptors for cytokines, chemokines, complement factors and growth factors (89). Some receptors are also dysregulated in obesity, which together with their adipokine ligands contribute to pathogenesis of MS. It has been demonstrated that human adipocytes express leptin receptors, both the long form (huOb-R) and the short forms (90). Two structurally distinct TNF-α receptors, TNFR1 and TNFR2 have been identified in AT. Most biological effects of TNF-α such as lipolysis and insulin resistance are mediated by TNFR1 but not TNFR2 (34; 91). The soluble IL-6 receptor (sIL-6R), ligand binding IL-6R (IL-6Rα) and its signal transduction component, glycoprotein 130 (gp130) are expressed in adipocytes (92; 93). Long-term combined treatment with IL-6/sIL-6R gradually suppresses ApN release from human adipocytes. IL-6 alone is unable to influence ApN synthesis, and the presence of sIL-6R is necessary to disclose IL-6 bioactivity in human adipocytes (94). The gp130 is a common signal transducer of IL-6 family, which typically activates STAT3 via the Janus kinase (JAK). gp130-mediated signaling is involved in adipocyte differentiation and function (95). One receptor of VEGF, VEGFR1 mediates the increased angiogenic action of PlGF in AT of obese mice (67), while blockade of the other receptor, VEGFR2 limits diet-induced expansion of AT in mice (96). The adiponectin receptors will be described in the next section.

The signaling pathways, which are activated by the adipokines in AT include those which are commonly involved in inflammation such as the NF-κB, JNK and JAK/STAT pathways. Activation of NF-κB and JNK signaling pathways is mainly responsible for insulin resistance (97).

3. White adipose tissue remodeling during obesity

In obesity, white AT is remodeled dynamically by adipocyte hypertrophy (increased size), hyperplasia (increased number), macrophage infiltration, endothelial cell proliferation and fibrosis (64; 98-100). This remodeling contributes to low-grade inflammatory state in white AT.

3.1 Adipocyte changes

Expansion of adipose tissue mass is a major characteristic of obesity. Enlarged fat cells (hypertrophy) is thought to be the most important determinant whereby fat depots increase in adults (101). Adipocyte hypertrophy, which commonly occurs in obese state at relatively early stage, has been speculated to initiate AT inflammation by triggering initial secretion of critical monocyte chemotactic factors (102). Moreover, adipocyte hypertrophy results in altered production of some adipokines which adversely affect the remodeling of AT (103; 104). Increased adipocyte number (hyperplasia) also contributes to fat mass expansion. Obese subjects have higher total adipocyte number than lean individuals (101). However, Spalding *et al*. have shown that the increased adipocyte number of obese subjects is set during childhood and adolescence and stays constant in adulthood. Any alterations in fat mass during adulthood are merely credited to alterations in adipocyte hypertrophy (101). Obesity is also associated with a higher adipocyte turnover due to an elevated rate of cell death, which is a prominent phagocytic stimulus for macrophage infiltration (105; 106). Increased adipocyte death is accompanied by a subsequent increased emergence of smaller adipocytes, thus restoring the adipocyte number (106). One speculation is that adipocyte hypertrophy, which commonly occurs in obese state at relatively early stage, may initiate AT inflammation by triggering macrophage infiltration into AT. Once macrophages infiltrate into AT and get activated, they become the major source of inflammatory molecules and continue to attract more macrophages to further amplify the harmful cycle (102). However, recent study proposed that B cells may infiltrate AT very early in response to high-fat feeding and modulate T cells which may contribute to the recruitment, differentiation and activation of macrophages.

3.2 Macrophage and lymphocyte infiltration

Macrophages recruited into obese AT are responsible for the majority of AT proinflammatory cytokine and chemokine secretion. Eventually, a positive feedback cycle is formed whereby infiltrating macrophages recruit and sustain the presence of new macrophages, thus resulting in further propagation of inflammation (2). Increased levels of macrophage markers are

observed in AT of obese mouse models (107). Large clusters of macrophage-related inflammatory genes are positively correlated with body mass in human and mice (53). Macrophage infiltration is reduced after surgery-induced weight loss in morbidly obese subjects (69). The majority of macrophages in obese AT aggregates in "crown-like structures", surrounding dead (necrotic-like) adipocytes and scavenging adipocyte debris (69; 105).

Besides increasing macrophage number, obesity also induces a phenotypic switch in AT macrophage polarization. Macrophages migrating to mouse AT upon high fat feeding differ from those that reside under normal diet conditions (73). Resident or M2 ('alternatively activated') macrophages are characterized by the expression of the chitinase-like protein Ym1, Arginase (an enzyme that blocks iNOS activity) and the production of anti-inflammatory cytokines (e.g. IL-10, IL-1Rα), while recruited or M1 ('classically activated') macrophages express the CD11c surface marker, iNOS and produce high levels of pro-inflammatory cytokines (e.g. TNF-α, IL-6) (108). Diet-induced obesity leads to a shift in the activation state of AT macrophages from an M2-polarized state in lean animals that may protect adipocytes from inflammation to an M1 proinflammatory state. This shift in the M2/M1 balance is due to the migration of inflammatory monocytes from the circulation to macrophage clusters surrounding dead adipocytes, and not to the conversion of resident M2 macrophages (109). However, obesity does not induce a strict M1 or M2 polarization in human AT (98; 110).

In addition to macrophages, recent studies have revealed the infiltration of other immune cells that may orchestrate AT remodeling. These include subsets of the lymphocyte lineage, including CD8+ and CD4+ T cells, Tregs, Natural killer T cells (NKT) and B cells. Early lymphocyte accumulation in AT of diet-induced obesity (DIO) mice may precede macrophage infiltration and insulin resistance. Interestingly, a few studies have reported that B cell infiltration into AT in response to high-fat diet, is earlier than T cell or macrophage infiltration and prior to the appearance of insulin resistance (111).

3.3 Endothelial cell activation

In obesity, endothelial cells are highly activated by macrophages and adipocyte secretory products (adipokines) to form new vascular network which is crucial for pathogenic adipogenesis and further expansion of AT. In humans, the proportion of endothelial cells (Vv in % adipose tissue) is 10-fold higher in omental AT from obese (average BMI ~41kg/m^2) than from lean subjects (64). Activation of endothelial cells results in intracellular signaling pathways leading to the production, in a coordinated regulated fashion, of a series of cell

adhesion molecules (CAMs), as well as chemokines and cytokines, which guide leucocytes into underlying tissues and co-stimulate them to become fully activated (112). For example, circulating levels of soluble ICAM-1 (intracellular adhesion-1), VCAM-1 (vascular cell adhesion molecule-1) and endothelial cell selective E-selectin are increased in obese adults and decreased after weight loss (113). Moreover, angiogenesis and adipogenesis are functionally linked. In animal models of genetic or induced obesity, the expansion of AT is associated with active angiogenesis, whereas inhibition of angiogenesis prevents AT development (114; 115).

3.4 Extracellular matrix overproduction

During AT hypertrophy, the extracellular matrix (ECM) is overproduced to accommodate the excessive growth and limit further expansion. Up-regulated ECM components, including collagen types (notably fibrillar collagens I and III) and glycoproteins (integrins, laminin, fibronectin, elastins) lead to AT fibrosis, a characteristic of inflamed tissue. This may ultimately impair cell-cell contact and adipocyte biology. Histological examination of subcutaneous AT has shown that the percentage of fibrosis was increased by ~3-fold in obese subjects compared to lean ones and remained high three months after bariatric surgery, in spite of the regression of the local inflammatory phenomena. This may indicate some degree of irreversibility in the evolution of obesity (100). The excessive stiffness of ECM in obese AT are mediated by fibrinolytic plasminogen, plasmin, matrix metalloproteinase (MMP), a disintegrin and metalloprotease (ADAM), and tissue inhibitors of MMP (TIMP) systems. Plasminogen activator inhibitor-1 (PAI-1) is highly expressed in obese AT (116). MMPs are over-expressed in AT during obesity with increased matrix degradation (increased plasticity) (117; 118). In addition, the cysteine proteases, Cathepsins, may also contribute to the remodeling process through their proteolytic activity toward extracellular elastins and collagens. Expression of adipose Cathepsin K and S is increased in obesity (119; 120) and decreases after weight loss (121).

4. Mechanisms leading to adipose tissue inflammation in obesity

AT remodeling that is pathologically accelerated in the obese state may trigger metabolic and hypoxic stress, leading to activation of multiple inflammatory signaling pathways (Figure 3) (4). Herein, I am going to highlight several prominent mechanisms that coordinately lead to AT inflammation.

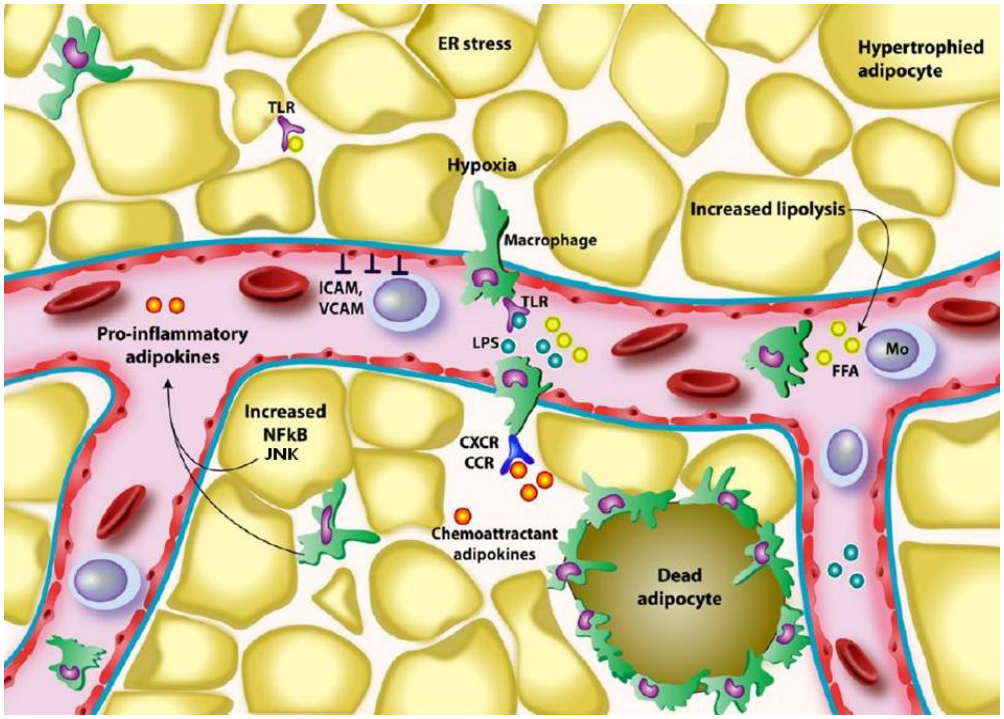

Figure 3: Scheme for the mechanisms leading to obesity-induced inflammation. Adipocyte hypertrophy initiates a state of cellular stress (hypoxia and ER stress), resulting in upregulation of pro-inflammatory adipokines. Some of these are chemokines that bind to specific receptors (CCR and CXCR) of monocytes (Mo)-macrophages to recruit them into AT. Increased pro-inflammatory adipokines also upregulate the expression of endothelial adhesion molecules (e.g., ICAM-1 and VCAM-1), that bind integrins of monocytes to further recruit them. In turn, these activated macrophages secrete pro-inflammatory adipokines. Additionally, increased lipolysis by hypertrophied adipocytes and metabolic endotoxemia (increased circulating LPS) contribute to inflammation. Saturated FFA and LPS bind to the TLR4 on the surface of both adipocytes and macrophages and further activate NF-κB and JNK signaling. Local inflammation worsens and propagates systemically via adipokines (4; 37).

4.1 Hypoxia

In obesity, the growing AT is submitted to hypoxia induced by hypoperfusion at the earliest stages of expansion. The reduced blood flow velocity per vessel seems to be the main cause of hypoxia rather than the enlarged cells, because in obese animals, hypoxia has been detected in small as well as in large adipocytes (122). Observations have proved that AT is poorly oxygenated in the obese state in humans and rodents (123; 124). This hypoxia could be a determinant mechanism to trigger obesity-induced inflammation in AT by altering expression of many adipokines. In several models of cultured adipocytes, hypoxia decreased mRNA levels of ApN, while increasing those of pro-inflammatory genes［Plasminogen activator inhibitor-1 (PAI-1), TNF-α, IL-1, IL-6, Macrophage migration inhibitory factor (MIF), MCP-1, Transforming growth factor β (TGF-β), leptin ...］together with those of hypoxia response genes［Hypoxia Inducible Factor 1-α (HIF-1α), Glucose transporter (GLUT-1), VEGF)］(122; 125; 126). In addition, some studies reported that hypoxia also up-regulates ECM

components including elastin, collagens I and III, as well as matrix metalloproteinases MMP2, MMP9 and TIMP1, thereby causing local fibrosis (127; 128). Thus, hypoxia-induced fibrosis in white AT may be another factor that contributes to local inflammatory responses. The molecular mechanisms underlying deregulated gene expression is related to activation of the transcription factors NF-κB, c-AMP-Response-Element-Binding protein (CREB) and HIF-1α, and to ER stress and posttranscriptional alterations (122; 125).

4.2 Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is responsible for much of a cell's protein synthesis and folding, but it has also a role in sensing cellular stress (129). Obesity results in conditions that increase the demand on the ER, such as hypoxia, excess of cytokines, lipids or glucose. Subsequently, the ER stress induces a complex response known as the unfolded protein response (UPR), which alters a cell's transcriptional and translational programs to cope with these stressful conditions and resolve the protein-folding defect (129). The UPR functions through three branches, denoted for the three stress-sensing proteins found in the ER membrane: PKR-like eukaryotic initiation factor 2a kinase (PERK), inositol-requiring enzyme-1 (IRE-1), and activating transcription factor-6 (ATF-6).

ER stress and the UPR lead to obesity-induced inflammation and metabolic abnormalities by several distinct mechanisms, including the activation of JNK-AP1 (activator protein 1) and IKK-NFκB pathways, induction of the acute-phase response, and the production of reactive oxygen species (ROS) (129). JNK activation by IRE-1α during ER stress is one key pathway to increase inflammation. IRE-1α can also activate the IKK-NF-κB inflammatory signaling pathway. The NF-κB pathway may also be activated through PERK signaling during the UPR. PERK-mediated phosphorylation of eIF2α inhibits the translation of IκB, a major negative regulator of NF-κB, thereby allowing the activation of NF-κB. Moreover, prolonged activation of the UPR may also generate oxidative stress, causing a toxic accumulation of ROS within the cell. This occurs as a result of the UPR-stimulated up-regulation of chaperone proteins involved in disulfide bond formation in the ER lumen. The enzymes responsible for forming disulfide bonds (Ero1p and Erv2p) perform oxidative reduction reactions that use molecular oxygen as the final electron recipient (130). This reduced molecular oxygen accumulates during UPR-increased protein folding and acts as cellular toxic ROS (131). It is well known that toxic ROS levels may elicit inflammatory response, thus making another connection between UPR activation and inflammation.

4.3 Lipotoxicity

Obesity is characterized by a positive energy balance, and the classical response to this nutrient oversupply is AT hypertrophy. However, the capacity for lipid storage in hypertrophied subcutaneous AT is limited. This limited storage capacity, coupled with the overstimulation of hormone-sensitive lipase, leads to massive increase in free fatty acid (FFA) release. The resulting spill-over of FFAs is the starting point of lipid deposition in visceral AT and non-adipose tissue. This metabolic toxicity is associated with increased pro-inflammatory cytokine production, which can induce the pro-inflammatory state. FFAs are potent ligands that activate Toll-like receptor (TLR) signaling (132). TLRs are a family of pattern-recognition receptors that play a critical role in the innate immune system by activating pro-inflammatory signaling pathways in response to microbial pathogens. AT expresses a broad spectrum of TLRs. In human AT, all TLR subtypes (TLR1–9) are expressed (133-135). TLR expression in AT is inducible by inflammatory stimulation and linked to downstream activation of NF-κB or JNK and subsequent release of pro-inflammatory cytokines and chemokines (Figure 4) (78; 133; 134). TLR4 has been proposed as a molecular link between lipids, inflammation and insulin resistance (136). TLR4-deficient mice and C3H/HeJ mice (which have a loss-of-function mutation in TLR4) are partially protected from fat-induced inflammation and insulin resistance in their visceral AT (136-139). Excess of FFAs treatment in adipocytes induces NF-κB-driven proinflammatory response via activation of TLR2 and TLR4 (140). In addition, activation of both TLR2 and TLR4 can mediate FFA-induced activation of JNK in adipose tissue of obese individuals (141). Stimulation of TLR1, TLR2/TLR6, TLR3 and TLR4 with their specific lipid ligands induces the expression of IL-6 and MCP-1 in murine adipocytes (133; 142). FFAs also increase the infiltration and activation of macrophages, especially the CD11c+ subset, thereby exacerbating their pro-inflammatory activity (143).

4.4 Metabolic endotoxemia

Excess dietary fat can cause moderate increases in plasma Lipopolysaccharide (LPS) concentrations. High-fat diet given to mice chronically increased plasma LPS concentration two to three times, a threshold that has been defined as metabolic endotoxemia (144). Obese subjects with type 2 diabetes have 76% higher circulating LPS than healthy controls (135) and this high level of LPS decreased significantly after surgical weight loss (145). Elevated plasma LPS levels result from increased absorption of LPS across the intestinal barrier triggered by high-fat diet. Recently, three underlying mechanisms have been proposed. First,

changes in gut microbiota environment. A high-fat diet changes gut microbiota toward a decreased number of bifidobacteria, a group of bacteria which has been shown to reduce intestinal LPS levels in mice and to improve the mucosal barrier function (144). Second, increased availability of chylomicrons. Because LPS has affinity for chylomicrons, chylomicron formation induced by long-chain fatty acids promotes LPS absorption (146). Third, increased permeability of the gut epithelium. Recent data has shown that obese and diabetic mice displayed impaired intestinal integrity and reduced expression of genes coding for proteins of the tight junctions such as ZO-1 and Occludin (147). Decreased response of glucagon-like peptide-2 (GLP2), decreased number of specific bacteria (e.g., Bifidobacterium spp.), as well the increased endocannabinoid system tone in obesity may contribute to the enhanced intestinal permeability (148-150).

LPS in plasma is transported by a specific acute phase response glycoprotein, the LPS-binding protein (LBP) to target tissues. LBP is a facilitator of LPS action. In murine adipocytes, LBP-bound LPS initiates inflammation via TLR4 and induces the secretion of proinflammatory cytokines via downstream activation of NF-κB or mitogen-activated protein kinases (MAPK) signaling pathways (151) (Figure 4). In addition, LPS induces lipolysis in 3T3-L1 adipocytes via TLR4, which liberates FFA into the circulation, resulting in systemic insulin resistance (152). In human adipocytes, stimulation by LPS increases TNF-α, IL-6 and MCP-1-release by NF-κB activation, and upregulates TLR2 expression. Newly expressed TLR2 further amplifies pro-inflammatory signals in AT (135).

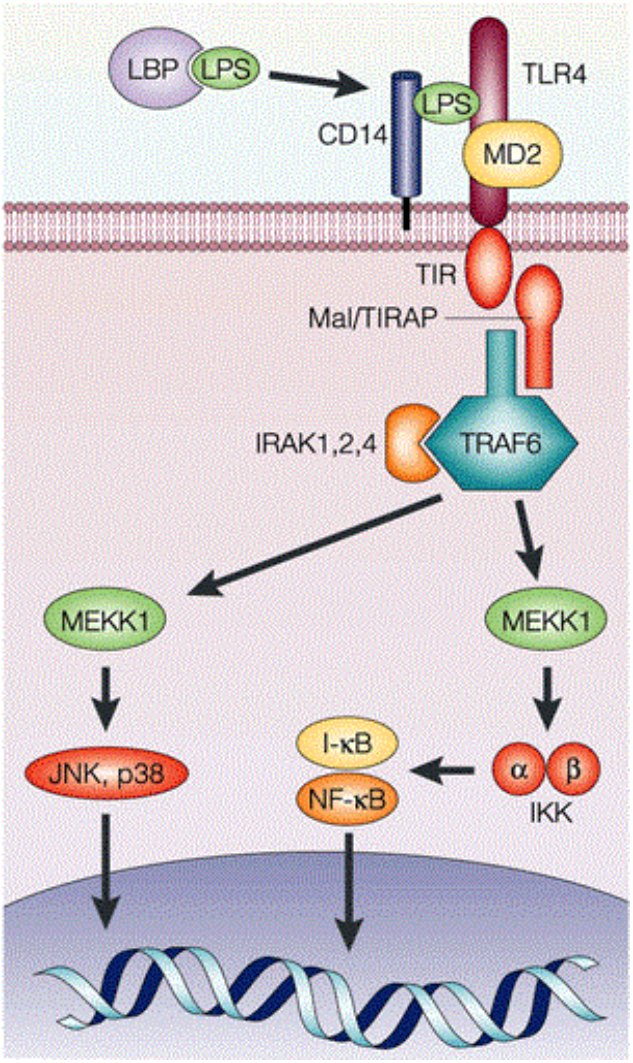

Figure 4: Signal transduction of Toll-like receptor 4 (TLR4). LPS forms a complex with LPS-binding protein (LBP), which binds to cell surface CD14. This then forms a complex with TLR4. On the cytoplasmic side of the membrane, TLR4 interacts with MyD88 (myeloid differentiation protein 88) and Mal/Tirap (myeloid differentiation protein 88 adaptor-like/Toll-IL-1-receptor-domain-containing adaptor protein), which then activates a cascade involving IRAK (IL-1 receptor associated kinase), TRAF6 (TNF-receptor-associated factor 6), and eventually NF-κB and MAPK, which result in transcriptional activation of inflammatory genes. IKK, IκB kinase; MEKK, mitogen-activated protein kinase/ERK kinase; TIR, Toll/Interleukin-1 receptor, the cytoplasmic domain of TLR-4; Mal/TIRAP, Toll-interleukin 1 receptor (TIR) domain containing adaptor protein; IRAK, Interleukin-1 receptor-associated kinase; TRAF6, TNF receptor associated factors 6 (153).

III. Adiponectin

1. Molecular structure

Adiponectin (ApN) is also named Acrp30 (adipocyte complement-related protein of 30kDa), AdipoQ (for its homology with the complement factor C1q), apM1 (adipose most abundant gene transcript 1) or GBP28 (gelatin binding protein of 28 kDa).

ApN, the most abundant adipokine, belongs structurally to the soluble defense collagen super-family sharing significant homology with collagen X, VIII and complement factor C1q. Full-length ApN protein is composed of a signal peptide, a variable N-terminal domain, followed by a collagenous domain and a C-terminal C1q-like globular domain (Figure 5). Full-length ApN can be processed by proteolytic cleavage into a smaller globular fragment (globular ApN) (Figure 5). The amino acid sequences of ApN are highly conserved, sharing over 80% identities among all the species so far (154). ApN in human or mouse serum forms a wide range of multimers including low molecular weight trimers (LMV, ～67kDa), medium molecular weight hexamers (MMV, ～150kDa) and high molecular weight oligomers (HMV,～300kDa) (Figure 5). HMW form has been shown to possess the most potent insulin-sensitizing activity (155). ApN circulates in the concentration range of ~3–30μg/ml in healthy individuals, with lower levels in males compared to females, which is mainly attributed to lower amounts of HMW form (156; 157). However, almost all ApN exists in plasma as full-length ApN, only a few globular ApN might circulate in human plasma (158).

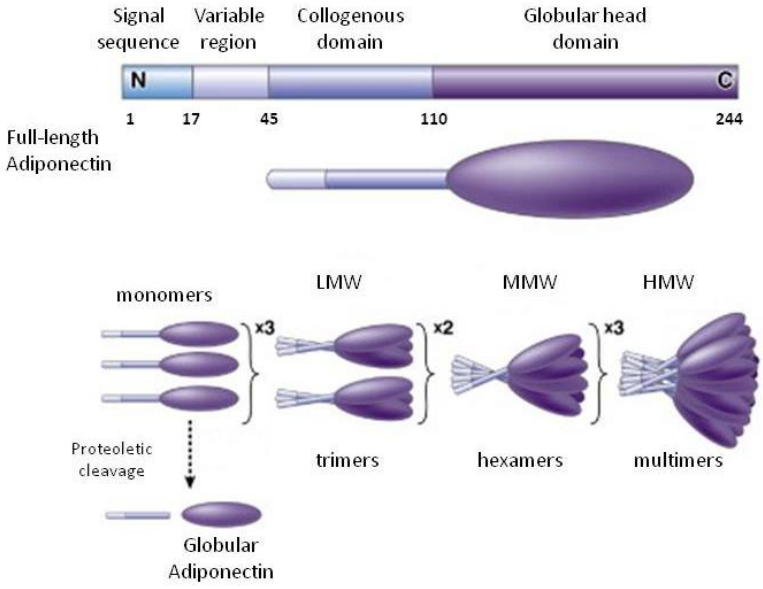

Figure 5: Schematic structure and multimerformation of adiponectin (ApN). LMW, low molecular weight; MMV, Medium molecular weight; HMW, high molecular weight. Adapted from (159)

2. Adiponectin receptors and signal transduction

2.1 Receptors

By now, 2 classical ApN receptors have been discovered and well described: adiponectin receptor 1 (AdipoR1) and receptor 2 (AdipoR2). Both AdipoR1 and AdipoR2 are surface membrane proteins with seven trans-membrane domains. Their N terminus is internal, and the C terminus is external, which is opposite to the topology of all reported G protein-coupled receptors. These two receptors are highly homologous in amino acids (67% identity). AdipoR1 is ubiquitously expressed and most abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in mouse liver. Scatchard plot analysis revealed that AdipoR1 is a high-affinity receptor for globular ApN, whereas AdipoR2 has with a relative higher affinity for full-length ApN (154) (Figure 6). Studies including our own data (160) have confirmed that most effects of ApN in muscle are mediated via AdipoR1(154; 160). Suppression of AdipoR1 with siRNA greatly reduced the stimulation of PPAR-α activity, fatty-acid oxidation and glucose uptake by globular ApN in C2C12 myocytes (161). In liver, AdipoR1 and AdipoR2 mediate different effects of ApN. Overexpression of AdipoR1 activates AMPK, thereby reducing hepatic glucose production; on the contrary, AdipoR2 predominantly activates PPAR-α signaling pathways, which induces fatty-acid oxidation. These findings have been further proved *in vivo*: AdipoR1-knockout (KO) mice have impaired glucose tolerance and insulin resistance; AdipoR2-KO mice have normal hepatic gluconeogenesis but increased insulin levels (162). Double transgenic AdipoR1/2-KO presented marked glucose intolerance and a more severe insulin resistance than the AdipoR1 or R2-KO models, together with an increased inflammation and oxidative stress (162). However, other studies on AdipoR-KO mice provided controversial data (163-165). With regard to AT, both receptors are expressed in this organ. AdipoR1 mRNA levels are approximately 10-fold higher than AdipoR2 in both cellular fractions. The presence of AdipoRs in both fractions suggests an autocrine/paracrine effect of ApN in AT. AdipoR1 expression in AT is reduced in obese subjects and is increased after weight loss (166).

In addition, Hug *et al.* isolated a third ApN receptor, T-cadherin, which binds to hexameric and high-molecular-weight species of ApN, but not to the trimeric or globular species. T-cadherin is a unique cadherin molecule that is anchored to the surface membrane, not through a trans-membrane domain but instead via a glycosylphosphatidyl inositol moiety. T-cadherin is expressed on vascular endothelial cells and smooth muscle where it is positioned to interact

with ApN (167). However, whether T-cadherin is a functional receptor for ApN or simply an ApN-binding molecule needs more evidence.

2.2 Adaptor proteins

Several ApN receptor binding proteins have now been identified. The best characterized is APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTB) domain, and leucine zipper motif) (168). APPL1 associates with both AdipoR1 and AdipoR2 and directly interacts with the N-terminal intracellular region of ApN receptors through its PTB domain (168) and sequentially activates downstream signaling (Figure 6). It has been shown that APPL1 expresses in various tissues, which mediates many effects of ApN including metabolic effects in liver, skeletal muscle (168-172) as well as cardio-protective effects in vascular endothelial cells (173; 174). In adipocytes, although the presence of APPL1 has been demonstrated, it is not established whether ApN signaling is mediated through APPL1. One study showed that APPL1 is involved in insulin-mediated Akt phosphorylation and subsequent glucose uptake in muscle and adipose tissues (175).

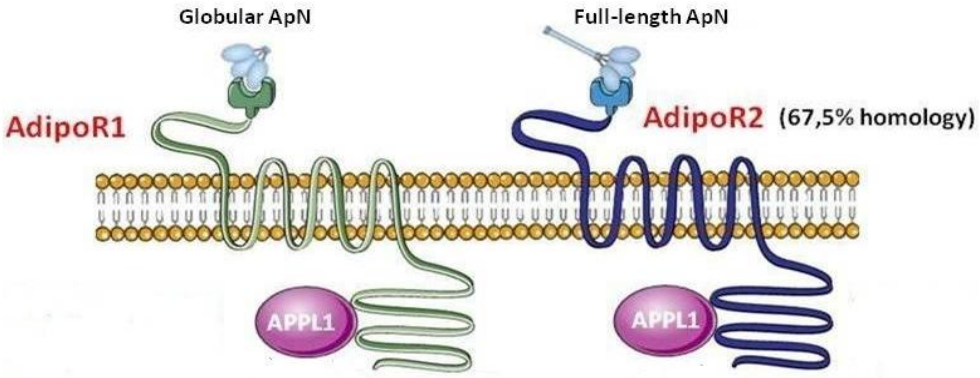

Figure 6: Schematic structure of adiponectin receptors (AdipoR1 and AdipoR2) and adaptor protein.
Adapted from (159)

2.3 Adiponectin signaling

Results from various *in vitro* and *in vivo* studies suggest the contribution of two major signaling pathways to ApN signal transduction. Both AMPK and PPAR-α pathways have been shown to mediate the metabolic properties of ApN (176). In the skeletal muscle and the liver, the modulation of the lipid and glucose metabolism by ApN is inhibited by blocking AMPK activation, indicating that stimulation of glucose utilization and fatty-acid oxidation by ApN occurs through activation of AMPK. Likewise, ApN activates PPAR-α in C2C12 myocytes *in vitro* and also increases the expression levels of PPAR-α *in vivo*, leading to increased fatty-acid oxidation and energy consumption(154). As AdipoRs are ubiquitously expressed, the ApN signaling is not restricted to muscle and liver. In endothelial cells, several *in vitro* studies have shown that ApN induces the production of nitric oxide (NO) as a consequence of increased endothelial NO synthase (eNOS) activity mediated by AMPK phosphorylation of eNOS (177-179). Moreover, ApN prevents the TNF-α-induced expression of vascular cell adhesion molecule-1 (VCAM-1), E-selectin, intracellular adhesion molecule-1(ICAM-1), IL-8 and reactive oxygen species (ROS) generation in human endothelial cells by its ability to promote signaling through cAMP-PKA (179; 180). Beyond its vasoprotective effects, ApN prevents the myocardium against ischemia-reperfusion injury through COX-2-mediated anti-inflammatory and AMPK-mediated anti-apoptotic mechanisms (179; 181). In macrophages, ApN attenuates the production of pro-inflammatory cytokines (TNF-α, IL-6) through suppressing NF-κB activation; it also acts to inhibit macrophage-to-foam cell transformation and reduce intracellular cholesteryl ester content by suppressing expression of class A scavenger receptor (SR-A) (179; 182). Both AdipoRs are expressed in AT. As yet, the signaling mechanisms underlying the autocrine effects of ApN on AT are still largely unknown. One study showed that activation of AMPK mediates the stimulatory effect of ApN on glucose uptake in primary mice adipocytes (183). Another group found that suppressed NF-κB activation is involved in the inhibitory effect of ApN on LPS-stimulated release of TNF-α and IL-6 in 3T3-L1 adipocytes (184) (Figure 7).

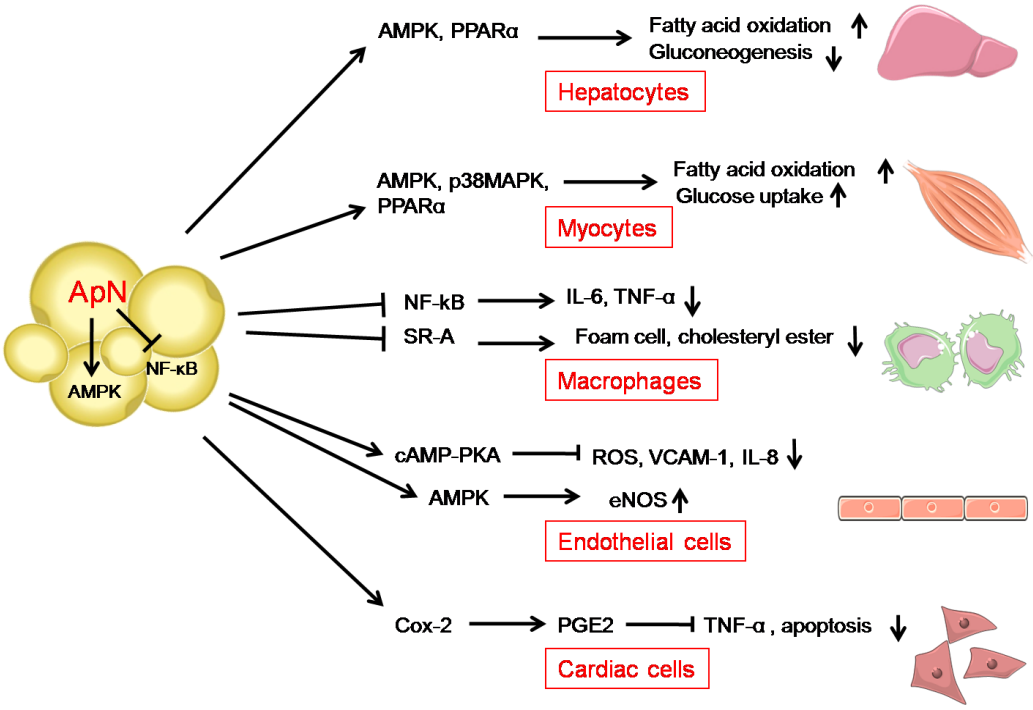

Figure 7: Adiponectin (ApN) signaling. Effects of ApN are mediated via various signaling pathways in different cell types. ApN activates AMPK and PPAR-α pathways in myocytes and hepatocytes, inducing fatty-acid oxidation and glucose uptake as well reducing hepatic glucose production. ApN inhibits production of ROS, IL-8 and VCAM-1 in endothelial cells via cAMP-PKA pathway. ApN also stimulates AMPK activation in endothelial cells, leading to increased activation of eNOS. In cardiac cells, ApN inhibits TNF-α production and cell apoptosis through its ability to stimulate the COX-2-PGE2 pathway. In macrophages, ApN attenuates production of pro-inflammatory cytokines through suppressing NF-κB activation, and inhibits foam cell formation by repressing SR-A expression. In AT, ApN stimulates AMPK activation and inhibits LPS-stimulated NF-kB activation. Arrows indicate stimulation, blunted arrows indicate inhibition. ROS, reactive oxygen species; SR-A, scavenger receptor; eNOS, endothelial nitric oxide synthase; Cox-2, Cyclooxygenase-2; PGE2, prostaglandin E2. Drawn from (179).

3. Regulation of adiponectin expression and secretion

3.1 Structure of adiponectin gene

The ApN gene is primarily expressed in white AT, but its expression has been further demonstrated in various tissues such as brown AT, skeletal muscle, liver, cardiac myocytes, bone forming cells, salivary gland, epithelial cells and placenta, and has also been detected in cerebrospinal fluid, although at incomparably lower levels (176). The Human ApN gene is localized on the chromosomal locus 3q27, while in mouse the gene is mapped to the telomere of the chromosome 16. The genomic structure of ApN gene, which is composed of three exons separated by two introns, is conserved between human and mouse species (185). Both mouse and human ApN promoters lack a TATA-box but contain multiple transcription-factor-binding sites, suggesting that the transcription of the ApN gene is subject to an elaborate regulation by diverse upstream signals. Among the different transcription-binding-sites, the promoter contains a PPRE (PPAR-responsive element), several C/EBP response elements, a LRH-RE (liver receptor homolog-1) and SREs (Sterol-regulatory element) (186) (Figure 8).

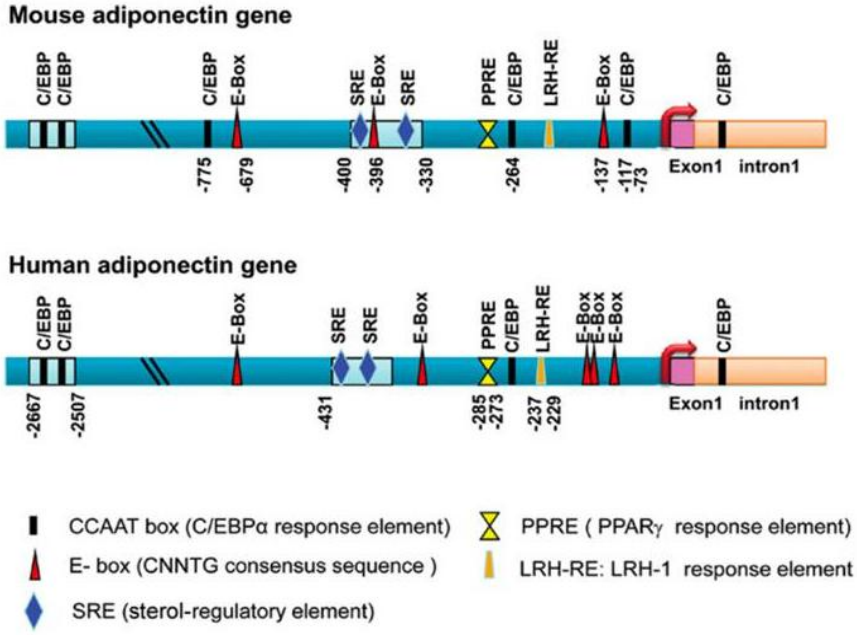

Figure 8: The basic structure of mouse and human adiponectin gene promoter. Both mouse and human adiponectin gene promoters contain one PPRE, one LRH-RE, two SREs, several c/EBPα enhancer elements and a different number of E-boxes (187).

3.2 Transcriptional regulation

Several transcription factors, cytokines have been demonstrated as important regulators for ApN gene expression (Figure 9).

Positive regulation PPARγ, which is mainly expressed in AT, leads to increased ApN gene transcription by binding to a PPRE site localized in ApN promoter. In adipocytes, point mutation of the PPRE markedly reduced the basal transcriptional activity and completely blocked thiazolidinedione (TZD)-induced transactivation of ApN promoter (188). Likewise, FoxO1 (Forkhead box O1), C/EBPα (CCAAT/enhancer-binding protein) and SREBPs are also positive regulators. FoxO1 forms a complex with C/EBPα, and this complex is stimulated by Sirtuin 1 (Sirt1), resulting in ApN promoter activation (189). C/EBPα interacts with the CCAAT-box motif and recruits co-activators such as CBP (CREB-binding protein) that, in turn, regulates the target gene. In C/EBPα-deficient adipocytes, ectopic expression of PPARγ only modestly increased the levels of ApN. But ApN mRNA level could be markedly enhanced by co-expression of both PPARγ and C/EBPα, suggesting that C/EBPα is required for full activation of the ApN gene promoter (190). SREBP-1c activates ApN gene promoter by binding to SREBP-responsive element which is required for basal promoter activity and has been identified in the human ApN promoter (191).

Negative regulation On the contrary, recent studies indicate that CREB (cAMP-response-element-binding protein) acts as transcriptional repressor of ApN gene expression. CREB indirectly inactivates ApN promoter through up-regulation of the transcriptional repressor ATF3 (Activating transcription factor 3) (192). In addition to CREB, several other transcription factors including NFAT (Nuclear factor of activated T-cells), AP-2β (Activating enhancer-binding protein) and Id3 (inhibitor of differentiation-3), have also been implicated in the negative regulation of ApN gene expression (70; 193-195).

Some inflammatory cytokines are also well documented to negatively regulate ApN mRNA abundance. TNF-α suppresses ApN gene expression through inhibition of transcription factors PPARγ, C/EBP and SREBP. The effect of TNF-α on ApN transcription is mediated by JNK signaling pathway, which has been shown to phosphorylate PPARγ and decrease its DNA-binding activity. IL-6 has been shown to down-regulate ApN mRNA levels through p44/42 MAPK pathway in 3T3-L1 adipocytes (193; 196). IL-18 treatment in 3T3-L1 adipocytes induces ERK1/2 (extracellular signal regulated kinase)-dependent phosphorylation and activation of NFATc4, the transcriptional repressor of ApN gene (193).

3.3 Post-translational regulation

ApN is synthesized as a single polypeptide and is then assembled in the ER (Endoplasmic reticulum) into multimeric forms. There are four conserved lysine residues in the collagenous domain of ApN, which are modified by hydroxylation and subsequent glycosylation, both of which are required for intracellular assembly of ApN multimers (Figure 9) (150; 197). Subsequently, the disulfide bond formed via Cys39 (mouse) or Cys36 (human) is critical for ApN to form higher molecular weight multimers based on its trimeric form. ApN multimerization and secretion by post-translational modification is critical for its biological function (Figure 9).

Positive regulation Ero1-Lα (ER membrane-associated oxidoreductase) and its associated protein ERp44 facilitate ApN's HMW formation and secretion by disulfide bond formation (198; 199). Another ER-associated protein DsbA-L (Disulfide-bond A oxidoreductase-like protein) has also been shown to promote ApN multimerization in 3T3-L1 adipocytes (200). PPARγ may not only stimulate ApN transcription as mentioned above, but also stimulate ApN multimerization by increasing expression of Ero1-Lα (198; 199).

Negative regulation ER stress, oxidative stress and pro-inflammatory cytokines such as TNF-α and IL-6 suppress ApN secretion by inhibiting expression of ER-associated proteins or inactivating PPARγ (201-205). As mentioned above, sexual hormone Testosterone has a negative effect on ApN serum levels, although the mechanisms involved have not been clarified. Insulin may also inhibit secretion of ApN. Hyperinsulinaemia induced by hyperinsulinemic euglycemic clamp significantly decreased human plasma ApN levels (206).

3.4 Hypoadiponectinemia in obesity and MS

Decreased mRNA expression and plasma levels of ApN are found in obese/diabetic mice and humans despite the increasing mass of AT. As the pro-inflammatory cytokines, ER stress, oxidative stress and hyperinsulinaemia are largely induced by obesity and MS, their negative regulation on ApN transcription, multimerization and secretion could contribute to the hypoadiponectinaemia associated with obesity and MS (187).

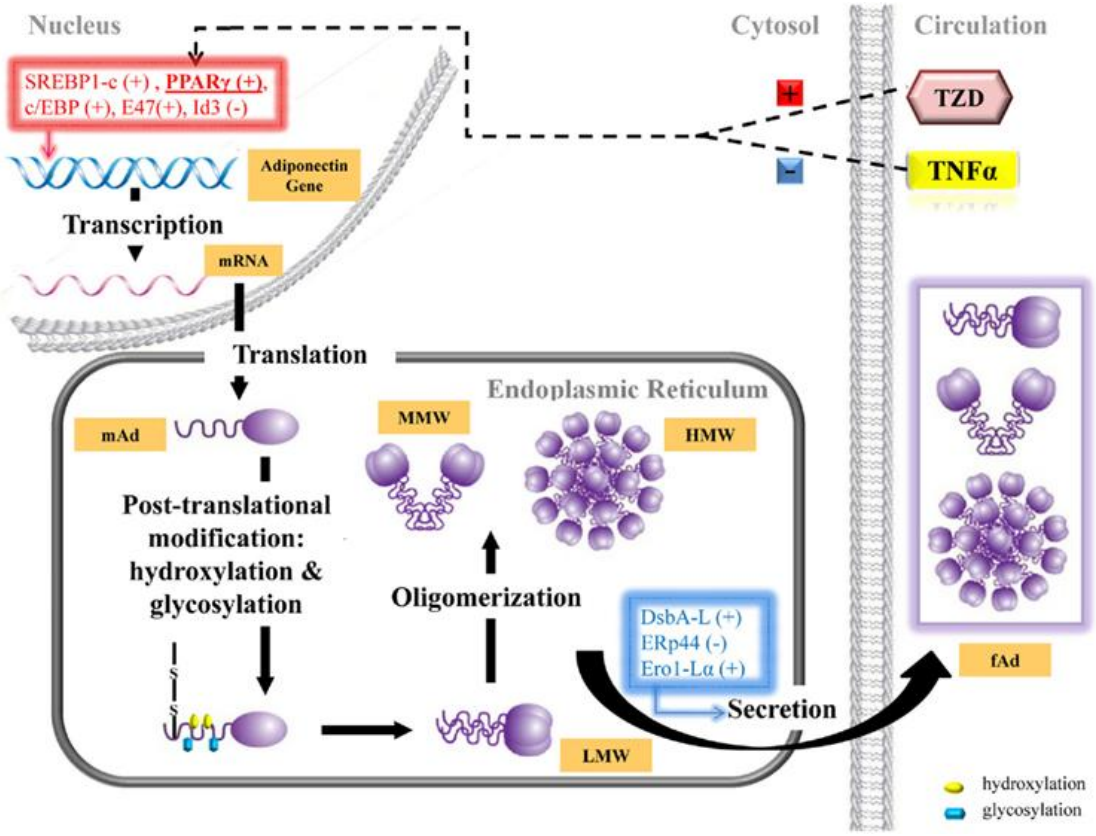

Figure 9: Schematic representation of the steps involved in transcription, translation, post-translational modification, oligomerization, and secretion of ApN. Several transcription factors (top left) which mediate ApN gene transcription are regulated to increase or decrease ApN expression. Monomeric ApN is posttranslationally modified and further oligomerized to form trimers (LMW), hexamers (MMW) and oligomeric (HMW) forms. Various mechanisms (bottom right) mediate this oligomerization and secretion resulting in secretion of HMW, MMW and LMW forms (207).

3.5 Adiponectin as a therapeutic target

Drugs like rosiglitazone and pioglitazone, belonging to the TZD class of PPARγ agonists and used for treatment of type 2 diabetes have been clinically and experimentally proven to be potent inducers of ApN production. By using ApN-KO mice, it has been shown that pioglitazone decreased insulin resistance and diabetes via ApN-dependent pathways in the liver and via ApN-independent mechanisms in skeletal muscle (208). Several studies suggest that the enhanced multimerization of ApN is a main effect of TZDs, besides the stimulation of ApN mRNA expression (209). Six months of metformin therapy in humans also leads to increased plasma ApN levels together with improved insulin sensitivity (210). It has been shown that metformin directly up-regulates ApN production in cultured human subcutaneous AT explants (211). The endocannabinoid receptor 1 antagonist, rimonabant has been developed as an anti-obesity drug due to its central effect leading to reduced food intake. Growing evidence including our own data has revealed that rimonabant also exerts peripheral

effect on several metabolic tissues including AT, thereby further improving several facets of the MS. We have shown that rimonbant directly up-regulated ApN in cultured human omental adipose explants and adipocytes. This may explain the increase in plasma ApN levels observed after rimonabant treatment of patients with the MS (212; 213).

4. Biological function of adiponectin

The biological actions of ApN are implicated in multiple tissues. Its pleiotropic properties play a role against various diseases including diabetes, non-alcoholic fatty liver disease (NAFLD), cardiovascular disease, obesity, and cancer (Figure 10) (214). ApN could thus be a therapeutic target for management of MS.

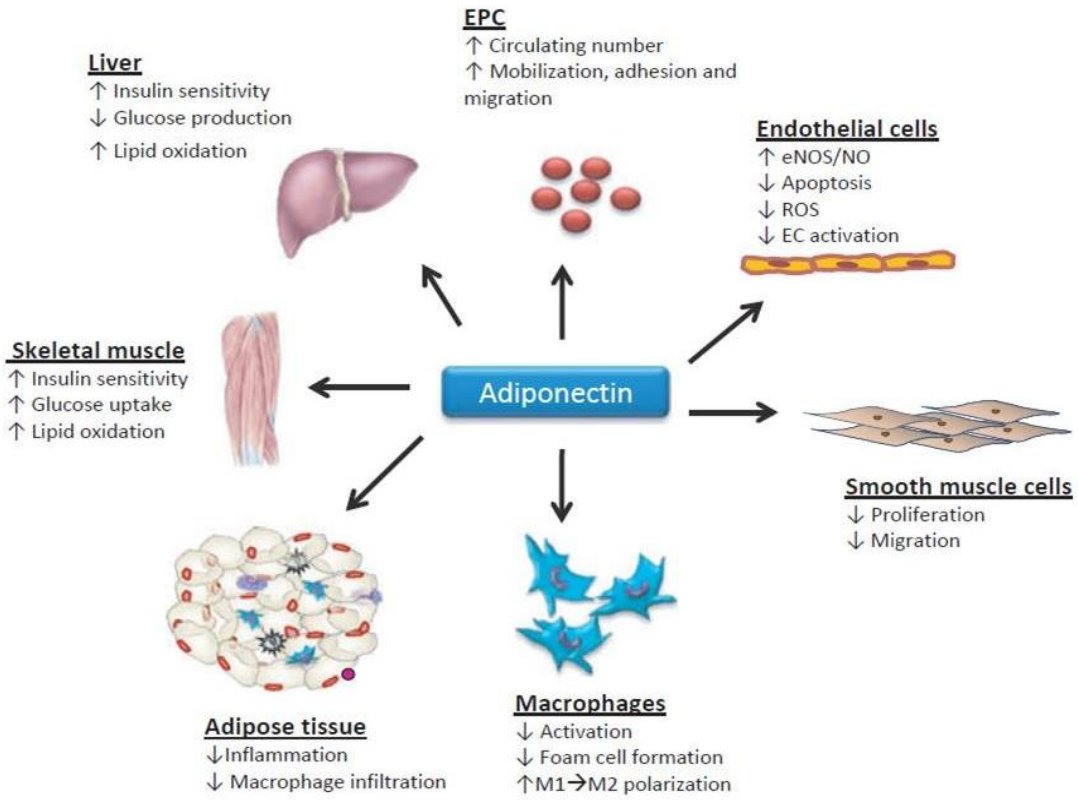

Figure 10: Adiponectin exerts beneficial effects on multiple targets. EC, endothelial cells; EPC, endothelial progenitor cells; NO, nitric oxide; ROS, reactive oxygen species (214).

4.1 Insulin-sensitizing action

Clinical data have shown that low plasma ApN concentration is associated with reduced insulin-stimulated tyrosine phosphorylation of insulin receptor in skeletal muscle of Pima Indians, and these low levels precede a decrease in whole body-insulin sensitivity (215). In cultured myocytes, ApN can increase both basal and insulin-stimulated glucose uptake by promoting GLUT4 translocation to the cell membrane through the activation of AMPK pathway (216). Improvement of insulin sensitivity may also be secondary to lower ectopic TG content. This may result from enhanced P38MAPK and PPAR-α activity and subsequent increased expression of molecules involved in both fatty-acid combustion and energy dissipation in muscle (217). These effects are summarized in transgenic mice overexpressing ApN: the transgene successfully corrected high-fat diet-induced insulin resistance in skeletal muscle and decreased TG content in the liver and skeletal muscle (218; 219), thereby further improving insulin sensitivity *in vivo.*

ApN also exerts an insulin-sensitizing role on liver. It decreases gluconeogenesis, thereby reducing glucose levels *in vivo*. This effect of ApN is mediated by increased phosphorylation of AMPK, which reduces the expression of gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (220). Like in skeletal muscle, ApN also protects hepatic insulin signaling against the hurt of TG excess through its ability to activate PPAR-α in hepatocytes (217). It is worth noting that hepatic insulin resistance is a key feature in the pathogenesis of NAFLD, which has been considered as the hepatic manifestation of the MS (221). There is increased evidence for a protective role of ApN on NAFLD. Low serum ApN levels have been reported to be associated with the presence of NAFLD, and serum ApN has been proposed in combination with other factors as a biomarker for nonalcoholic steatohepatitis (NASH), a component of NAFLD. Moreover, exogenous ApN depleted lipid accumulation and attenuated hepatic inflammation in animal models of NASH (222).

4.2 Cardiovascular protection

Extensive studies suggest that hypoadiponectinemia is associated with coronary heart disease (223-225) and hypertension (226; 227). Indeed, ApN deficiency more than conventional cardiovascular risk factors correlates to early atherosclerosis in obese children (228). Transgenic mice overexpressing ApN in ApoE-deficient background are protected against atherosclerosis (218). The molecular mechanisms by which ApN prevents the development of atherogenesis are mediated by (a) down-regulating adhesion molecules including ICAM-1,

VCAM-1 and E-selectin (223; 229-231); (b) attenuating growth factors-induced proliferation of vascular-smooth muscle cells (232); (c) inhibiting macrophage-to-foam cell transformation (182; 218) and (d) reducing vascular inflammation (229; 230) and oxidative stress (233). Moreover, ApN-knockout (ApN-KO) mice display larger infarct sizes and increased apoptosis resulting from myocardial ischemia-reperfusion injury (181); these mice also exhibit enhanced concentric cardiac hypertrophy in response to pressure over-load (234; 235).

Besides atherosclerosis and cardioprotection, ApN may also play crucial role in blood pressure by regulating the vascular tone. When fed a high-salt or an atherogenic diet, ApN-KO mice exhibited hypertension, which is associated with reduced eNOS production and impaired endothelium-dependent vasodilatation, while adenovirus-mediated recruitment of ApN reversed these abnormalities (236; 237). Recent studies suggest that ApN could affect blood pressure by a central action. ApN injection into cerebral ventricle in rats decreased blood pressure and sympathetic nerve activity through stimulation of the suprachiasmatic nucleus (238).

4.3 Anti-obesity effect

Decreased plasma ApN levels have been found in patients with obesity despite the increased adipose tissue mass (239; 240). The anti-obesity function of ApN could be explained by impaired preadipocyte differentiation as well as by increased energy expenditure (241; 242). When ApN is administered to rodents, body temperature and oxygen consumption increased possibly because of an increase in UCP1-2 in white and brown AT and UCP3 in skeletal muscle (243).

4.4 Anti-inflammatory effect

Hypoadiponectinemia negatively correlates with elevated plasma CRP levels in obese and diabetic subjects, suggesting that ApN is linked to obesity-induced chronic inflammatory state (244-246). ApN attenuates inflammatory responses to multiple stimuli by modulating signaling pathways in a variety of cell types (see Figure 7). Herein, I will focus on the anti-inflammatory effect of ApN on adipose tissue as it is the main purpose of my thesis. Before my thesis, only a few *in vitro* studies have investigated this field. It has been reported that ApN attenuated LPS-induced IL-6 and TNF-α release in primary pig adipocytes (140). The globular ApN was also found to reduce the secretion of several pro-inflammatory adipokines as shown by protein array analysis of culture medium from human mammary adipocytes differentiated *in vitro* (247).

4.5 Anti-cancer

Circulating ApN levels are lower in patients with breast, endometrial, prostate and colon cancer. It has been proposed that ApN may be a link between obesity and increased cancer risk. ApN can also act on tumor cells directly, as several cancer cell types express ApN receptors. The anticarcinogenic effects could be explained by the anti-proliferative effects of this adipokine (248).

5. Adiponectin transgenic mouse models

To address the chronic effects of ApN and the underlying mechanisms, several ApN transgenic mouse models have been created so far. Herein, I briefly summarize the characteristics of some of these mouse models.

5.1 Adiponectin overexpression targeted to white adipose tissue

The group of Scherer *et al.* generated a transgenic mouse model using a deletion mutant of ApN (lacking 13 repeats in the collagenous domain) under the control of the adipocyte-specific aP2 promoter. This mouse model exhibited a chronic 3-fold elevation of serum ApN, which improved hepatic insulin sensitivity. However, because of the ApN mutation, an unusual phenotype with bilateral exophthalmia and expansion of interscapular tissue was observed in those mice at a later age (219).

Our lab has also generated a transgenic mouse model overexpressing ApN specifically in white AT. In our model, the *native full-length* ApN cDNA was placed under the control of aP2 (Figure 11). These mice exhibited moderate overexpression of ApN exclusively in white AT and moderate enhanced ApN secretion in circulation with no changes in the distribution of ApN multimers (242). When fed a high-sucrose diet, five-month-old transgenic mice showed improved insulin sensitivity and lipid profile, they also had lower fat mass than WT littermates resulting from increased energy expenditure and impaired preadipocyte differentiation (242).

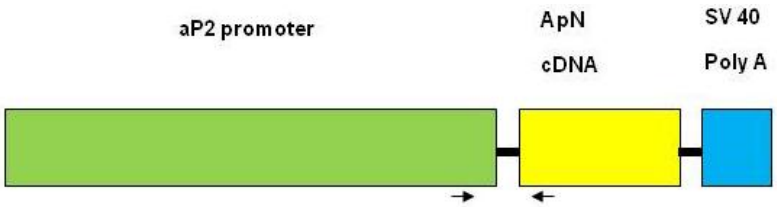

Figure 11: Schematic representation of the aP2 promoter/mouse ApNcDNA fusion gene. The black arrows represent the primers used for the identification of mice carrying aP2-ApN transgene (242).

5.2 Adiponectin overexpression targeted to liver

Some teams placed ApN under the control of a liver promoter, serum amyloid P component (SAP). Yamauchi *et al.* created a fusion gene comprising the human SAP promoter and mouse gApN coding sequences so that the overexpression of gApN was targeted to the liver. Plasma gApN concentrations were very high in transgenic mice. These mice showed partial amelioration of insulin resistance and hyperglycemia on a high-fat diet, but not of obesity (218). To study the effects of ApN on life span, the cDNA encoding human full-length ApN was also placed under the control of SAP. Interestingly, a prevention of premature death was observed in these mice, probably due to an attenuation of oxidative DNA damage (249).

5.3 Adiponectin knockout mice

The ApN-knockout mice (ApN-KO) used in our lab were generated by Maeda *et al*. (38). They disrupted the ApN gene by replacing exon 2 (which contains the translation initiation sites) with the neomycin-resistance gene. These mice are characterized by a complete lack of ApN mRNA in fat and ApN protein in plasma. When challenged with high-fat diet/high sucrose diet, ApN-KO mice developed insulin resistance, which is associated with increased TNF-α mRNA levels in AT (38).

IV. microRNAs

1. microRNA biogenesis and function

microRNAs (miRNAs) are endogenous ～22nt RNAs that can bind to the 3'-untranslated region (3'-UTR) of target mRNAs to repress mRNA expression at the posttranscriptional level. As a group, miRNAs may directly regulate expression of over 30% of human and mouse genes and more than 60% of human protein-coding genes have been under selective pressure to maintain pairing to miRNAs (250). miRNAs play important regulatory roles in broad biological processes in plants and animals.

1.1 microRNA genes

Most miRNA genes come from regions of the genome that are quite distant from previously annotated genes, implying that they derive from independent transcription units. Nonetheless, a minority (e.g. about quarter of human miRNA genes) are not transcribed from their own promoters but are instead processed from the introns of pre-mRNAs. Other miRNA genes (e.g. over half of the known Drosophila miRNAs) are clustered in the genome with an arrangement and expression pattern implying transcription as a multi-cistronic primary transcript. Nearly all of the cloned miRNAs are conserved in closely related animals, such as human and mouse, or *C. elegans* and *C. briggsae* (251).

1.2 microRNA biogenesis

miRNA genes are transcribed by either RNA polymerase II or III into primary miRNA transcripts (pri-miRNA) (Figure 12, step ①). In the nucleus, the pri-miRNA is cleaved resulting in a 60–70nt stem loop intermediate, known as the miRNA precursor or the pre-miRNA (Figure 12, step ②). This cleavage is performed by the RNase III enzyme Drosha and the DGCR8 (DiGeorge critical region 8) protein. The pre-miRNA is next actively transported from the nucleus to the cytoplasm by Exportin-5 in complex with Ran-GTP (Figure 12, step ③). In the cytoplasm, the pre-miRNA is further processed by another RNase III endonuclease, Dicer (Figure 12, step ④). Dicer together with TRBP (Tar RNA binding protein) cleaves off the loop of the pre-miRNA and produces a mature miRNA duplex, which comprises the functional guide strand and the passenger strand. However the miRNA duplex exists as a transit intermediate, it is subsequently separated by a helicase-like enzyme (yet to be identified). The functional miRNA strand is then incorporated into a ribonucleoprotein complex, known as RNA-induced silencing complex (RISC) (Figure 12, step ⑥), and the passenger strand is degraded (Figure 12, step ⑤). This functional asymmetry depends on the

thermodynamic stability of the base pairs at the two ends of the duplex: the miRNA strand with the less stable base pair at its 5' end in the duplex is loaded into RISC. The functional core component of RISC is the Argonaute protein (Ago), which can guide RISC to bind to the complementary sequence located in 3'-UTR of target mRNA and lead to mRNA degradation, destabilization or translational inhibition (Figure 12, step ⑦) (251; 252).

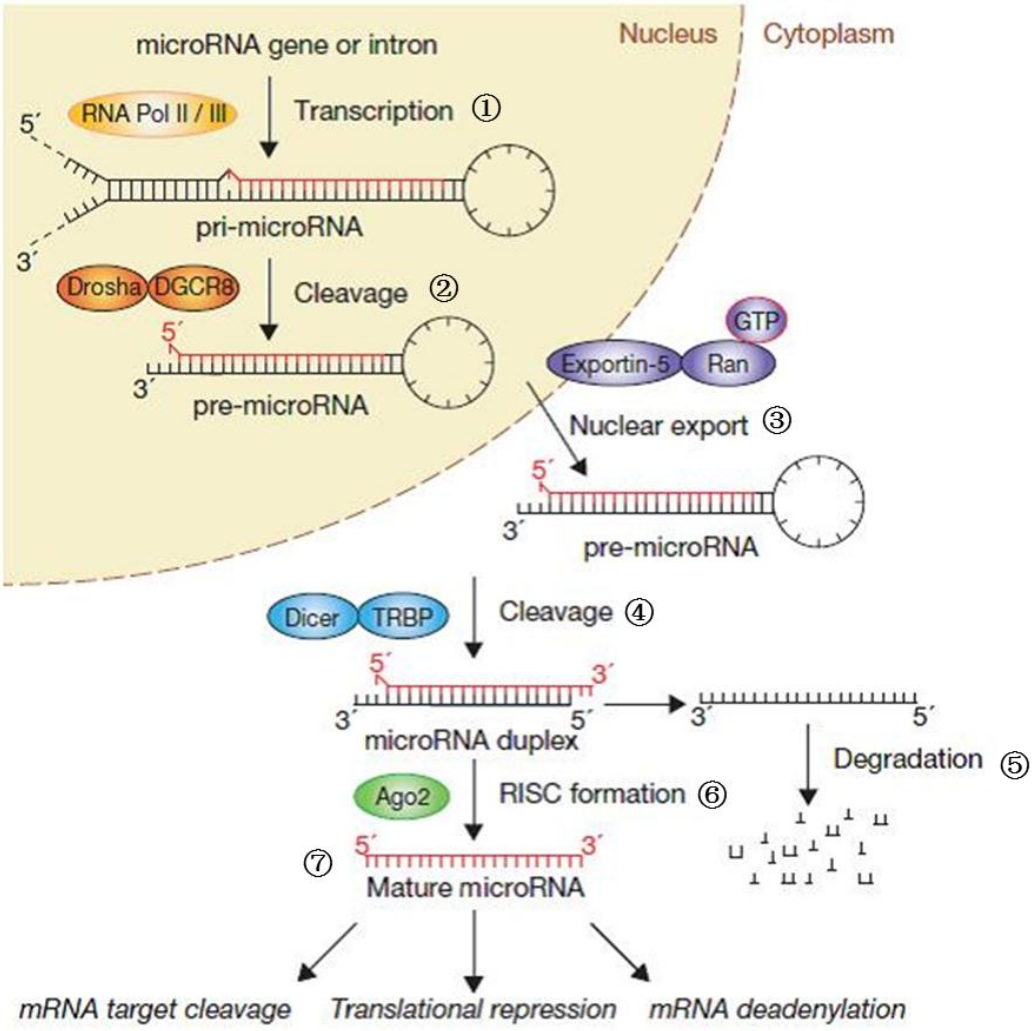

Figure 12: microRNA biogenesis. ① miRNA gene is transcribed into Pri-miRNA by RNA polymerase II or III. ② Pri-miRNA is cleaved into Pre-miRNA by the microprocessor complex Drosha–DGCR8 in the nucleus. ③ Pre-miRNA is transported from nucleus to cytoplasm by Exportin-5–Ran-GTP. ④ In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP (Tar RNA binding protein) cleaves the pre-miRNA hairpin to its mature length. ⑤ The miRNA duplex unwinds, the passenger strand (black) is degraded. ⑥ The functional strand of the mature miRNA (red) is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC). ⑦ Ago2 guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation (252).

1.3 microRNA function: mRNA posttranscriptional repression

Once incorporated into a cytoplasmic RISC, the miRNA can direct the RISC to down-regulate gene expression by two main posttranscriptional mechanisms: mRNA degradation or translational inhibition. Over the past few years, it has been proposed that the miRNA will specify mRNA degradation if the mRNA has sufficient complementarity to the miRNA, otherwise the miRNA will repress mRNA translation (251). Perfect complementarities mainly exist in plants, while in animals all but a few targets lack the extensive paring for cleavage. Thus, animal miRNAs were originally thought to mainly repress target translation by slowing the ribosomes or degrading newly synthesized polypeptides, with little or no influence on mRNA abundance, whereas the reverse was thought to be true in plants. Now, however, numerous studies have provided strong evidence that the predominant repression mode in animals is miRNA-mediated mRNA degradation. This evidence comes from studies on specific miRNA-target pairs and, more generally, from transcriptome studies showing that the abundance of miRNA targets inversely correlates with the level of the miRNA (253). One recent study has compared mRNA abundance and translational rates in human embryonic kidney (HeK)-293T cells transfected with miR-124: it revealed that mRNA degradation accounted for about 75% of the changes observed in protein synthesis, regardless of the magnitude of the regulation (254). Likewise, the group of Bartel *et al.* found, by using ribosome profiling, that mammalian miRNAs caused a decrease in steady-state mRNA levels, which can explain most of the reduction (84%) in protein production (255). The mechanism of miRNA-mediated target mRNA degradation is proposed to be a 5'-to-3' mRNA decay pathway. mRNAs are primarily deadenylated by the deadenylase complex, and then decapped by the decapping enzymes. Decapped mRNAs are ultimately degraded by the major cytoplasmic 5'-to-3' exonuclease XRN1 (Figure 13). The role of mRNA decay factors in miRNA-mediated mRNA destabilization is evidenced by the observation that the abundance of miRNA targets increases when these factors are depleted or antagonized (253).

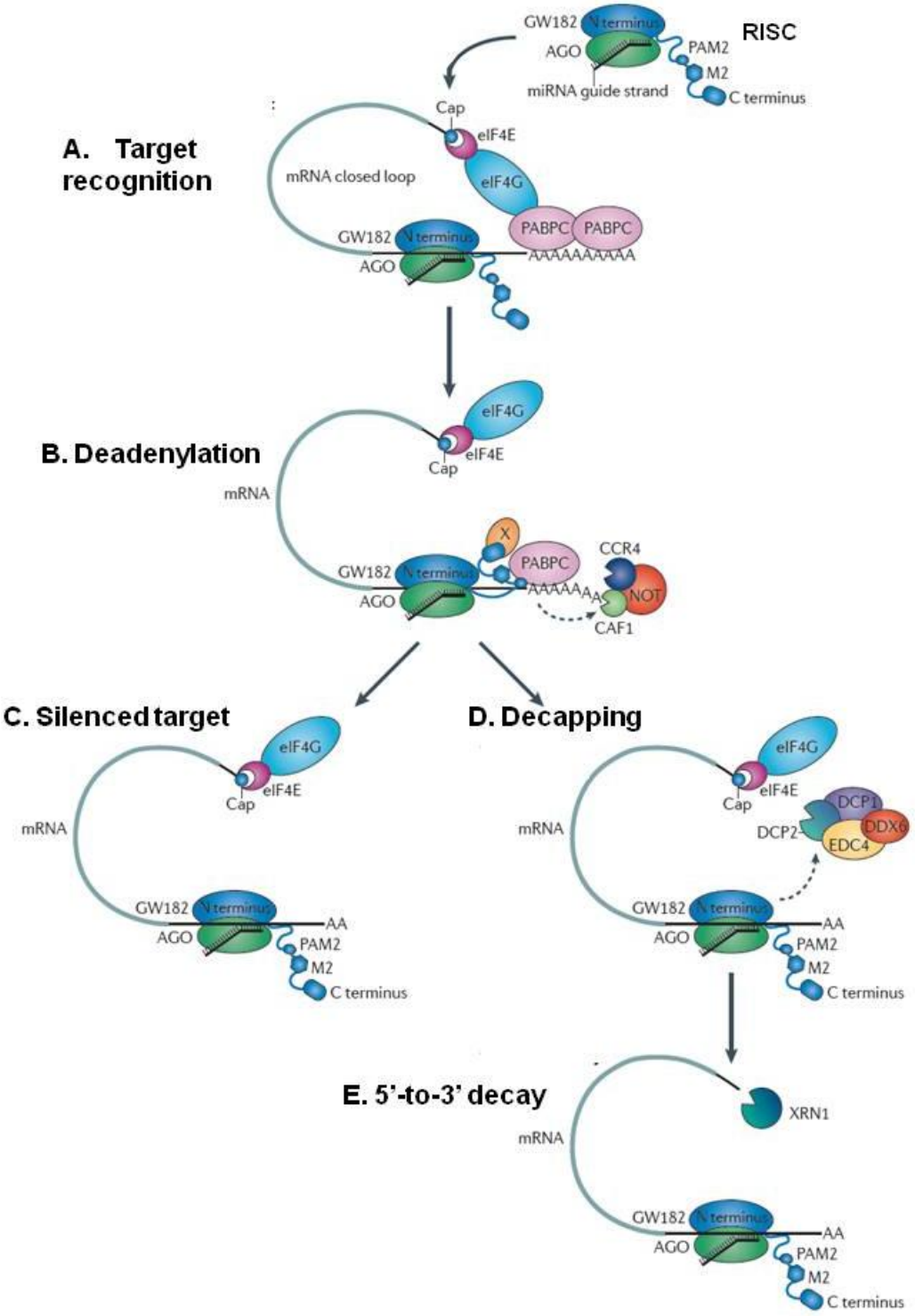

Figure 13: Mechanisms of miRNA-mediated gene silencing in animals. The minimal requirements for microRNA (miRNA)-mediated gene silencing in animals are: an Argonaute protein (Ago), a GW182 trinucleotide-repeat-containing protein, cytoplasmic poly(A)-binding protein (PABPC), components of the major deadenylase complex (CAF1, CCR4 and the NOT complex), the decapping enzyme DCP2 and several decapping activators (for example, DCP1, EDC4 and DDX6, also known as RCK). **A.** miRNA loaded into RISC recognizes its mRNA target by base-pairing to partially complementary binding sites in the mRNA 3'UTR. **B.** AGO interacts with GW182, which in turn interacts with PABPC bound to the mRNA poly (A) tail. The AGO-GW182 complex directs the mRNA to deadenylation. **C.** Depending on the cell type and/or specific target, deadenylated mRNA can be stored in a translationally repressed state. **D, E.** In animal cell cultures, deadenylated mRNA is decapped and rapidly degraded by the major 5'-to-3' exonuclease XRN1 (253).

1.4 microRNA targets prediction

miRNA target genes can be computationally predicted. The computational algorithms are developed based on several principles (256): (1) Base pairing pattern in the binding sites. (2) Thermodynamic stability of miRNA-mRNA hybrid, which is assessed by calculating free-energy (ΔG) of the putative binding. (3) Comparative sequence analysis to check whether target sequences are evolutionarily conserved across species. (4) Examination of the presence of multiple target sites and take the number of target sites into account for prediction.

The most established and widely used miRNA target prediction algorithms are TargetScan (257), PicTar (258) and miRanda (259; 260). TargetScan integrates thermodynamics-based modeling of miRNA-mRNA interactions and comparative sequence analysis to predict miRNA targets conserved across multiple genomes; its estimated false-positive rate varies between 22 % and 31%. PicTar checks the alignments of 3' UTRs for those displaying seed matches to miRNAs, and filters the retained alignments based on their thermodynamic stability. This algorithm has been shown to correctly identify some known miRNA targets and its false positive rate is estimated to be around 30%. miRanda selects target genes based on sequence complementarities, free energies of RNA-RNA duplexes and conservation of target sites in related genomes. miRanda was described to correctly identify 9 out of 10 currently characterized target genes, and its false-positive rate is around 24% (256).

Since computational methods are not perfect, the predicted miRNA targets should be validated experimentally in biological systems. A gene reporter assay may be useful to check directly the interaction between the miRNA and its target mRNA. Additionally, quantitative real-time PCR (RT-qPCR), Western blot, or in situ hybridization are often performed to examine the expression of predicted miRNA target genes in either *in vitro* or *in vivo* systems where the miRNA has been overexpressed or knocked out (256).

2. Role of microRNA in inflammation

I shall briefly review the role of miRNAs in the immune system.

Role of microRNAs in adaptive immunity Studies conducted by many groups have demonstrated that miRNA regulation is indispensable for stable immune process. miRNAs appear to have a key role in the differentiation of B-cells. Ablation of whole miRNAs by

deletion of Argonaute 2 (Ago2) or Dicer impairs pre-B-cell differentiation and the succeeding peripheral B-cell generation (261). Several individual miRNAs have also been reported to be involved in the B-cell biology. For example, over-expression of miR-17~92 cluster enhances B-cell proliferation and survival (262), while miR-150 profoundly affects early B-cell differentiation and mature B-cell responses (263).

miRNAs have been shown to be key regulators of the T-cell lineage induction pathways. Deletion of Dicer at an early stage of T cell development compromised the survival of T cell lineage (264). The best evidence for miRNA playing a role in specific developmental stages of T-cell differentiation is from miR-181, which, as well as reducing the number of T cells in haematopoietic over-expression systems (265), increases the sensitivity of T-cell receptor signaling (266).

In addition, miRNAs also play pivotal roles in the induction, function and maintenance of the regulatory T cell (Treg) lineage. miRNAs can enhance thymic and peripheral induction of Treg cells (264; 267). Dicer-deficient Treg cells almost lost their suppressive capacity and anergic profile (267).

Role of microRNAs in innate immunity Several miRNAs (including miR155, miR146a, miR21, miR9) have been consistently found to be rapidly induced by innate immune activation (e.g. Toll-like receptor), indicating that they may regulate the innate immune response (268). Target prediction analyses indicate that up to a half of innate immune genes could be under the direct regulation of miRNAs. A study on 613 genes, which regulate immunity utilizing a computational approach, identified 285 genes as miRNA targets. Major targets include transcription factors, cofactors and chromatin modifiers whereas upstream factors, such as ligands and receptors (cytokines, chemokines…) were, in general, poor or non-targets (269).

The mechanisms by which miRNAs regulate cytokine expression include direct regulation by binding to seeding sites in mRNA 3'-UTR and indirect regulation. Computational analyses predict that most cytokines lack miRNA target sites in 3'-UTRs (269). For example, of the interleukin genes (IL1-29) studied, 9 had predicted miRNA binding sites. Of 20 interleukin receptor genes examined, only 2 had high probability miRNA target sites. These findings suggest the regulation of cytokine genes by miRNAs occurs often via indirect mechanism. Recent studies indicate indeed that miRNAs could indirectly regulate cytokine genes via AU-

rich elements (ARE) located in the mRNA 3'-UTR by targeting ARE-binding proteins (Figure 14). ARE are the cis-acting structural RNA motifs that are important determinants of cytokine message stability. ARE-mediated mRNA degradation is regulated by a number of trans-acting factors, called the ARE-binding proteins (which include TTP, AUF1 and members of the HUR family) (270). These ARE-binding proteins are heavily predicted targets of miRNAs (271). Repression of several ARE components by miRNAs may alter the levels of inflammatory cytokines as well as of other immune genes (269; 271).

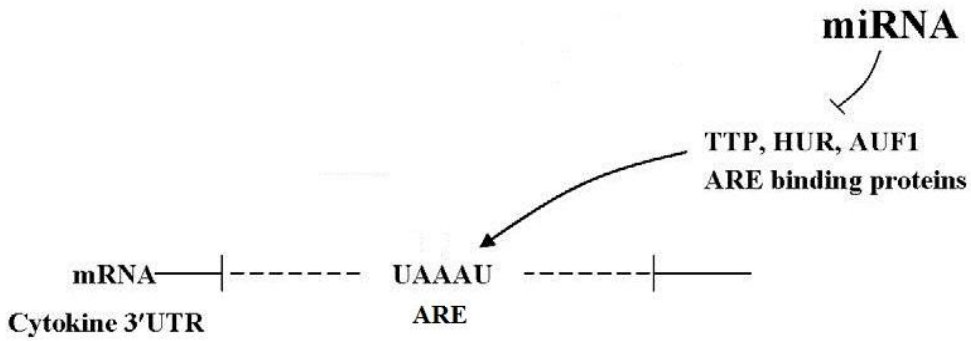

Figure 14: Scheme for indirect regulation of cytokine genes by miRNAs. Cytokine mRNA 3'UTR have AU-rich element (ARE) site. miRNAs potentially repress the expression of the ARE binding proteins (TTP, HUR, AUF1), which interact with ARE site to alter cytokine mRNA levels. Adapted from (271).

3. Role of microRNAs in obesity and metabolic syndrome

3.1 microRNAs regulate adipocyte differentiation and mature adipocyte function

Adipogenesis is a multi-step process involving a cascade of transcription factors and cell-cycle proteins regulating gene expression and leading to adipocyte development (272). There are two key steps to mature adipocyte development: determination and terminal differentiation. In the determination step, multipotent mesenchymal stem cells (MSCs) are induced to become preadipocytes, which are morphologically similar to MSCs but have lost the ability to differentiate into non-adipocyte cell types. Following commitment to the adipocyte lineage, preadipocytes undergo terminal adipocyte differentiation, acquire a mature adipocyte morphology, and express mature adipocyte markers such as ApN, leptin and glucose transporter type 4 (GLUT4) (273) (Figure 15).

The first evidence for participation of individual miRNAs in "adipose tissue" came from a genetic screen in Drosophila. Xu *et al.* found that miR-14 knockout flies displayed significant larger lipid droplets and a roughly two-fold increase in triglyceride content in adipocytes, thereby suggesting the importance of miR-14 in lipid metabolism of mature adipocytes (274). However its mammalian homologue has not yet been discovered. Subsequent studies carried in mammals showed that knocking down Dicer (the essential enzyme for miRNA maturation) in mouse preadipocytes dramatically impaired lipogenesis and downregulated adipocyte markers (275; 276). Moreover, Ortega *et al.* profiled the global changes of miRNA expression during adipogenesis using miRNA array in human preadipocytes and mature adipocytes. They showed that expression of approximately 70 miRNAs (8.8%) significantly differed between premature and mature adipocytes (277). These findings imply that miRNAs may be indispensable for controlling adipocyte development (278). Some miRNAs are able to accelerate adipocyte differentiation, while some miRNAs appear to act as negative regulators of adipogenesis.

microRNAs and acceleration of adipocyte differentiation Several miRNAs have been identified to accelerate adipocyte differentiation (Figure 14). A screening study of adipogenic miRNAs in human subcutaneous preadipocytes revealed that inhibition of miR-143 suppressed the adipogenic markers and triglyceride accumulation, indicating the promoting role of miR143 in normal adipocyte differentiation (279). Conversely, ectopic miR-143 expression during differentiation of 3T3-L1 preadipocytes resulted in increased triglyceride accumulation (280). Several evolutionarily conserved signaling pathways, such as wingless-related MMTV integration site (WNT) and TGF-β signaling disfavor adipogenic fate in MSCs (278). As an example, ectopic expression of Wnt10b in a murine stromal cell line favors osteogenesis at the expense of adipogenesis (278). In a high-throughput microarray analysis performed in 3T3-L1 cells, 18 miRNAs might promote adipogenesis by repressing WNT signaling. Among them, miR-210 blocked the Transcription factor 7-like 2 (TCF7L2), which is a key transcription factor triggering the downstream responsive genes of WNT signaling (281). Another miRNA, miR-21 also promotes adipogenesis; miR-21 is a negative regulator of TGF-β signaling in human adipose-tissue-derived mesenchymal stem cells (hASCs) by silencing TGF-β receptor 2 (TGFBR2) (282).

microRNAs and suppression of adipocyte differentiation Some studies have focused on miRNAs which appear to act as negative regulators of adipocyte differentiation (Figure 14). One report showed that overexpression of miR-448 in 3T3-L1 preadipocyte reduced the

expression of adipogenic genes and triglyceride accumulation. The anti-adipogenic effect of miR-448 was mediated by silencing the Krüppel-like factor 5 (KLF5), a transcription factor that induces the key adipogenic transcription factor PPARγ (283). miR-27a and miR-27b are also negative regulators of adipogenesis, and both have been shown to directly suppress the PPARγ mRNA (284-286). Likewise, miR-31 directly suppresses the adipogenic transcription factor C/EBPα mRNA (287). Another miRNA, let-7 has been reported to reduce protein of high-mobility group AT-hook 2 (HMGA-2) (288), a transcription factor that regulates growth and proliferation. Mice lacking HMGA-2 have marked reduction in adiposity (289), while ectopic introduction of let-7 into 3T3-L1 cells inhibited clonal expansion as well as terminal differentiation (288).

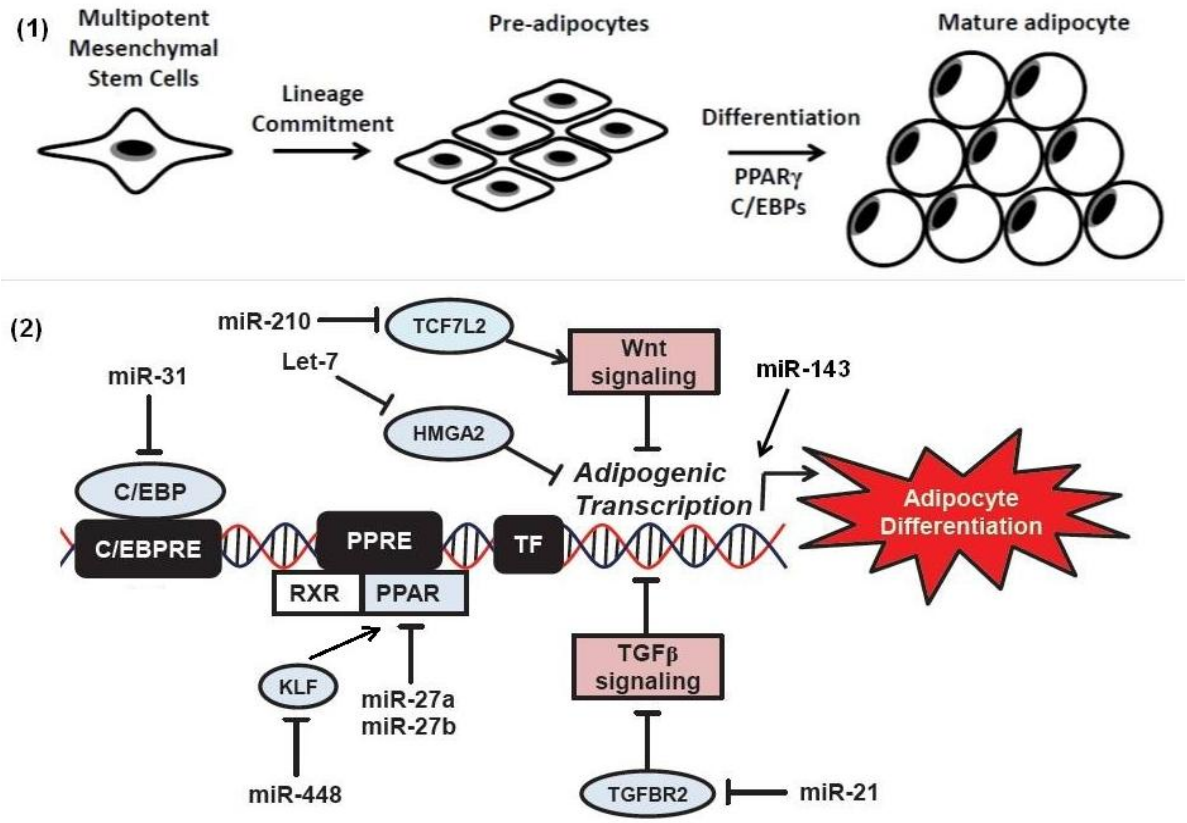

Figure 15: miRNAs in regulation of adipocyte differentiation. (1) Fat cell development: Multipotent mesenchymal stem cells can develop into lineage committed pre-adipocytes. PPAR and C/EBP transcription factors co-ordinate adipogenic gene expression during terminal differentiation into lipid storing mature adipocytes. (2) Mammalian miRNAs regulate target genes during adipogenesis. Adapted from (290)**.** Wnt, Wingless-related MMTV integration site; TCF7L2, Transcription factor 7-like 2; HMGA2, High-mobility group AT-hook 2; KLF, Kruppel-like factor 5; TGFBR2, TGF-β receptor 2.

microRNAs and regulation of mature adipocyte function There are several miRNAs that have been implicated as regulators of mature adipocyte metabolic functions. Recently, miR-27a was found to be able to accelerate lipolysis, while miR-143 could promote triglyceride

accumulation in porcine adipocytes (291). Some miRNAs are also associated with adipose tissue insulin sensitivity. Overexpression of miR-103/107 in mouse adipocytes impaired insulin signaling and insulin-stimulated glucose uptake by directly targeting caveolin-1, a critical regulator of the insulin receptor. Silencing caveolin-1 resulted in diminishing the number of insulin receptors in caveolae-enriched plasma membrane microdomains, thereby reducing downstream insulin signaling (292). Likewise, overexpression of miR-29 in 3T3-L1 adipocytes largely repressed insulin-stimulated glucose uptake, presumably through inhibiting Akt activation (293).

3.2 Adipose depot specific expression of microRNAs

Kloting *et al.* performed a global miRNA gene expression assay in different fat depots of overweight or obese subjects (n=15). Their study showed that miRNA expression is adipose depot-specific. Expression of 16 miRNAs differed between omental and subcutaneous fat with higher expression in omental fat (294). Another study identified 136 miRNAs, which were significantly differentially expressed between abdominal and gluteal adipose tissue of human subjects (295). Taken together, these data suggest that miRNA expression differences may contribute to intrinsic differences between specific fat depots.

3.3 Deregulation of microRNAs in adipose tissue during obesity and the metabolic syndrome

Xie *et al*. showed an inverse correlation of miRNA expression during adipocyte differentiation (3T3-L1 cells) and mouse obesity. miRNAs up-regulated during adipogenesis tended to be downregulated in the obese state, and *vice-versa* (280; 296). A recent study also identified 21 miRNAs, which were differentially expressed in epididymal AT between lean mice fed a standard diet and mice rendered obese by a high-fat diet (297). Ortega *et al.* performed miRNA array on human subcutaneous adipose tissue: 50 of the 799 miRNAs tested (6.2%) significantly differed between obese (n=9) and lean (n=6) subjects (277). Among these 50 miRNAs, 17 were highly correlated with BMI and metabolic parameters (fasting glucose and/or triglycerides) (277). These data are concordant with those obtained in overweight or obese subjects by Kloting *et al.* They showed significant correlations between the expression of selected miRNAs and both AT morphology and key metabolic parameters, including visceral fat area, HbA1c, fasting plasma glucose, and circulating leptin, adiponectin, interleukin-6 (294).

Chronic inflammation in AT is a key feature of obesity and the MS. Inflammation *per se* could alter AT miRNA levels. TNF-α treatment of 3T3-L1 adipocytes mimicked the changes of miRNA expression observed in AT of obese mice (280). However, only a few individual miRNAs have been reported as playing a crucial role in this inflammatory state. Expression of miR-221 and miR-222 has been positively correlated to TNF-α and negatively correlated to ApN expression in white AT of mice (298). Yet, the direct effects of both miRNAs on these pro- or anti-inflammatory adipokines are still unraveled. Very recently, Zhuang *et al.* have demonstrated that miR-223 played a crucial role in modulating macrophage polarization in a pattern that protects mice from diet-induced adipose tissue inflammation and systemic insulin resistance. Consequently, miR-223 deficient mice fed a high-fat diet exhibited increased AT inflammation characterized by enhanced pro-inflammatory activation of macrophages (299).

3.4 Roles of microRNAs in other metabolic tissues

Besides adipose tissue, the regulatory roles of miRNAs have also implicated in other metabolically related tissues (Figure 16) (296).

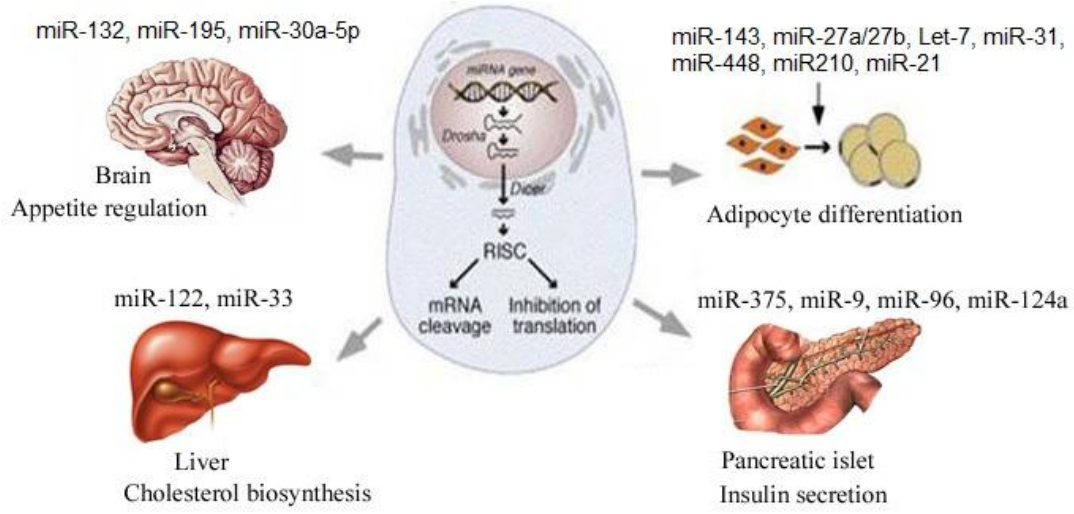

Figure 16: Regulation of metabolic pathways by miRNAs. The schematic illustration summarizes the current evidence from the literature for the function of miRNAs in the metabolism of different tissues (296; 300).

microRNAs in liver biosynthesis of cholesterol Multiple researchers have highlighted the important role that miRNAs play in hepatic lipid metabolism. miR-122 is a liver-specific miRNA involved in cholesterol and lipid metabolism and in hepatitis C virus replication (296) (301). Krützfeldt *et al.* provided evidence to support miR-122 as a key regulator of the cholesterol biosynthetic pathway; in particular they observed that the expression of at least 11

genes involved in cholesterol biosynthesis was decreased in antagomir-122-treated mice, including hydroxy-3-methylglutaryl-CoA-reductase (Hmgcr), a rate-limiting enzyme of endogenous cholesterol biosynthesis (302). Very recently, the role of miR-33 (a/b) has raised attention. miR-33 (a/b) are intronic miRNAs embedded in the sterol-response-element-binding protein genes SREBP2 and SREBP1, that repressed expression of the cholesterol transporter ABCA1, a key regulator of HDL biogenesis. Antagonizing miR-33a raised plasma HDL levels over 12 weeks in non-human primates, thereby opening new therapeutic strategy for reducing cardiovascular disease risk (303).

microRNAs in pancreatic β-cell biology miRNAs are necessary for proper pancreatic islet development as demonstrated in a mouse model lacking the miRNA processing enzyme Dicer selectively in the pancreas. Pancreatic-specific, Dicer-null mice display gross defects in all endocrine pancreatic lineages, with insulin-producing β cells being the most affected (304). miR-375 play a key role in pancreatic islet development. Deletion of miR-375 in mice causes a marked decrease in β-cell mass, which results in severe insulin-deficient diabetes in mature β cells, miR-375 also plays a negative regulatory role in glucose-induced insulin secretion. miR-375 is therefore important for several features of β cells, such as insulin expression and secretion as well as β-cell proliferation and adaptation to insulin resistance (304; 305). Additionally, at least three other miRNAs play a critical role in insulin exocytosis in β-cells. miR-9 and miR-96 in β-cells negatively regulate insulin exocytosis (306; 307), while miR-124a enhances insulin release at low-glucose concentrations but reduces high glucose-induced secretion (308).

microRNAs and neural factors promoting obesity miRNAs have recently been shown to be differentially expressed in brain tissue and have been linked to the regulation of neural factors specific to obesity, in particular the control of appetite. These miRNAs are also differentially expressed in neural signaling to liver, muscle, pancreas and gastrointestinal tract, to influence metabolism (296)**.** We will focus on a hypothalamic brain-derived neurotrophic factor (BDNF). BDNF plays a role of energy balance and has been implicated in the development of obesity (309). Low levels of BDNF have been linked to increased appetite and obesity (296). Both miR-30a-5p and miR-195 have been shown to target specific sequences surrounding the proximal polyadenylation site within the BDNF 3′-untranslated region on chromosome 11p13. Furthermore, neuronal overexpression of miR-30a-5p and miR-195 resulted in down-regulation of BDNF protein (296; 310; 311).

4. Therapeutic relevance of microRNAs

4.1 microRNAs as diagnostic tools

Recent studies have demonstrated that miRNAs can be secreted into the circulation. Chen *et al.* screened serum miRNAs of healthy Chinese subjects and found over 100 and 91 serum miRNAs in male and female subjects, respectively. Yet, how these miRNAs make their way into the circulation is still mysterious (312). The levels of miRNAs in serum are stable, reproducible, and consistent among individuals. This raises the potential of miRNAs as novel biomarkers for many disorders. Multiple reports have noted the potential clinical use of blood miRNAs for the diagnosis of cancer (313). For example, Heneghan *et al.* identified a systemic miRNA profile diagnostic of breast cancer, based largely on circulating miR-195 levels. Their results far highlight miR-195 as a potentially ideal breast tumor marker; circulating levels reflect tumor miR-195 levels, correlate with tumor size and stage of disease, decrease to basal level two weeks post-curative tumor resection, and miR-195 is not elevated in blood from patients with other malignancies (prostate, colon, renal, melanoma) (314). miRNAs can also serve as potential biomarkers for the metabolic syndrome. Heneghan *et al.* showed that circulating levels of miR-17-5p and miR-132 were significantly decreased in obese individuals compared to non-obese individuals and reflected miRNA expression in omental fat. The miRNA expression in blood and omental fat from obese patients correlated significantly with BMI, fasting blood glucose, and glycated hemoglobin (315).

4.2 microRNAs as therapeutic tools

The knowledge that miRNAs mediate potent and specific gene silencing makes them attractive therapeutic targets. “Silencing” of key miRNA, or “replacement” of certain tissue-specific miRNA whose expression is known to be decreased, are potential therapeutic interventions.

Techniques Several techniques have been set up to use the therapeutic potential of miRNAs. miRNAs can be deactivated and silenced by anti-miRNA oligo-nucleotides (AMOs), "miRNA sponges" and "miRNA masking" (316) (Figure 17). AMOs are synthetic antisense oligonucleotides that competitively inhibit the interaction between miRNAs and their mRNA targets. The most widely employed types of AMOs are 2’-O-methyl AMO, 2’-O-methoxyethyl AMOs and Locked Nucleic Acids (LNAs) (317). Given that miRNAs have been observed to function often in clusters in pathological processes, the "miRNA sponges"

has been developed to knockdown multiple miRNAs. This technique requires a vector encoding large quantities of transcripts (anti-miRNAs) which display numerous and tandem binding sites for a cluster of miRNAs of interest. The "miRNA masking" is an alternative miRNA-knockdown strategy to the AMO approach, with the advantage of targeting miRNAs in a gene-specific manner. Unlike an normal AMO, which binds to the endogenous miRNA directly, a miRNA mask binds to the miRNA's binding site located in the 3'-UTR of its target mRNA, thereby avoiding off-target effects (316).

Conversely, with regard to those miRNAs with decreased expression in disease, the fundamental principle strategy is to restore their expression. This can be achieved through miRNA mimicry or plasmid/viral vector-encoded miRNA replacement. miRNA mimics are small chemically altered double-stranded RNA molecules that imitate endogenous miRNAs (316) (Figure 17). Plasmid/viral vectors encoding miRNAs are encouraging strategies to replace miRNA *in vivo*, with good transduction efficiency and minimal toxicity (318; 319).

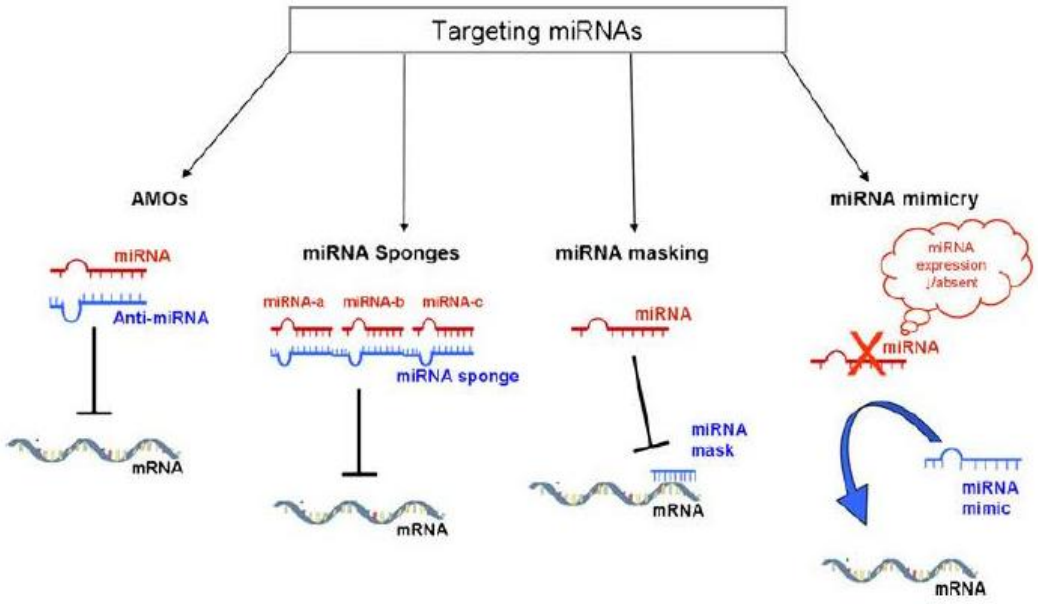

Figure 17: Strategies of miRNA manipulation and potential miRNA therapeutic strategies. Endogenous miRNAs can be down-regulated by anti-miRNA AMOs (anti-miRNA oligonucleotide), miRNA sponges, and miRNA-masking. AMOs can bind to complementary miRNAs and induce either duplex formation or miRNA degradation. MiRNA sponges exhibit multiple miRNA binding sites, resulting in the ability to simultaneously sequester multiple miRNAs. MiRNA masks are complementary to the 3′UTR of the target miRNA, resulting in competitive inhibition of the downstream target effects. The effects of miRNAs can be restored by introducing synthetic miRNAs (miRNA mimicry) (316).

microRNAs and therapeutics To date, the greatest efforts have been made in exploring the potential application of miRNAs therapeutics in cancer and liver disorders. Gain or loss of function of individual miRNAs have been reported in almost every solid and hematological

cancer, with therapeutic suppressing effect in tumor cell proliferation, progression of tumors and the metastatic process (316). The liver specific miR-122 has been proved to regulate cholesterol biosynthesis and hepatitis C virus replication. Silencing of miR-122 by intraperitoneal administration of high affinity LNA anti-miR-122 has resulted in dose-dependent lowering of plasma cholesterol in mice and non-human primates (monkeys). This was achieved without significant adverse reactions or hepatic toxicity (302; 320). Also, the intravenous injection of the drug SPC3649 (containing LNA anti-miR-122) is currently undergoing Phase I clinical trials for treatment of human hepatitis C infection (www.laboratorytalk.com/news/saa/saa104.html).

4.3 Potential challenges in miRNA-based therapy

Although significant advances have been made in miRNA-based therapy, various challenges remain to be overcome before clinical use. First, one individual miRNAs may have multiple potential targets, which may coordinate or antagonize each other's functions. In addition, miRNA-target interactions depend not only on the sequence of the target site but also on the cellular context, in which the interactions occur. This complexity explains the difficulties in predicting the spectrum of side effects and toxicity profiles, which may be associated with miRNA-based therapeutics. Second, replacement of miRNA may potentially lead to oversaturation of endogenous miRNAs pathway. High levels of exogenous miRNA can compete with the endogenous miRNA biogenesis processing, leading to cell toxicity (321; 322). Another critical issue is the site-specific, safe, effective and repeat delivery of miRNA *in vivo*. The systemic intravenous delivery of viral or liposomal-mediated miRNA oligonucleotides have been well achieved in cancer and liver disease. However, targeting certain miRNA in other specific tissues is poorly achieved due to the invasive nature of access for repeat delivery and lack of cell-specific up-take. Restoration of tissue-specific miRNA expression could be a more rational approach for managing local abnormalities with more efficiency and precision and with less systemic side effects (316; 322).

Chapter 2: Objectives

The lab has generated a transgenic mouse model with targeted overexpression of ApN into white AT. ApN has emerged has a potent anti-inflammatory factor.

Since AT chronic inflammation is considered to build the common soil for the development of the MS, I took advantage of this transgenic model to examine whether ApN regulates the secretory profile of downstream adipokines and induces a shift of the immune balance in adipocytes and/or SVCs toward a less inflammatory phenotype. Next, I hypothesized that miRNAs regulated by ApN may mediate some of the anti-inflammatory effects of ApN on AT by directly targeting genes involved in inflammatory pathways (Figure 18).

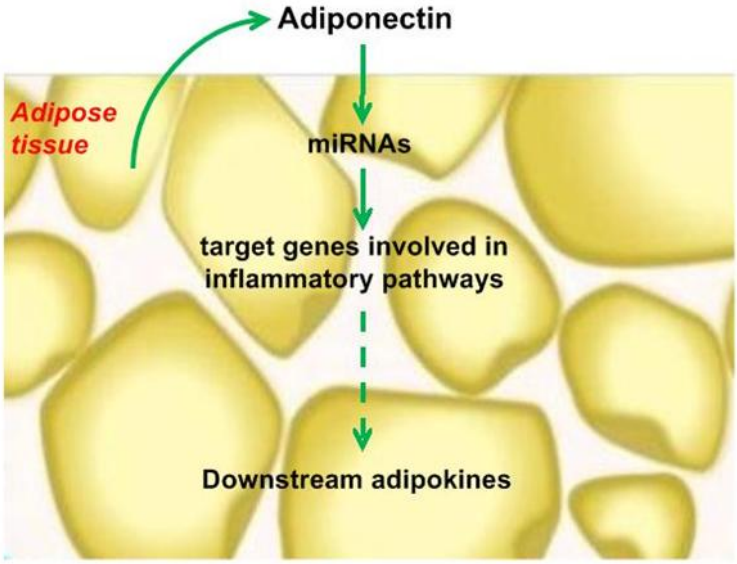

Figure 18: Hypotheses of my thesis

I therefore investigated the effects of ApN on the secretion of downstream adipokines (article 1) and the expression/role of miRNAs (article 2).

Objective 1

Article 1: Ge Q, Ryken L, Noel L, Maury E, Brichard SM 2011 Adipokines identified as new downstream targets for adiponectin: lessons from adiponectin-overexpressing or -deficient mice. Am J Physiol Endocrinol Metab 301:E326-E335

To investigate *in vivo* the auto/paracrine effects of ApN on adipose tissue secretory function. I took advantage of our unique mouse model overexpressing ApN (ApN-Overex) to investigate the effect of ApN on the secretion of downstream adipokines by cytokine antibody

array profiling and subsequent ELISA measurements. These experiments were carried out on young mice studied before emergence of any metabolic confounding factors (such as improvement of insulin action or decrease of fat mass). I further examined whether a reverse profile occurred in ApN-knockout (ApN-KO) mice.

Objective 2

Article 2: Ge Q, Gerard J, Noel L, Scroyen I and Brichard SM. MicroRNAs Regulated by Adiponectin as Novel Targets for Controlling Adipose Tissue Inflammation. Endocrinology 2012

To explore whether miRNAs regulated by ApN *in vivo* contribute to the anti-inflammatory effects of ApN in AT and whether these miRNAs represent novel mechanisms for controlling AT inflammation. I used the AT of the same ApN-Overex and ApN-KO mouse models to identify several miRNAs specifically regulated by ApN *in vivo* by microRNA expression profiling and subsequent RT-qPCR. The biological functions of miRNAs were predicted by computational algorithm and were further validated by gain- or-loss of function approaches in 3T3-F442A adipocytes *in vitro* as well as in *de novo* formed fat tissue *in vivo*.

Additional paper

Article 3: Ge Q, Maury E, Rycken L, Gerard J, Noel L, Detry R, Navez B and Brichard SM. Endocannabinoids regulate adipokine production and the immune balance of omental adipose tissue in human obesity. Int J Obes (Lond) 2012.

The objectives of this additional study were to assess whether the modulation of the cannabinoid type 1 receptor (CB1R) directly regulates *in vitro* the production of ApN and other adipokines by omental adipose tissue (OAT) of obese subjects, to establish in which cellular fraction of OAT the effects of CB1R blockade take place and to unravel the underlying mechanisms.

Because this study was carried out in a different model to the first two ones, its results and the related discussion do not represent the core of my thesis, but are presented as an annex at the end of this work.

Chapter 3: Results

Am J Physiol Endocrinol Metab 301:E326-E335

Adipokines identified as new downstream targets for adiponectin: lessons from adiponectin-overexpressing or deficient-mice

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Running title: Adipokines identified as new targets for adiponectine

Precis : Adipokines identified as new downstream targets for adiponectin by secretome analysis: studies in adiponectin-overexpresing or- deficient mice

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ABSTRACT

Adipokines play a central role in the pathogenesis of the metabolic syndrome. Among them, Adiponectin (ApN), a master regulator of immune and fuel homeostasis is decreased. Identifying downstream adipokines targeted by ApN may help in deciphering this syndrome. We have generated transgenic mice allowing persistent and moderate overexpression of ApN (ApN-Overex) specifically in white adipose tissue (AT). We took advantage of this model to unravel the adipokine secretion profile triggered by ApN. AT was fractionated into adipocytes and stromal-vascular cells (SVC), which were cultured for 8 h. Profiling of secretory products by antibody arrays and subsequent ELISAs showed that the secretion of 3 pro-inflammatory factors (IL-17B, IL-21, TNF-α) and 3 hematopoietic growth factors (GF)(thrombopoietin and granulocyte-(macrophage)-colony-stimulating-factors) was reduced in adipocytes of ApN-Overex mice when compared to wild-type mice. In SVC of these mice, besides the hematopoietic GFs, the secretion of another GF (vascular endothelial GF receptor 1, VEGFR1), 2 chemokines (RANTES, ICAM-1) and 2 pro-inflammatory factors (IL-6, IL-12p70) was reduced as well. Only one cytokine was oversecreted by SVC of ApN-Overex mice: Interleukin 1 receptor 4 (IL-1R4/ST2), which may exhibit anti-inflammatory properties. Most of these changes in secretion were due to corresponding changes in mRNAs. A reverse profile of adipokine expression was observed in ApN-KO mice. In conclusion, ApN regulates *in vivo* the secretion of downstream adipokines, thereby inducing a shift of the immune balance in both adipocytes and SVC toward a less inflammatory phenotype. These downstream adipokines may be new therapeutic targets for the management of the metabolic syndrome.

Key words: adiponectin, inflammation, adipocytes, stromal-vascular cells, secretome

INTRODUCTION

Adipose tissue (AT) secretes a number of bioactive peptides, collectively named adipokines. They may exert endocrine actions on distant organs or autocrine/paracrine actions on AT. They play a central role in energy and vascular homeostasis as well as in immunity (1). Virtually all known adipokines are dysregulated in obesity, type 2 diabetes and metabolic syndrome leading to overproduction of deleterious adipokines and hyposecretion of defensive ones, such as ApN. Such a dysregulation triggers the development of a low-grade pro-inflammatory state, which is considered to build the common soil for the development of obesity-linked disorders (1). Resetting the immunological balance in adipose tissue may be a crucial approach for the future management of the metabolic syndrome. Adipokines may be potential therapeutic targets.

ApN has emerged as a master regulator of immune/inflammatory and fuel homeostasis(2). Besides its vascular protection (3), ApN exerts anti-inflammatory effects on other organs such as liver, colon, cardiac or skeletal muscles (4-7). As yet, the autocrine and paracrine anti-inflammatory effects of ApN on its own production site have been scarcely described (8-10). Moreover, its effects on adipose tissue secretory functions have only been reported in a few numbers of *in vitro* studies (8; 9). Thus, identifying downstream adipokines targeted by ApN *in vivo* may be instrumental in highlighting its action on AT.

We have generated a transgenic mouse model allowing persistent and moderate overexpression of ApN (ApN-Overex) specifically in white adipose tissue by using the aP2 promoter (11). We took advantage of this unique *in vivo* model to unravel the adipokine secretion profile triggered by ApN in AT. We also examined whether a reverse profile occurred in ApN-knockout (ApN-KO) mice (10).

RESEARCH DESIGN AND METHODS

Animals

Male ApN-Overex mice and their wild-type (WT) littermates were housed in groups of 2 to 4 at a constant temperature (22 C) with a fixed 12-h light, 12h-dark cycle. The two groups of mice were matched for weaning body weight. They were weaned to a high-sucrose diet (HS) (TD00220; Harlan, Horst, Netherlands) as mouse phenotype and degree of transgene expression were characterized in those conditions (11). In an additional experiment, male ApN-KO mice, which exhibit a complete lack of ApN in fat and plasma, and their WT controls (mice of the same genetic background, raised together with ApN-KO mice, but which were not their littermates) were also used. These animals were housed in the same conditions as ApN-Overex mice and received the same diet from weaning.

Body weight was measured every week. At the end of the experiments, mice were sacrificed by decapitation (between 0800 and 1000 h). Tail vein blood samples were saved before sacrifice. Inguinal fat pads (the adipose tissue site where the effects of the transgene ApN were the most pronounced; (11)) were quickly removed, weighed and either directly used for culturing or frozen in liquid nitrogen and stored at -80 C for subsequent experiments.

The University Animal Care Committee approved all procedures.

Quantification of circulating parameters

Blood glucose was measured using a glucometer (MediSense Precision Xtra Plus®, Abott-Medisense, Louvain-la-Neuve, Belgium). Plasma insulin was measured by RIA (kit from Linco Research, St Charles, MO, USA) and plasma triglycerides by a colorimetric method (kit from BD Biosciences, Erembodegem, Belgium).

Adipose tissue immunohistochemistry and morphometry

Adipose tissue samples were fixed in 10% formaldehyde for 24 h and embedded in paraffin. Five µm thick sections were processed, using a rat monoclonal antibody directed against mouse F4/80, a macrophage marker (AbD, Serotec, Dusseldorf, Germany), according to the manufacturer‘s recommendations. Sections were pretreated in a microwave oven in Tris-citrate buffer (pH 6.5) for one cycle of 3 min at 750 W and 3 cycles of 3.5 min at 350 W. Binding of the antibody was detected by applying for 30 min at room temperature a second antibody, which was a rabbit anti-rat immunoglobulin conjugated to peroxidase labelled polymer (En Vision +, Dako, Copenhagen, Denmark). Peroxidase activity was revealed with

DAB substrate (3,3'-Diaminobenzidine, Dako, Copenhagen, Denmark) which produces a brown staining. For quantification, the number of immunolabelled macrophages per microscopical field (density) was counted, at magnification X 400, on 20 fields for each mouse (7). Adipocytes areas were measured by an image analyzer system (MOP-Vidioplan; Kontron, Eching, Germany); at least two hundred adipocytes, randomly chosen in 7-8 fields per section were counted for each mouse (12).

Culture of isolated adipocytes and stromal-vascular cells (SVC)

We used an established protocol (13; 14), with slight modifications, to study the release of adipokines by isolated cells of AT. This protocol has been validated for secretome analysis: under those experimental conditions, neither tissue fractionation nor culturing per se did alter adipokine expression levels (13).

Briefly, fresh inguinal adipose tissue (0.3 g) was finely minced and incubated in a shaking waterbath at 37°C in 750 µl Krebs-BSA buffer (KRAB) containing collagenase A (Roche Diagnostics, Vilvoorde, Belgium; 2.5 mg/0.3 g tissue) for 15 minutes. The digested tissue was filtered through a metallic mesh and then centrifuged at 400 *g* for 1 minute. The supernatant (containing the adipocytes) and pellet [containing the stromal-vascular cells (SVC)] were separated and washed 3 times in KRAB. The pellet (SVC) was resuspended in 80 µl KRAB. Isolated cells were then cultured in 48 well plates. Experiments were performed in duplicate. 80 µl packed adipocytes or 40 µl SVC were distributed per well, which contained 700 µl MEM supplemented with 1% BSA and 1/500 (v/v) antibiotics (Primocin, InvivoGen, USA). Cells were allowed to stabilize for 1 h in this medium; next, the medium was renewed and the cells were cultured for 8 h. After culturing, aliquots of medium were stored at -20 C, and cells were harvested and stored at -80°C. DNA content was measured in cell samples as described before (13).

Cytokine antibody arrays and ELISAs or RIA on adipocyte- or SV cell-conditioned media

Screening for cytokines secreted by cultured adipocytes or SV cells was performed by hybridizing medium with antibody-coated membranes according to the protocol supplied by the manufacturer [RayBio Mouse Cytokine Antibody Array C Series 2000, a kit combining membranes of Array III, VI and V and allowing the simultaneous detection of 144 cytokines (cat. no. AAM-CYT-2000, for details see http://www.raybiotech.com/manual/Antibody%20Array/AAM-CYT-2000.pdf (RayBiotech, Inc. Parkway Lane, USA)]. Briefly, 1 ml medium was incubated with arrayed antibody supports

for 1 h 30 min at room temperature; membranes were then washed and incubated with the mix of biotin-conjugated antibodies for another 1 h 30 min. After washing, horseradish peroxidase-conjugated streptavidin was added to the membranes for 1 h at room temperature. Spots intensities on membranes were quantified by scanning densitometry (Gel-Doc2000, Bio-Rad Laboratories, UK Ltd), analyzed with Quantity One^{TM} (Bio-Rad). Non-conditioned media (containing 1% BSA) were used as negative controls, as already described (13). Signals were normalized to internal positive controls present on each membrane (see Fig. 2), and then expressed as intensity units per µg DNA in each cellular fraction. The experiments on both genotypes of mice were always carried out simultaneously.

The cytokines identified by protein antibody arrays as differentially secreted between the two genotypes of mice ($P < 0.1$) were further quantified by specific ELISAs: Chemokine (C-X-C motif) ligand 5 (Cxcl5) (LIX), Granulocyte colony-stimulating factor (GCSF), Granulocyte macrophage colony-stimulating factor (GM-CSF), Growth arrest-specific 6 (Gas6), Interleukin-6 (IL-6), Interleukin-21 (IL-21), Interleukin-17B (IL-17B), Interleukin-12 (IL-12p70), Interleukin 1 receptor 4 (IL-1R4/ST2), Inter-cellular adhesion molecule 1(ICAM-1), Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES), Tumour Necrosis Factor-α (TNF-α), Thrombopoietin (TPO), Vascular Endothelial Growth Factor Receptor-1 (VEGFR1) (all from RayBiotech, Parkway Lane, USA) except for IL-12p70, IL-1R4 from R&D Systems Europe Ltd., Abingdon, United Kingdom). However, we did not quantify TIMP-2 by this method, because its function on AT is not clear and there was no commercial kit available. On the other hand, we measured TNF-α by ELISA, which actually escaped detection by arrays, because of the known relationships between TNF-α and ApN (10) and our past experience [the background generated by non-conditioned medium may mask the low amounts of some adipokines secreted by cultured cells (such as TNF-α)] (13). ApN was measured in media by a RIA kit (Linco Research).

RNA extraction and Real-time quantitative polymerase chain reaction (RTQ-PCR)

Total RNA from cells or tissue was extracted by using TriPure Isolation Reagent (Roche Diagnostics, Vilvoorde, Belgium). Total RNA (0.2 µg - 2 µg) were reverse transcripted as described (15). RTQ-PCR primers were designed using Primer Express Software (Applied Biosystems; see Table 2). For VEGFR1 and IL-1R4 mRNAs, the sets of primers used did not discriminate between soluble or transmembrane receptor isoforms and thus, global gene expression was measured. Total RNA equivalents (4 ng - 40 ng) were

amplified with iQSyber Green Supermix (Bio-Rad) containing 300 nM of each specific primer using iCycler iQ Real Time PCR detection System (Bio-Rad). Briefly, the threshold cycles (Ct) were measured in duplicate. ΔCt values were calculated in every sample for each gene of interest as follows: $Ct_{\text{gene of interest}} - Ct_{\text{reporter gene}}$ with cyclophilin as the reporter gene. Relative changes in the expression level of one specific gene (ΔΔCT) were calculated as ΔCT of the test group minus ΔCT of the reference group, and then presented as $2^{-\Delta\Delta Ct}$.

Western blot

Adipose tissue was homogenized in a lysis buffer (Cell Signaling Technology, BIOKÉ, Leiden, Netherlands) supplemented with 100 mM NaF and 1% protease inhibitor cocktail (Active Motif, Rixensart, Belgium). Proteins (30 µg) were dissolved in Laemmli buffer, subjected to SDS-PAGE under reducing and heat-denaturating conditions and then transferred to PVDF membrane. The following antibodies were used for immunodetection: anti-AMPKα, Phospho-AMPKα (Thr172), anti-p44/42 MAPK (Erk1/2), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-SAPK/JNK, Phospho-SAPK/JNK (Thr183/Tyr185). Each antibody was used according to the manufacturer's instructions (Cell Signaling Technology, BIOKÉ, Leiden, Netherlands). Signals were revealed by enhanced chemiluminescence. Band intensities were quantified by scanning densitometry (Gel-Doc2000, Bio-Rad Laboratories, UK Ltd), analyzed with Quantity One™ (Bio-Rad) and normalized to Actin band intensity.

NF-kB activity

The TransAM NF-κB p65 transcription factor assay kit (Active Motif, Rixensart, Belgium) was used, as already described (14). Binding of p65 NF-κB transcription factors was detected by ELISA. 10 µg adipose tissue proteins, extracted as indicated for Western blots, were used per well. Samples were run in duplicate.

Presentation of the results and statistical analysis

The results are means ± SEM for the indicated numbers of mice. Comparisons between two different groups were carried out using two-tailed unpaired Student's t-test. Differences were considered statistically significant at $P<0.05$. For the screening by cytokine antibody arrays, the differences that were statistically significant ($P<0.05$) and those that were "borderline" ($P<0.1$) were considered for subsequent analysis in order-to avoid false-negatives due to the low number of mice used in this screening.

RESULTS

Overexpression of ApN reduced body weight, adiposity and improved lipid and glucose parameters

Because ApN over-expression targeted to white adipose tissue resulted in reduced adiposity and improved metabolic profile (11), we first examined the evolution of these parameters. Inguinal fat pad was studied because the expression of the transgene was the most pronounced in this adipose site (11).

ApN overexpression did not influence body weight, fat pad weight, plasma triglycerides and glucose homeostasis as well as insulin sensitivity in 8- and 10-wk-old mice (Table 1). However, all these parameters were significantly modified from the age of 12 wks: adiposity and circulating triglycerides were reduced while insulin action was enhanced, as expected (11) (Table 1). Therefore, we used 10-wk-old mice to study the specific and direct effects of ApN on adipose tissue secretory function without the interference of any obvious confounding factors. We also checked that adipocyte size was similar in 10-wk-old ApN-Overex mice and their WT littermates by morphometry analysis of adipose tissue sections. There was also no difference in macrophage number between the two genotypes of mice as shown by immunochemistry using the specific macrophage marker F4/80 (Table 1).

Table 1. Characteristics of ApN-Overexpressing mice

	8 weeks		10 weeks		12 weeks	
	WT	ApN-Overex	WT	ApN-Overex	WT	ApN-Overex
Body weight (g)	21.5 ± 1.0	22.3 ± 0.7	25.1 ± 1.0	24.7 ± 1.0	28.3 ± 0.5	25.4 ± 0.6*
Inguinal adipose tissue						
Fat pad weight (mg)	277 ± 25	338 ± 20	387 ± 50	342 ± 22	522 ± 38	302 ± 24**
Relative weight (% body wt)	12.9 ± 1.1	15.2 ± 0.9	15.4 ± 1.9	13.8 ± 0.5	20.5 ± 0.7	12.5 ± 1.4**
Adipocyte size (μm^2)	—	—	73.6 ± 6.9	74.2 ± 11.8	—	—
Macrophage density	—	—	2.6 ± 0.4	2.7 ± 0.5	—	—
Circulating parameters						
Plasma triglycerides (mg/dl)	48.3 ± 3.9	43.3 ± 6.0	58.9 ± 5.9	53.6 ± 2.7	61.4 ± 3.1	50.7 ± 2.2*
Blood glucose (mg/dl)	156 ± 7	149 ± 9	164 ± 9	144 ± 13	178 ± 9	154 ± 5*
Plasma insulin (ng/ml)	0.56 ± 0.06	0.54 ± 0.07	0.59 ± 0.07	0.5 ± 0.07	1.18 ± 0.10	0.81 ± 0.06*
Insulin sensitivity index (G/I; mg/ng)	2.90 ± 0.27	2.96 ± 0.37	2.88 ± 0.21	3.01 ± 0.26	1.54 ± 0.10	1.91 ± 0.07*

ApN-Overexpressing (ApN-Overex) mice and their wild-type (WT) littermates were studied at the age of 8, 10 and 12 weeks. Blood was sampled at 0800 h-1000 h. Organ weight refers to pairs of inguinal fat pads, and relative organ weight is expressed as % body weight (i.e. as gram per gram body weight X 100). Adipocyte size is estimated from mean adipocyte area. Macrophage density corresponds to mean macrophage number per microscopical field, at magnification X 400. The index of insulin sensitivity (G/I) is calculated as Glucose / Insulin (mg/ng) (16). Data are means ± SEM for 5-6 mice per group. *P < 0.05, **P < 0.01 vs. WT; —, not measured.

ApN production by adipocytes and SVCs of ApN-Overex mice

ApN gene was overexpressed in both cellular fractions of adipose tissue in transgenic mice compared to WT mice (Fig. 1A). However, the overall expression of ApN was much higher in adipocytes than in SVC (which contain macrophages), in line with the fact that the transgene was placed under control of aP2, which is much more abundantly (~10.000-fold) expressed in adipocytes than in macrophages (17). Accordingly, the levels of ApN secreted in culture medium were higher in ApN-Overex mice than in WT mice, with again a more abundant secretion by adipocytes (Fig. 1B).

Gene expression levels of ApN receptors (AdipoR1 and AdipoR2) were measured in both cellular fractions and did not markedly vary between mice of the two genotypes (data not shown).

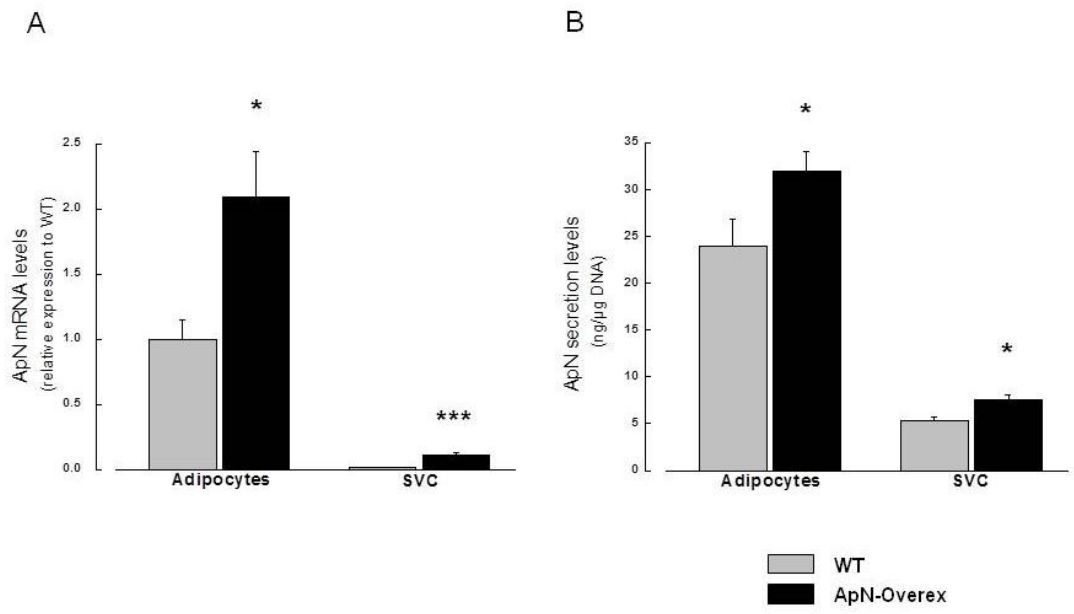

Fig. 1. ApN production by adipocytes and stromal-vascular cells (SVC) from ApN-overexpressing (ApN-Overex) mice. Adipocytes and SVC were isolated from adipose tissue of transgenic mice and wild-type (WT) littermates and cultured independently for 8 h. (A) ApN gene expression was quantified by RTQ-PCR, normalized to the levels of cyclophilin and presented as relative expression compared to WT adipocytes. (B) ApN secreted in medium was measured by RIA and expressed as ng per µg DNA in each cellular fraction. Values are means ± SEM for 7-8 mice per group. **P* <0.05, ****P* <0.001 or less *vs.* WT mice.

Adipokine secretion profiling in ApN-Overex mice

Medium from 8-h cultured adipocytes or SVC were screened by cytokine antibody arrays (Fig. 2). Among the 144 cytokines tested, more than 40 were secreted by each cellular

fraction in mice of both genotypes. The secretory profile of adipocytes showed that 9 cytokines were or tended to be less secreted by ApN-Overex mice than WT mice (Fig. 3A, P<0.1 or less). In the SV fraction, 10 cytokines were or tended to be differentially secreted between mice of the two genotypes (Fig. 3B, P<0.1 or less). These cytokines belong to 5 families: pro-inflammatory factors, growth factors, chemokines, anti-inflammatory factors and extracellular matrix/ growth arrest factors (Fig. 2).

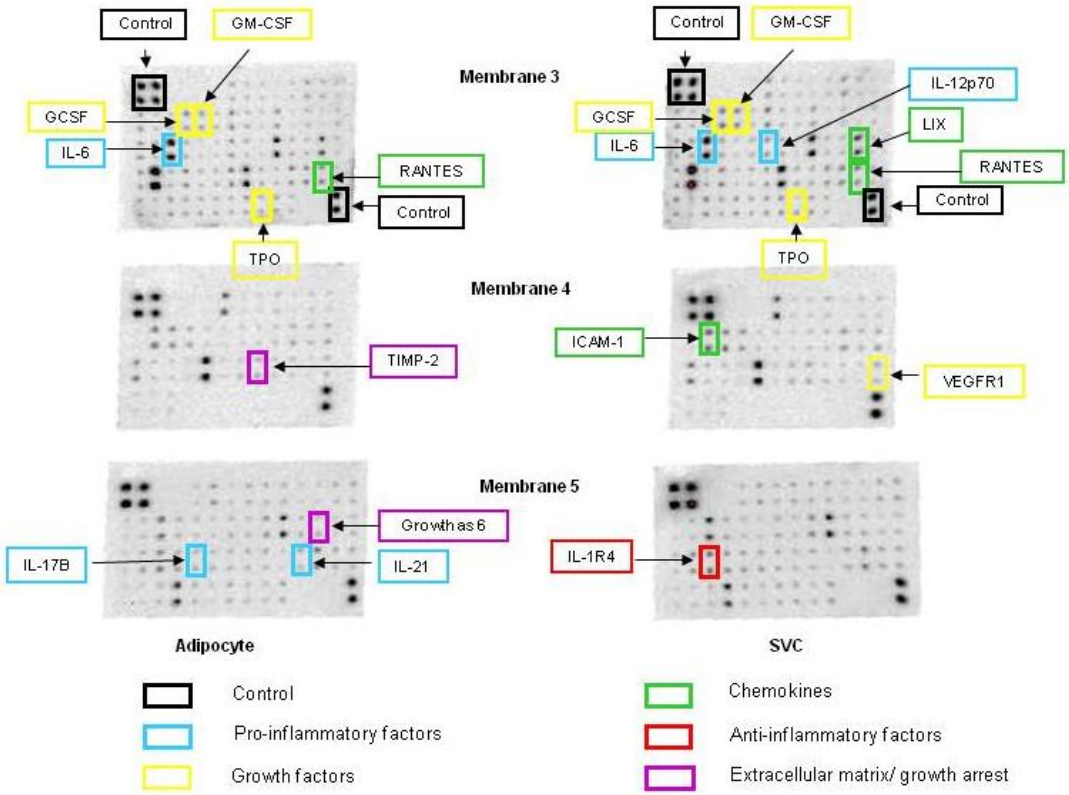

Fig. 2. Cytokine antibody array membranes used to detect adipokines secreted by each cellular fraction of adipose tissue. A set of 3 Raybiotech membranes, which tests up to 144 cytokines was used and probed with 8-h conditioned medium. This figure illustrates the representative arrays of adipocyte- or SVC-conditioned medium from one WT mouse. Highlighted adipokines represent the factors, which turned out to be differentially secreted between mice of the two genotypes. Each colour corresponds to a specific factor family.

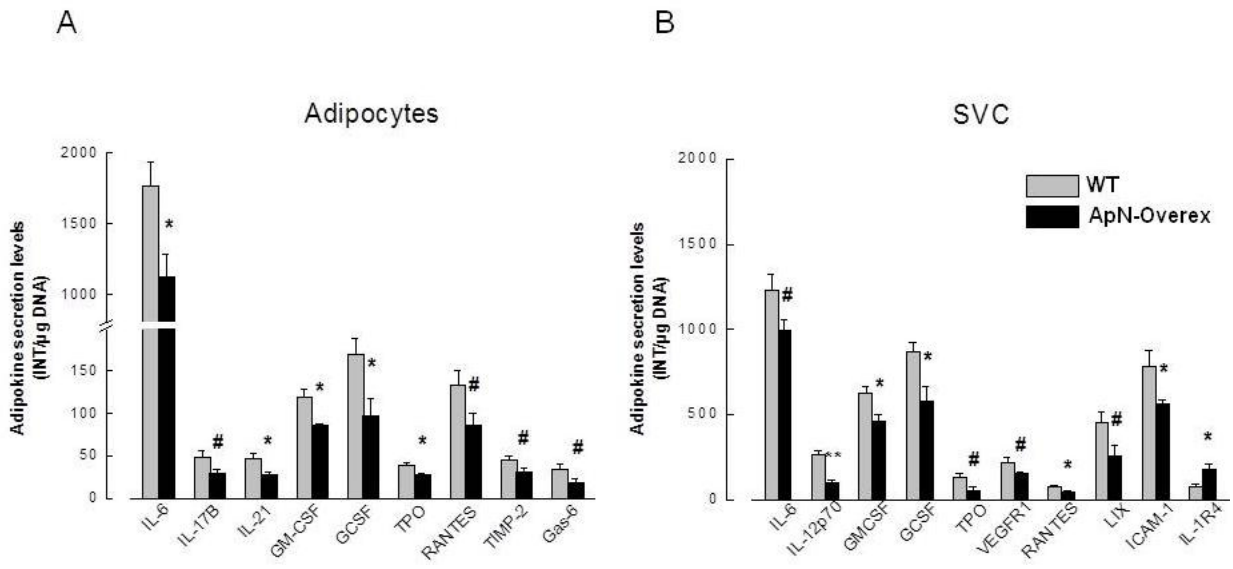

Fig. 3. Screening secretome of adipocytes and SVC from ApN-overex mice. Adipocytes and SVC were cultured independently for 8 h. Conditioned medium were incubated with the sets of cytokine antibody arrays like those shown in figure 2. Adipokine levels were measured by chemiluminescence, normalized to internal positive controls and expressed as intensity units (INT) per µg DNA in each cellular fraction. Only the adipokines, which differed (or tended to differ) between the two genotypes of mice are shown in the histograms. (A) Adipocyte secretome screening: 9 adipokines were or tended to be less secreted by ApN-Overex mice than WT mice. (B) SVC secretome screening: 10 adipokines were or tended to be differentially secreted between ApN-Overex and WT mice. Values are means ± SEM for 4 mice per group. **P* < 0.05, ***P* <0.01 *vs.* WT mice. Differences, which tended to be, but were not, statistically significant, are also indicated on this figure (*# P* <0.1) and were considered for subsequent ELISA analysis.

These adipokines (i.e. differently secreted based on cytokine array screening; P<0.1 for ApN-Overex *vs.* WT) were further quantified by specific ELISAs. TNF-α was also measured by this method. Only the adipokines that turned out to be different after ELISA quantification are shown on Fig .4 (P <0.05 or less for ApN-Overex *vs*. WT).

In adipocyte-conditioned media, the secretion of 3 pro-inflammatory factors (IL-17B, IL-21, TNF-α) and 3 hematopoietic growth factors (TPO; GM-CSF and G-CSF) was reduced in ApN-Overex mice compared to WT mice (Fig. 4A). In SVC-conditioned media of transgenic mice, besides the 2 hematopoietic growth factors (GM-CSF and G-CSF), the secretion of a receptor for VEGF (VEGFR1), 2 chemokines (RANTES, ICAM-1) and 2 pro-inflammatory factors (IL-6, IL-12p70) was reduced as well. Only one cytokine was oversecreted by SVC of ApN-Overex mice: IL-1R4 that exhibits anti-inflammatory properties (Fig. 4B).

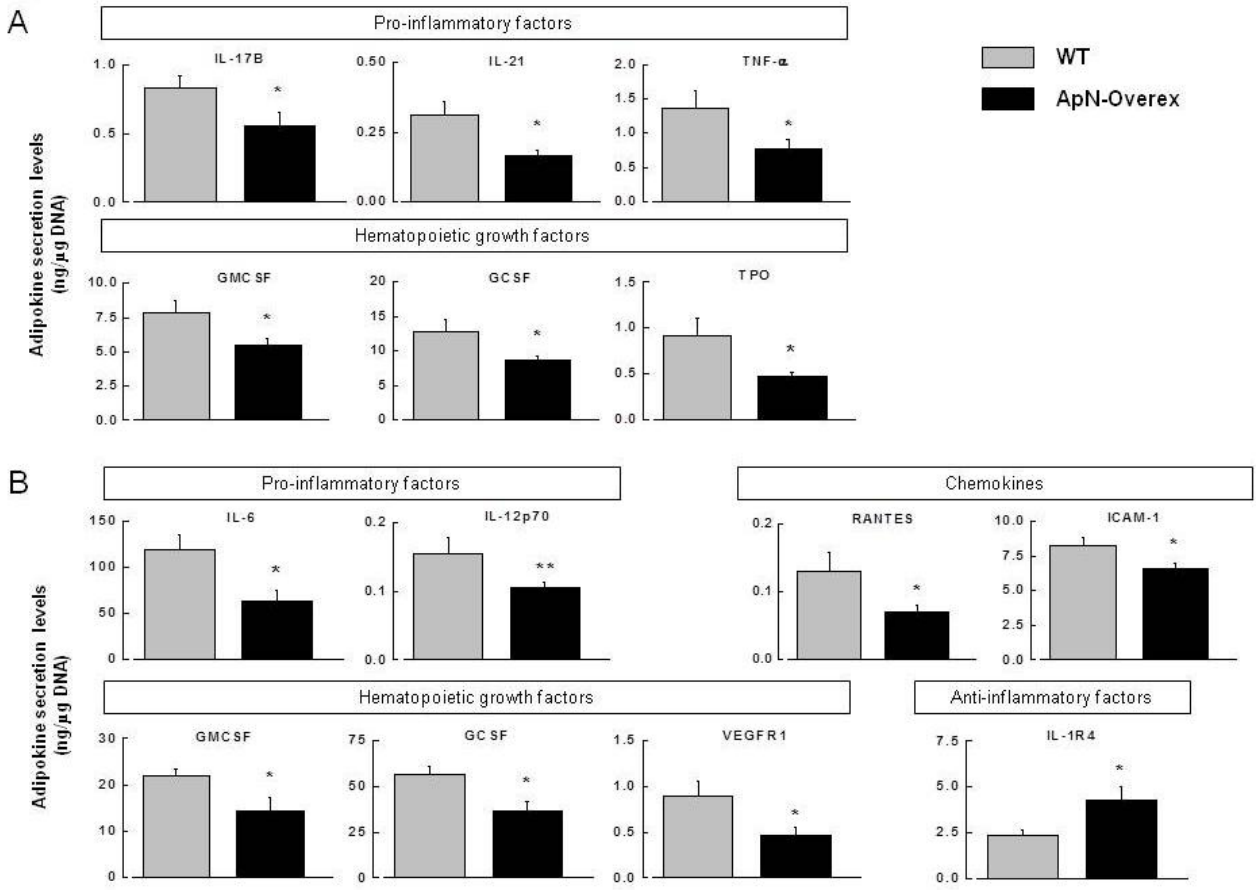

Fig. 4. Quantification of adipokine levels secreted by adipocytes (A) and SVC (B) from ApN-overex mice. Adipokines, which were (or tended to be) differentially secreted between the two genotypes of mice, were firstly identified by cytokine antibody arrays (see figures 2 and 3). Afterwards, adipokine concentrations in culture medium were quantified by specific ELISAs and expressed in ng per µg DNA. Values are means ± SEM for 5-6 mice per group. **P*<0.05 *vs.* WT mice.

Gene expression of adipokines in adipocytes and SVC of ApN-Overex mice

To investigate whether the adipokine secretion changes were in part mediated by pre-translational mechanisms, we quantified gene expression. Most adipokines exhibited a similar pattern between mRNA abundance and secretion levels. Thus, in adipocytes, mRNA levels of most adipokines shown on Fig.4 were down-regulated in ApN-Overex mice (Fig. 5A). In SVC, mRNA levels of most adipokines were down-regulated as well, while IL-1R4 mRNA was up-regulated (Fig. 5B). The attenuated inflammatory response of SVC is consistent with a phenotypic switch of macrophages (18). Thus, in SVC, the abundance of a M1 marker, CD11c, which characterizes a pro-inflammatory macrophage phenotype was halved in ApN Overex-mice (relative expression to WT: 0.56±0.09 *vs.* 1.00±0.13, n=6 per group; P<0.05), while the mRNA abundance of a M2 marker, Macrophage Galactose N-acetyl-galactosamine-specific Lectin-1 (Mgl1), which characterizes an anti-inflammatory phenotype was increased

two-fold (2.16±0.46 *vs.* 1.00±0.26, n=6 per group; P<0.05), in line with the opposite phenotype reported in ApN-KO mice (19)

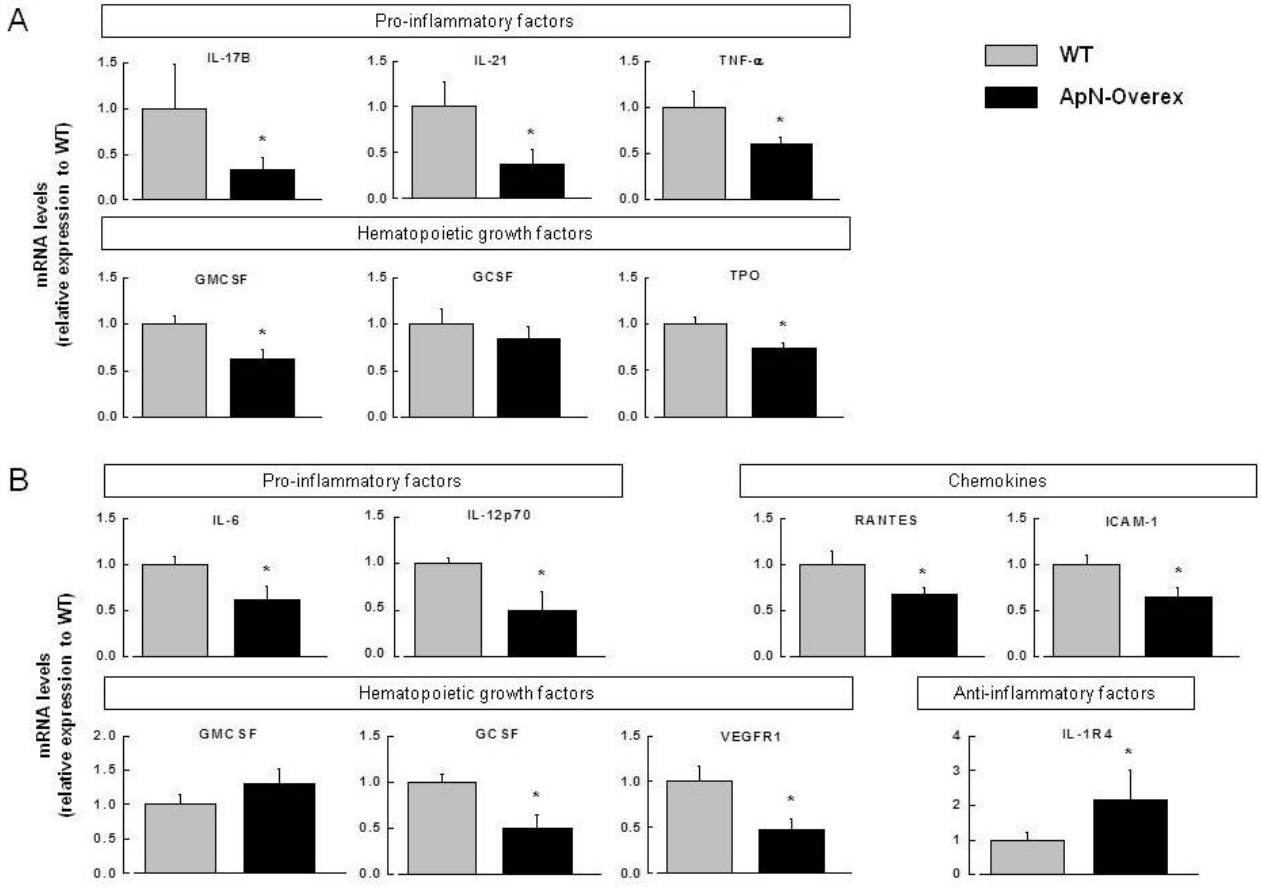

Fig. 5. Gene expression of adipokines in adipocytes (A) and SVC (B) from ApN-Overex mice. mRNA levels of the adipokines, which were measured by ELISA in figure 4, were quantified by RTQ-PCR. Values were normalized to the levels of cyclophilin and presented as relative expression compared to WT mice. Results are presented as means ± SEM for 6-7 mice per group.*P< 0.05 *vs.* WT mice.

Gene expression of adipokines in adipocytes and SVC of ApN-KO mice

We next explored whether ApN was the specific regulator of these pre-translational changes and measured mRNA levels of these cytokines in ApN-deficient mice of the same age and receiving the same diet. It should be noted that, at this age, there were also no differences in body weight, fat pad weight, circulating glucose and triglycerides levels between ApN-KO mice and their WT controls (data not shown). In adipocytes, TPO mRNA was increased in ApN-KO mice (P<0.01 *vs.* WT) (Fig. 6A) and thus showed a reverse pattern of expression when compared to ApN-Overex mice. In SVC, gene expression of IL-12p70, ICAM-1 and VEGFR1 was increased in ApN-KO mice while IL-1R4 mRNA was decreased

as expected (Fig. 6B). Thus, some adipokines displayed a reverse gene expression profile in ApN-KO mice compared to ApN-Overex mice.

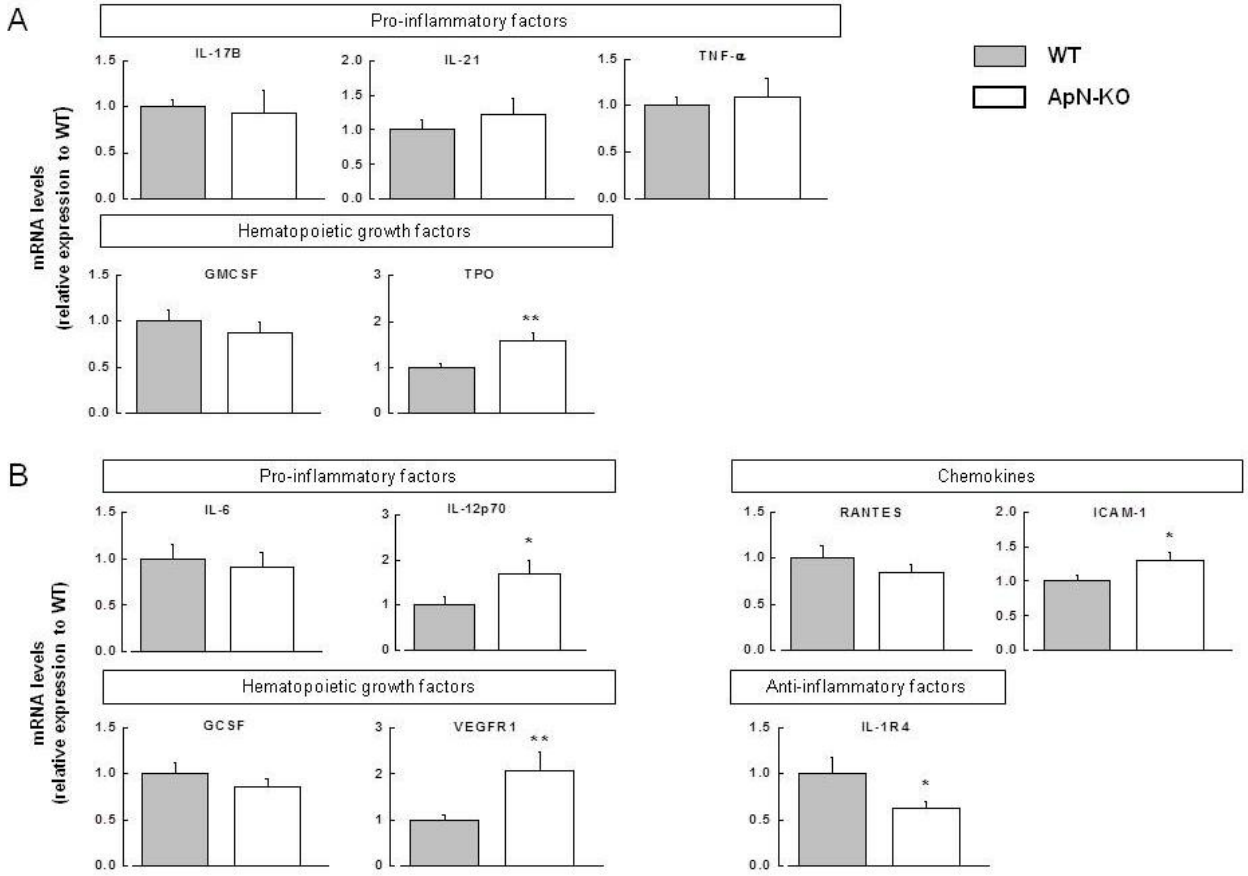

Fig. 6. Gene expression of adipokines in adipocytes (A) and SVC (B) from ApN-KO mice. The adipokines whose gene abundance was altered in ApN-overex mice (see figure 5) were tested in both cellular fractions of ApN-KO mice. Adipokine mRNA levels were quantified by RTQ-PCR, normalized to the levels of cyclophilin and presented as relative expression compared to WT values. ApN-KO mice were of the same age, gender as ApN-Overex mice and were submitted to the same diet for the same time. Results are presented as means ± SEM for 6-7 mice per group.*P< 0.05, **P<0.01 *vs.* WT mice.

NF-kB and JNK, ERK1/2, AMPK activity in adipose tissue of ApN-overex mice

Several molecular pathways, particularly those involving activation Nuclear Factor kappa-light-chain-enhancer of Activated B cells (NF-κB), c-Jun NH2-terminal protein kinase (JNK) and Extracellular Signal-Regulated kinases (ERK1/2) may play crucial roles in linking obesity to low-grade inflammation (20-22). In addition, AMP-activated protein kinase (AMPK) is a well-established downstream event of ApN in AT (23). We therefore investigated these pathways to unravel the mechanisms underlying the anti-inflammatory properties of ApN.

Gene expression of IκBα, an endogenous inhibitor of NF-κB activity was increased ~2-fold (P<0.001) in adipose tissue of ApN-Overex mice compared to WT mice, and this was accompanied by ~ 20% decrease of NF-κB activity (P< 0.01 *vs* WT) (Fig. 7A). ERK1/2 phosphorylation was also reduced by ~35% in ApN-Overex mice (Fig. 7B). However, neither JNK protein nor JNK phosphorylation was affected by ApN (data not shown). Interestingly, both AMPK protein expression and phosphorylation were increased in adipose tissue of ApN-Overex mice by 60% and 40%, respectively (P<0.05 for both) (Fig. 7C). Thus, the anti-inflammatory effect of ApN on adipose tissue was associated with reduction of NF-κB and ERK1/2, and activation of AMPK.

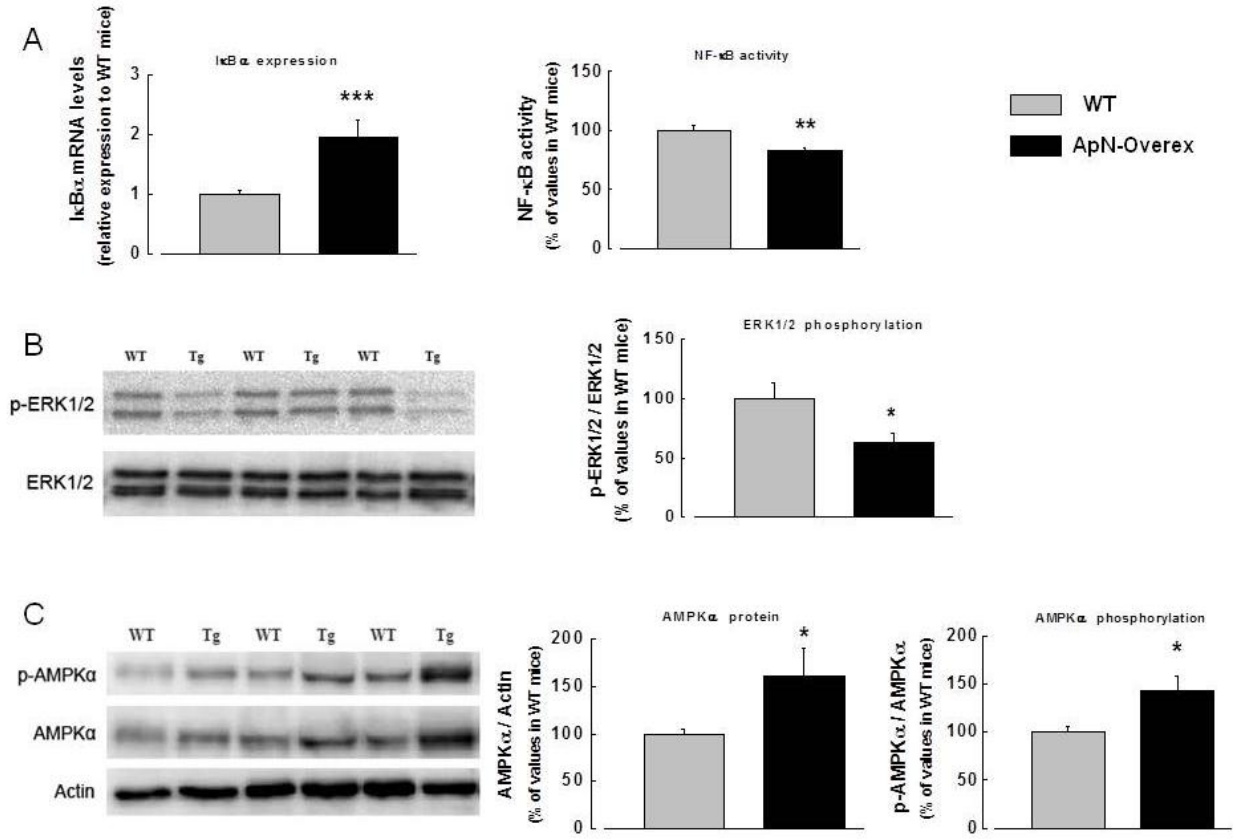

Fig. 7. Evaluation of NF-κB, ERK1/2 and AMPK pathways in adipose tissue of ApN-Overex mice. Adipose tissue was freshly isolated from ApN-Overex and WT mice. (A) Gene expression of IκBα was quantified by RTQ-PCR, normalized to the levels of cyclophilin and presented as relative expression compared to WT values. NF-κB (p65) DNA-binding activity was measured by ELISA in whole tissue protein extracts; results were expressed as percentages of values in WT mice. (B) Phosphorylation of ERK1/2 was measured by Western blot analysis. ERK1/2 phosphorylation was normalized to total ERK1/2 protein levels and these ratios were expressed as percentages of WT values. A representative blot is shown on the left. (C). Phosphorylation of AMPK was measured by Western blot analysis (a representative blot is also shown on the left). All data from these blots were normalized to actin levels. Both total AMPKα protein and the ratio p-AMPKα to total APMKα were increased in ApN-Overex mice, the data being presented as percentages of WT mice. Results shown herein (A-C) are the means ± SEM for 5 mice per group. **P*<0.05, ***P*<0.01, ****P*<0.001 *vs.* WT mice.

DISCUSSION

The aim of this study was to investigate *in vivo* the effects of ApN on adipose tissue secretory function before the emergence of confounding factors. We used a unique transgenic mouse model generated in our lab, which exhibits a moderate over-expression of native full-length ApN targeted to white AT with no changes in the distribution of ApN multimers (11). Five-month-old transgenic mice were characterized by reduced adiposity and adipocyte size. The mechanisms responsible for this phenotype involved increased energy expenditure and altered adipocyte differentiation (11). These mice also showed improved insulin sensitivity and lipid profile when challenged by a high-sucrose diet (11). As yet, the *in vivo* molecular modifications triggered by ApN before any changes in adiposity, insulin action and circulating lipids are still unravelled in AT. We therefore used 10-wk-old mice to study the direct and specific effect of ApN on AT secretory function. We also used ApN-KO mice of the same age, which similarly showed no difference in body weight, fat mass and metabolic parameters when compared to WT controls.

Auto/paracrine effects of ApN on AT secretion have been reported *in vitro* by some groups. ApN attenuated LPS-induced IL-6 and TNF-a release in primary pig adipocytes (9). The globular domain of ApN also reduced the secretion of several cytokines as shown by protein array analysis of culture medium from human mammary adipocytes differentiated *in vitro* (8). However, these *in vitro* effects of ApN could not completely reflect the actual *in vivo* conditions and merely focused on one cellular fraction of AT. Herein, we show that ApN downregulated the secretion of 6-7 cytokines in both cellular fractions of AT, and upregulated one cytokine in the SVC fraction *ex vivo*. Alternatively, ApN may have prevented the immune deregulation that occurred with increasing adiposity and ageing. It should also be stressed that the lower secretion of adipokines by transgenic mice was explained neither by a reduced yield of cell number (as data were actually normalized per DNA) nor by a reduction of fat cell size as adipocyte size was actually similar in transgenic and WT mice.

By using cytokine antibody array and ELISA approaches, in addition to already known adipokines, we identified several adipokines as newly secreted by AT and/or regulated by ApN. Besides decreasing TNF-α and IL-6 whose role in propagating inflammation and inducing insulin resistance is well known (1), ApN donwregulated (or prevented the stimulation of-) 3 other pro-inflammatory factors (i.e. 3 interleukins: IL12, IL17B, IL21) whose function on- or presence in- AT is still poorly documented. Yet, these 3 interleukins

play a role in initiating and shaping immune-inflammatory responses (24-26). IL12 is also thought to contribute to the development of atherosclerosis and its circulating levels were increased in patients with type 2 diabetes (27). The chemokines, ICAM-1 and RANTES as well as the granulocyte (macrophage)-growth factors (GCSF and GM-CSF) are implicated in adipose tissue inflammation/immunity by recruiting monocytes/macrophages and T cells (28-31). RANTES could also be implicated in type 2 diabetes development (32). We have previously shown that the megakaryocytic growth factor, TPO was oversecreted by omental adipose tissue of obese subjects (13) and that its circulating levels were increased in obesity and negatively correlated with adiponectinemia (33). Herein, we extend those data by demonstrating that TPO is unambiguously regulated in adipocytes by ApN excess or deficiency *in vivo*. Because high systemic TPO levels could participate in the pathogenesis of the acute coronary syndrome (34), it is tempting to speculate that suppressed TPO may further link ApN to cardiovascular protection. Like TPO, ApN specifically downregulated VEGFR1. This downregulation is of interest since inactivation of a VEGF homologue, which specifically binds VEGFR1 led to impaired adipose tissue development via reduced angiogenesis in obese rodents (35). The only adipokine that was specifically upregulated by ApN in the present study was IL-1R4, a member of the Toll-like 1 receptor family. However, we cannot exclude the possibility that some other anti-inflammatory adipokines, like IL-10, whose levels were very low by cytokine antibody arrays (our own data) may have escaped to the detection. Herein, both soluble secreted form and total gene expression of IL-1R4 were enhanced by ApN. IL-1R4 may act as anti-inflammatory factor: it suppressed inflammatory responses induced by LPS both *in vitro* and *in vivo* (36), while IL-1R4 deficient mice produced elevated concentrations of pro-inflammatory cytokines and failed to develop endotoxin tolerance (37). Recently, a protective role for IL-1R4 has been proposed in atherosclerosis (38) and obesity as well (39). IL-1R4 protected obese mice against the development of AT inflammation and related metabolic disorders (39). Thus, several adipokines identified as downstream targets of ApN may be instrumental in further deciphering the pathogenesis of the metabolic syndrome.

After identifying these new secretory products, we examined whether their regulation occurred at the pre-translational level. We thus measured the mRNA levels of all these adipokines. Gene expression pattern of most adipokines roughly followed the secretion pattern, indicating a pre-translational effect of ApN. Expression of some genes like TPO, IL-12p70, VEGFR1, ICAM-1 and IL-1R4 further showed a reverse pattern in the ApN lacking

condition, implying that ApN was the specific regulator of these changes. However, the expression of some genes was not affected in ApN-deficient mice. Yet, the two models of mice were not exactly the opposite of each other. In ApN-KO mice, there is a complete and generalized lack of ApN and some compensatory mechanisms may operate and mask the expected repercussions especially on AT (e.g. these mice do not develop obesity (10)). In ApN-Overex mice, the overexpression is primarily targeted to white AT and remains moderate (i.e. within a physiological range).

To unravel the mechanisms underlying the anti-inflammatory effects of ApN on AT, we explored several potential intracellular signalling pathways linking inflammation/immune response to obesity. Obesity and high-fat diet are known to activate IKKβ/NF-κB, JNK and ERK1/2 pathways in adipose tissue (22; 40). On the other hand, APMK activation is a crucial link in the signalling effects of ApN in metabolically responsive tissues including AT (23); thus, we also explored the AMPK pathway. ApN has been reported to inhibit LPS-stimulated activation of NF-κB, JNK and ERK1/2 (or related MAP kinase) in cultured porcine or human blood-derived macrophages (41; 42). We confirmed *in vivo* that ApN indeed suppressed the activation of NF-κB and ERK1/2 in whole white AT, although it did not affect JNK protein or phosphorylation. We also found that ApN enhanced both AMPK protein expression and phosphorylation. Most studies focused on ApN-induced AMPK phosphorylation and hardly characterized the relationship between ApN and AMPK protein expression. The increased AMPK protein expression found herein is likely to result from chronic exposure to ApN. Noticeably, AMPK activity has recently been found to be reduced in omental adipose tissue of obese subjects and to be associated with markers of inflammation (43). Hence, the decreased activity of NF-κB and ERK1/2 as well the increased expression and activity of AMPK were associated with- and were likely to contribute to- the protective effect of ApN against inflammation into AT.

In conclusion, ApN regulates in vivo the secretion of several newly identified downstream adipokines, thereby inducing a shift of the immune balance in both adipocytes and SVC toward a less inflammatory phenotype. These downstream adipokines may be new therapeutic targets for the management of the metabolic syndrome.

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Reference List

1. **Maury E and Brichard SM**. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol Cell Endocrinol* 314: 1-16, 2010.

2. **Guerre-Millo M**. Adiponectin: an update. *Diabetes Metab* 34: 12-18, 2008.

3. **Ouchi N and Walsh K**. Adiponectin as an anti-inflammatory factor. *Clin Chim Acta* 380: 24-30, 2007.

4. **Asano T, Watanabe K, Kubota N, Gunji T, Omata M, Kadowaki T and Ohnishi S**. Adiponectin knockout mice on high fat diet develop fibrosing steatohepatitis. *J Gastroenterol Hepatol* 24: 1669-1676, 2009.

5. **Nishihara T, Matsuda M, Araki H, Oshima K, Kihara S, Funahashi T and Shimomura I**. Effect of adiponectin on murine colitis induced by dextran sulfate sodium. *Gastroenterology* 131: 853-861, 2006.

6. **Tao L, Gao E, Jiao X, Yuan Y, Li S, Christopher TA, Lopez BL, Koch W, Chan L, Goldstein BJ and Ma XL**. Adiponectin cardioprotection after myocardial ischemia/reperfusion involves the reduction of oxidative/nitrative stress. *Circulation* 115: 1408-1416, 2007.

7. **Jortay J, Senou M, Delaigle A, Noel L, Funahashi T, Maeda N, Many MC and Brichard SM**. Local induction of adiponectin reduces lipopolysaccharide-triggered skeletal muscle damage. *Endocrinology* 151: 4840-4851, 2010.

8. **Dietze-Schroeder D, Sell H, Uhlig M, Koenen M and Eckel J**. Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 54: 2003-2011, 2005.

9. **Ajuwon KM and Spurlock ME**. Adiponectin inhibits LPS-induced NF-kappaB activation and IL-6 production and increases PPARgamma2 expression in adipocytes. *Am J Physiol Regul Integr Comp Physiol* 288: R1220-R1225, 2005.

10. **Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T and Matsuzawa Y**. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* 8: 731-737, 2002.

11. **Bauche IB, El Mkadem SA, Pottier AM, Senou M, Many MC, Rezsohazy R, Penicaud L, Maeda N, Funahashi T and Brichard SM**. Overexpression of adiponectin targeted to adipose tissue in transgenic mice: impaired adipocyte differentiation. *Endocrinology* 148: 1539-1549, 2007.

12. **Gilson H, Schakman O, Kalista S, Lause P, Tsuchida K and Thissen JP**. Follistatin induces muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin and activin. *Am J Physiol Endocrinol Metab* 297: E157-E164, 2009.

13. **Maury E, Ehala-Aleksejev K, Guiot Y, Detry R, Vandenhooft A and Brichard SM**. Adipokines oversecreted by omental adipose tissue in human obesity. *Am J Physiol Endocrinol Metab* 293: E656-E665, 2007.

14. **Maury E, Noel L, Detry R and Brichard SM**. In vitro hyperresponsiveness to tumor necrosis factor-alpha contributes to adipokine dysregulation in omental adipocytes of obese subjects. *J Clin Endocrinol Metab* 94: 1393-1400, 2009.

15. **Delaigle AM, Jonas JC, Bauche IB, Cornu O and Brichard SM**. Induction of adiponectin in skeletal muscle by inflammatory cytokines: in vivo and in vitro studies. *Endocrinology* 145: 5589-5597, 2004.

16. **Tran TT, Gupta N, Goh T, Naigamwalla D, Chia MC, Koohestani N, Mehrotra S, McKeown-Eyssen G, Giacca A and Bruce WR**. Direct measure of insulin sensitivity with the hyperinsulinemic-euglycemic clamp and surrogate measures of insulin sensitivity with the oral glucose tolerance test: correlations with aberrant crypt foci promotion in rats. *Cancer Epidemiol Biomarkers Prev* 12: 47-56, 2003.

17. **Furuhashi M, Fucho R, Gorgun CZ, Tuncman G, Cao H and Hotamisligil GS**. Adipocyte/macrophage fatty acid-binding proteins contribute to metabolic deterioration through actions in both macrophages and adipocytes in mice. *J Clin Invest* 118: 2640-2650, 2008.

18. **Bourlier V and Bouloumie A**. Role of macrophage tissue infiltration in obesity and insulin resistance. *Diabetes Metab* 35: 251-260, 2009.

19. **Ohashi K, Parker JL, Ouchi N, Higuchi A, Vita JA, Gokce N, Pedersen AA, Kalthoff C, Tullin S, Sams A, Summer R and Walsh K**. Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. *J Biol Chem* 285: 6153-6160, 2010.

20. **Vitseva OI, Tanriverdi K, Tchkonia TT, Kirkland JL, McDonnell ME, Apovian CM, Freedman J and Gokce N**. Inducible Toll-like receptor and NF-kappaB regulatory pathway expression in human adipose tissue. *Obesity (Silver Spring)* 16: 932-937, 2008.

21. **Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M and Hotamisligil GS**. A central role for JNK in obesity and insulin resistance. *Nature* 420: 333-336, 2002.

22. **Ito A, Suganami T, Miyamoto Y, Yoshimasa Y, Takeya M, Kamei Y and Ogawa Y**. Role of MAPK phosphatase-1 in the induction of monocyte chemoattractant protein-1 during the course of adipocyte hypertrophy. *J Biol Chem* 282: 25445-25452, 2007.

23. **Wu X, Motoshima H, Mahadev K, Stalker TJ, Scalia R and Goldstein BJ**. Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. *Diabetes* 52: 1355-1363, 2003.

24. **Brombacher F, Kastelein RA and Alber G**. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol* 24: 207-212, 2003.

25. **Yamaguchi Y, Fujio K, Shoda H, Okamoto A, Tsuno NH, Takahashi K and Yamamoto K**. IL-17B and IL-17C are associated with TNF-alpha production and contribute to the exacerbation of inflammatory arthritis. *J Immunol* 179: 7128-7136, 2007.

26. **Monteleone G, Pallone F and Macdonald TT**. Interleukin-21 as a new therapeutic target for immune-mediated diseases. *Trends Pharmacol Sci* 30: 441-447, 2009.

27. **Wegner M, Winiarska H, Bobkiewicz-Kozlowska T and Dworacka M**. IL-12 serum levels in patients with type 2 diabetes treated with sulphonylureas. *Cytokine* 42: 312-316, 2008.

28. **Sengenes C, Miranville A, Lolmede K, Curat CA and Bouloumie A**. The role of endothelial cells in inflamed adipose tissue. *J Intern Med* 262: 415-421, 2007.

29. **Keophiphath M, Rouault C, Divoux A, Clement K and Lacasa D**. CCL5 promotes macrophage recruitment and survival in human adipose tissue. *Arterioscler Thromb Vasc Biol* 30: 39-45, 2010.

30. **Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, Bouillot JL, Bouloumie A, Barbatelli G, Cinti S, Svensson PA, Barsh GS, Zucker JD, Basdevant A, Langin D and Clement K**. Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* 54: 2277-2286, 2005.

31. **Kim DH, Sandoval D, Reed JA, Matter EK, Tolod EG, Woods SC and Seeley RJ**. The role of GM-CSF in adipose tissue inflammation. *Am J Physiol Endocrinol Metab* 295: E1038-E1046, 2008.

32. **Herder C, Haastert B, Muller-Scholze S, Koenig W, Thorand B, Holle R, Wichmann HE, Scherbaum WA, Martin S and Kolb H**. Association of systemic chemokine concentrations with impaired glucose tolerance and type 2 diabetes: results from the Cooperative Health Research in the Region of Augsburg Survey S4 (KORA S4). *Diabetes* 54 Suppl 2: S11-S17, 2005.

33. **Maury E, Brichard SM, Pataky Z, Carpentier A, Golay A and Bobbioni-Harsch E**. Effect of obesity on growth-related oncogene factor-alpha, thrombopoietin, and tissue inhibitor metalloproteinase-1 serum levels. *Obesity (Silver Spring)* 18: 1503-1509, 2010.

34. **Senaran H, Ileri M, Altinbas A, Kosar A, Yetkin E, Ozturk M, Karaaslan Y and Kirazli S**. Thrombopoietin and mean platelet volume in coronary artery disease. *Clin Cardiol* 24: 405-408, 2001.

35. **Lijnen HR, Christiaens V, Scroyen I, Voros G, Tjwa M, Carmeliet P and Collen D**. Impaired adipose tissue development in mice with inactivation of placental growth factor function. *Diabetes* 55: 2698-2704, 2006.

36. **Sweet MJ, Leung BP, Kang D, Sogaard M, Schulz K, Trajkovic V, Campbell CC, Xu D and Liew FY**. A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. *J Immunol* 166: 6633-6639, 2001.

37. **Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, O'Neill LA and Liew FY**. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat Immunol* 5: 373-379, 2004.

38. **Miller AM, Xu D, Asquith DL, Denby L, Li Y, Sattar N, Baker AH, McInnes IB and Liew FY**. IL-33 reduces the development of atherosclerosis. *J Exp Med* 205: 339-346, 2008.

39. **Miller AM, Asquith DL, Hueber AJ, Anderson LA, Holmes WM, McKenzie AN, Xu D, Sattar N, McInnes IB and Liew FY**. Interleukin-33 induces protective effects in adipose tissue inflammation during obesity in mice. *Circ Res* 107: 650-658, 2010.

40. **Shoelson SE, Herrero L and Naaz A**. Obesity, inflammation, and insulin resistance. *Gastroenterology* 132: 2169-2180, 2007.

41. **Wulster-Radcliffe MC, Ajuwon KM, Wang J, Christian JA and Spurlock ME**. Adiponectin differentially regulates cytokines in porcine macrophages. *Biochem Biophys Res Commun* 316: 924-929, 2004.

42. **Folco EJ, Rocha VZ, Lopez-Ilasaca M and Libby P**. Adiponectin inhibits pro-inflammatory signaling in human macrophages independent of interleukin-10. *J Biol Chem* 284: 25569-25575, 2009.

43. **Gauthier MS, O'Brien EL, Bigornia S, Mott M, Cacicedo JM, Xu XJ, Gokce N, Apovian C and Ruderman N**. Decreased AMP-activated protein kinase activity is associated with increased inflammation in visceral adipose tissue and with whole-body insulin resistance in morbidly obese humans. *Biochem Biophys Res Commun* 404: 382-387, 2011.

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MicroRNAs regulated by Adiponectin as novel targets for controlling adipose tissue inflammation

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Running title: Adiponectin and microRNA in adipose tissue inflammation

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ABSTRACT

Low-grade pro-inflammatory state contributes to the metabolic syndrome (MS). Adiponectin (ApN), which is reduced in the MS, has emerged as a master regulator of inflammation/immunity. We wished to identify whether microRNAs (miRNAs) may mediate the anti-inflammatory action of ApN on adipose tissue (AT). miRNA expression profiling was performed in mice overexpressing ApN specifically in AT (ApN-Overex) and in wild-type controls. Role of specific miRNAs was analyzed by gain-of or loss-of function approaches in 3T3-F442A (pre)-adipocytes and in *de novo* AT formed from engineered 3T3-F442A preadipocytes transplanted in nude mice. miRNA expression was compared in omental AT of lean and obese subjects. Expression of miR532-5p and miR1983 was down-regulated, while that of miR883b-5p and miR1934 was up-regulated in AT of ApN-Overex mice. We focused on miR883b-5p, which was identified by computational analysis as being involved in inflammatory pathways. miR883b-5p overexpression down-regulated a lipopolysaccharide (LPS) facilitator, LPS-binding protein (LBP) in 3T3-F442A cells, while miR883b-5p blockade had reverse effects. miR883b-5p blockade also abolished the protective effects of ApN on pro-inflammatory adipokine induction. These data were recapitulated in *de novo* AT where miR883b-5p silencing induced LBP production and tissue inflammation. Eventually, miR883b-5p expression was down-regulated in AT of obese subjects. We identified several novel miRNAs which are regulated by ApN in AT *in vivo*. miR883b-5p, which is up-regulated by ApN represses LBP production and Toll-like receptor-4 signaling, acting therefore as a major mediator of the anti-inflammatory action of ApN. These novel miRNAs may open new therapeutic perspectives for the MS.

INTRODUCTION

Adipose tissue (AT) secretes adipokines, which play central roles in energy and vascular homeostasis as well as in immunity. Deregulation of these adipokines triggers the development of a low-grade pro-inflammatory state, which is considered to build the common soil for the pathogenesis of obesity-linked disorders (1; 2). Resetting the immunological balance in AT may be a crucial approach for the future management of the metabolic syndrome.

Adiponectin (ApN), which is decreased in the metabolic syndrome, has emerged as a master regulator of inflammation/immunity in various tissues (3; 4), including AT, its own production site. We have recently shown, thanks to our transgenic mice overexpressing ApN specifically in white AT (5), that ApN regulates *in vivo* the secretory profile of downstream adipokines, decreasing those with pro-inflammatory properties while up-regulating those with anti-inflammatory action (6). These changes were specific: they occurred before the emergence of any metabolic confounding factors (such as improvement of insulin action or decrease of fat mass). Moreover, a reverse profile of expression was observed for most adipokines in ApN-knockout (ApN-KO) mice (6). Yet, the mechanisms by which ApN shifts the immune balance of adipocytes toward a less inflammatory phenotype are not fully elucidated.

MicroRNAs (miRNAs) are small non-coding RNAs that control gene expression by inducing target mRNA degradation or blocking translation (7; 8). A growing body of evidence indicates that deregulation of miRNAs is closely associated with obesity-related metabolic disorders including type 2 diabetes and atherosclerosis (9; 10). Specific miRNAs have been implicated in adipogenesis and mature adipocyte function (11). However, the involvement of miRNAs in AT inflammation has been scarcely investigated. As yet, only few miRNAs have been identified as relevant in this field. miR-132 has been reported to activate NF-κB and the transcription of IL-8 and MCP-1 in primary human preadipocytes and in *in vitro* differentiated adipocytes (12). Very recently, miR-223 has been identified as a crucial regulator of macrophage polarization in mouse AT, its deficiency promoting AT inflammation (13). Whether miRNAs mediate the potent anti-inflammatory/immunomodulatory action of ApN on AT is still unsettled.

The aim of the present work was to address this question. To this end, we first took advantage of our transgenic mice overexpressing ApN to identify miRNAs regulated by ApN

in AT *in vivo*. Second, we characterized the function of these novel miRNAs by *in vitro* and *in vivo* experiments. Finally, we preliminary examined whether these miRNAs were abnormally expressed in human obesity.

RESEARCH DESIGN AND METHODS

Animals

Ten weeks-old male mice overexpressing ApN (ApN-Overex) specifically in white AT under the control of the aP2 promoter and their wild-type (WT) littermates were housed at a constant temperature (22°C) with a fixed 12h-light, 12h-dark cycle. They received a high-sucrose diet (TD00220; Harlan, Horst, Netherlands) from weaning, as mouse phenotype and degree of transgene expression were characterized in those conditions (5). Male ApN-KO mice, which exhibit a complete lack of ApN in fat and plasma, and their WT controls (mice of the same genetic background, raised together with ApN-KO mice, but which were not their littermates) were used for comparison. These animals were housed in the same conditions as ApN-Overex mice and received the same diet from weaning. At 10 weeks of age, mice were sacrificed and inguinal fat pads (the adipose tissue site where the effects of the transgene ApN were the most pronounced (5)) were quickly removed, weighed, frozen in liquid nitrogen and stored at -80°C for subsequent experiments. Some morphological and laboratory characteristics of these mice have already been described (6). In an additional set of experiments, we used WT mice to compare miRNA expression between inguinal and epididymal fat as well as between both fractions (adipocytes *vs.* stromal-vascular cells (SVC)) of the inguinal depot, the tissue being fractionated and processed as described (6).

For *de novo* fat pad formation, 6 weeks-old male Balb/c Nude mice (Charles River, Les Oncins, France) were used: these mice were kept in micro-isolation cages and received a Western diet to promote fatness for 4 weeks (TD88137, Harlan) (14). At the end of the experiment, blood glucose was measured with a glucometer (MediSense Precision Xtra Plus®, Abott-Medisense, Louvain-la-Neuve, Belgium) and tail vein blood samples were saved. The *de novo* formed fat pad was removed, weighed and stored at -80°C.

The University Animal Care Committee approved all procedures.

Subjects

Omental adipose tissue was obtained from 6 lean (BMI: 24.6±0.7 kg/m^2) and 6 obese subjects (BMI: 42.3±4 kg/m^2) undergoing elective abdominal surgery after an overnight fast, as described (15). Obesity was defined as a BMI of ≥ 30 kg/m^2. The two groups of participants were sex- (3 men/3 women per group) and age-matched (59±3 yr *vs.* 52±1 yr); 4

subjects (one lean and three obese) were diagnosed with type 2 diabetes. Patients provided written informed consent and the study protocol had the approval of the local Ethical Committee of Saint-Luc University Hospital.

MicroRNA array profiling

Total RNA of inguinal AT was extracted by the mirVana™ miRNA isolation kit (Life Technologies, Gent, Belgium). 1µg RNA from experimental samples and from a common reference mouse sample was labelled with Hy3™ and Hy5™ fluorescent probes, respectively (Exiqon, Vedbaek, Denmark). Each pair of labelled experimental and reference samples was mixed and hybridized to the miRCURY™ LNA Array (version 5th Generation; Exiqon), which contains 4 replicates of each of the 694 mouse-specific miRNA probes (based on mirBASE v15.0). The quantified signals were background corrected and normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm within each array, to adjust for any intensity-dependent dye bias.

Direct miRNA or mRNA quantification by real-time quantitative polymerase chain reaction (RT-qPCR)

For miRNA quantification, RNA was isolated as described above. 0.2 µg total RNA were reverse transcribed by using the NCode^{TM} VILO^{TM} miRNA cDNA Synthesis Kit (Life Technologies). 2 ng of total RNA equivalents were amplified with iQSyber Green Supermix (Bio-Rad Laboratories, UK Ltd) using commercial miRNA-specific forward primers (Qiagen, Venlo, The Netherlands) and a reverse universal primer (provided in the NCode^{TM} VILO^{TM} miRNA cDNA Synthesis Kit).

For mRNA quantification, RNA was extracted and revere transcribed, and RT-qPCR was performed with designed primers (supplementary Table 1), as described earlier (6).

Cyclophilin, 18srRNA or RNU6B (Qiagen) were used as reporter genes. Relative changes in the expression level of one specific gene were presented as $2^{-\Delta\Delta Ct}$ (6).

In silico functional profiling of target genes

Potential miRNA target genes were predicted using the miRanda algorithms implemented by the MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm). The biological function of target genes was annotated using GENECODIS database by integrating Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) Biological Process (www.genecodis.dacya.ucm.es) (16; 17).

Culture of 3T3-F442A adipocytes

Mouse 3T3-F442A preadipocytes were seeded at 1×10^5 cell/ml and grown at 37 C in 5% CO_2 in DMEM containing 1 g/l glucose 10% fetal calf serum, 8 mg/l biotin and 1/500 (v/v) antibiotics (Primocin, InvivoGen, Cayla, Toulouse, France). Two days after confluence (day 0), adipocyte differentiation was induced by an adipogenic cocktail medium, as previously described (5). At day 9, more than 90% of the cells had changed their morphology and accumulated fat droplets as evidenced by Oil Red O staining. In some experiments, preadipocytes or mature adipocytes (day 9) were treated with or without 5 µg/ml mouse recombinant full-length ApN (Biovendor GmbH, Heidelberg, Germany) for 24h.

Transfection of miR883b-5p Mimic or Anti-miR in 3T3-F442A adipocytes

Synthetic double-stranded oligonucleotide mimicking mature endogenous miR883b-5p (miR-Mimic, 50nM), miR-mimic negative control (AllStars Negative Control, 50 nM), Anti-miR883b-5p single-stranded oligonucleotide (Anti-miR, 100 nM) or Anti-miR negative control (miScript Inhibitor Negative Control, 100nM) (all from Qiagen) were delivered into 3T3-F442A preadipocytes or mature adipocytes (day 7), which had been previously cultured overnight in antibiotic-free medium. Delivery was performed by using Dharmafect 3 siRNA transfection reagent (Dharmacon, Lafayette, CO). The miR-1 Mimic (endogenous miR-1 is undetectable in 3T3-F442A cells) was used as positive control to confirm transfection efficiency. The medium was replaced with a fresh medium 24h post transfection in all cases. The cells and the medium were collected 48h post transfection.

In some experiments, 24 h after transfection of Anti-miR or negative control, the medium was renewed, then cells were treated with or without 10 µg/ml ApN for 24 h, with

recombinant TNF-α (50ng/ml; Biovendor GmbH) added or not to the medium for the last 18 h.

Blockade of miR883b-5p in de novo formed fat tissue

3T3-F442A preadipocytes were transfected with a plasmid pEZX-AM02 containing the Anti-miR883b-5p sequence or a control plasmid containing a scrambled sequence (GeneCopoeia LabOmics, Nivelles, Belgium) using Lipofectamine^{TM} 2000 Transfection Reagent (Life Technologies). Stably transfected cells were selected by 500 ng/ml Puromycin (Sigma-Aldrich, Belgium). The 10^7 transfected 3T3-F442A preadipocytes were re-suspended in 200 µl PBS and injected subcutaneously in the back of Balb/c Nude mice. After 4 weeks, the *de novo* formed tissue was removed. A sample was fixed in 4% formaldehyde for 24 h and embedded in paraffin. 5µm-thick sections were stained with haematoxylin-eosin-safran (HES). Adipocytes areas were measured by an image analyser system (MOP-Vidioplan; Kontron, Eching, Germany), as described (6; 18).

LPS-binding protein (LBP) quantification

LBP concentrations in cultured medium, plasma and tissue homogenates were measured by a commercial ELISA kit (Biometec, Greifswald, Germany) according to the manufacturer's instructions. *De novo* formed tissue was homogenized in a lysis buffer (Cell Signalling Technology, BIOKÉ, Leiden, The Netherlands) supplemented with a 1% protease inhibitor cocktail (Roche Diagnostics, Vilvoorde, Belgium). LBP in tissue homogenates was normalized to protein content in each sample.

Presentation of the results and statistical analysis

The results are means ± SEM for the indicated numbers of mice or subjects or independent cultures. Comparisons between two groups were carried out using two-tailed unpaired Student's t-test. Comparisons of at least 3 groups were performed by one-way ANOVA followed by the Dunnett's or Newman-Keuls' test, when appropriate. The differences were considered statistically significant at $P < 0.05$, except for the *in silico* functional analysis. In this case, computed P values were adjusted using the FDR method of

Hochberg and Benjamini (19) to control the false discovery rate, and only corrected P values <0.01 were considered significant .

RESULTS

miRNA expression profiling in adipose tissue of ApN-Overex mice

White AT from 10-week-old ApN-Overex mice and from their WT littermates was profiled by miRNA arrays. At this age, there were still no differences in body weight, fat mass and adipocyte size, as well as no alterations in glucose, insulin and triglycerides levels between mice of the two genotypes. Mice were therefore studied before the emergence of any metabolic confounding factors (6).

Among the 694 miRNAs tested, the expression of 289 miRNAs was detectable in both groups, while the expression of ~ 51 miRNAs was differently regulated between ApN-Overex mice and WT mice ($P<0.05$). Among them, 9 miRNAs were very significantly regulated by ApN ($P\leq0.01$ *vs.* WT; Fig. 1, on the left of the dashed line), while 3 others were significantly regulated ($P<0.05$ *vs.* WT) and had established roles in AT function (10; 20) (Fig. 1, on the right of the dashed line).

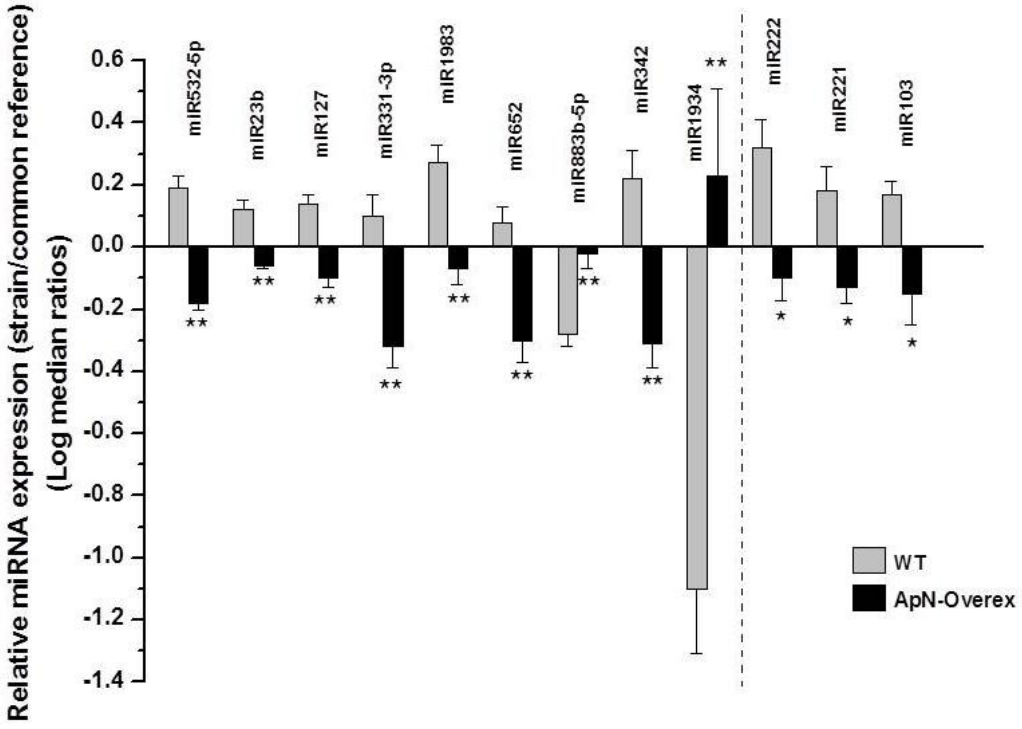

Fig.1. miRNA expression profiling in adipose tissue from ApN-Overex or wild-type (WT) mice. miRNA expression was screened in inguinal adipose tissue from ApN-Overex mice and WT littermates by using microarrays, which contain probes for 694 mouse-specific miRNAs. 9 miRNAs, which showed highly significant differences ($P\leq0.01$) between the two genotypes of mice, are presented on the left of the dashed line. The other 3 miRNAs presented on the right also exhibited significant differences (albeit at $P<0.05$) but were already known to influence adipose tissue function. Values are log-median ratio intensities for each sample relative to the common reference. Positive values indicate higher expression in the experimental sample than in the common reference and vice versa for negative values. Results are means ± SEM for 3 mice per group. *$P<0.05$, **$P\leq0.01$ *vs.* WT mice.

These 12 miRNAs were checked and quantified by RT-qPCR. Four out of the 12 miRNAs were validated by RT-qPCR as being regulated by ApN overexpression *in vivo*: two miRNAs (miR532-5p and miR1983) were down-regulated in ApN-Overex mice by ~40% and ~50% respectively (Fig. 2A), whereas two others (miR883b-5p and miR1934) were up-regulated by ~50% (Fig. 2A).

miRNA expression in adipose tissue of ApN-KO mice

These four miRNAs were next quantified in AT of ApN-KO mice of the same age and sex and receiving the same diet. The expression of two miRNAs, miR1983 and miR1934 showed a reverse pattern when compared to that of ApN-Overex mice. The expression of miR1983 was thus up-regulated in ApN-KO mice (Fig. 2B), while the expression of miR1934 was dramatically down-regulated to almost undetectable levels (Fig. 2B). The expression of miR1934 seems therefore to be dependent on the presence of ApN.

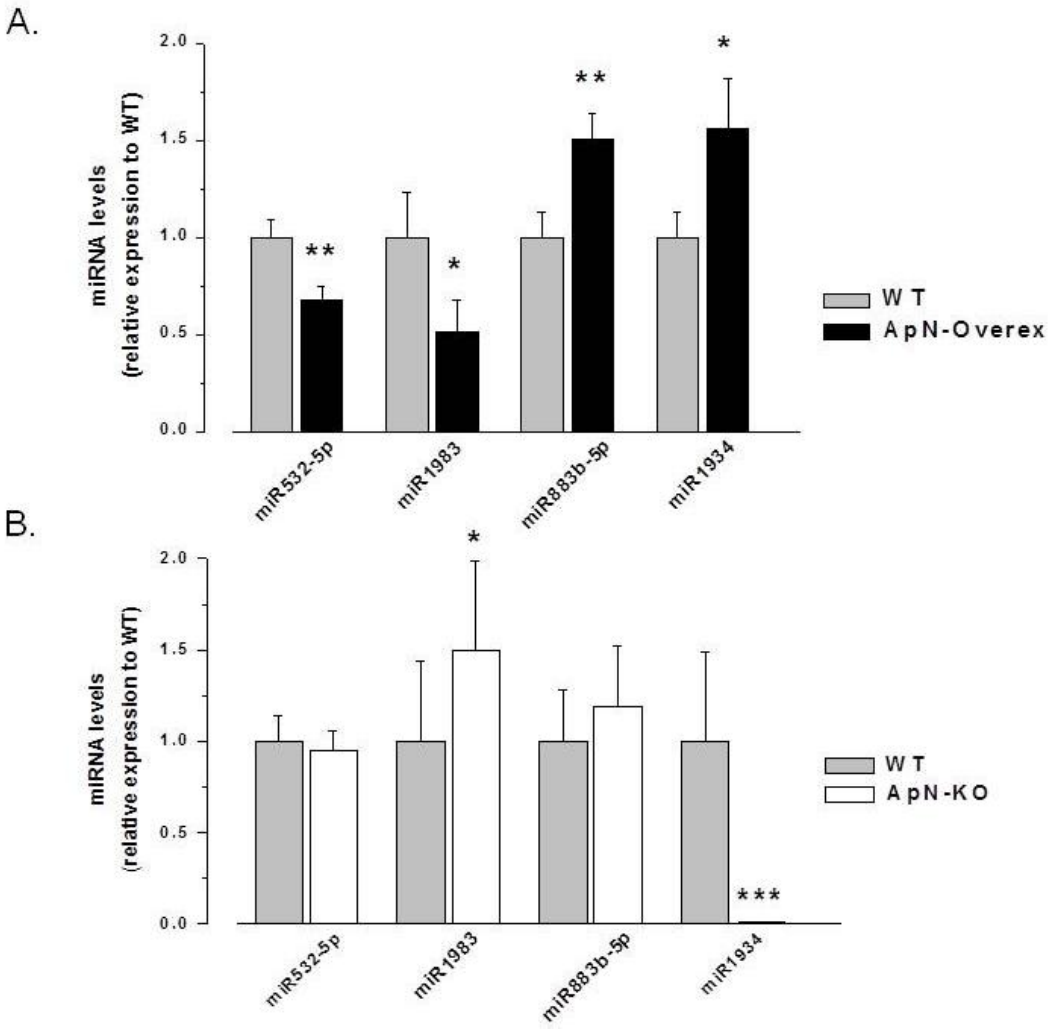

Fig.2. Quantification of miRNA expression in adipose tissue of ApN-Overex (A) and ApN-KO mice (B). The 12 miRNAs, which were first identified as differentially expressed between two genotypes of mice by microRNA array screening (see Fig.1; ApN-Overx *vs.* WT mice) were further quantified by RT-qPCR (A). Only miRNAs exhibiting genotype differences validated by RT-qPCR are shown in the histograms of A panel. These miRNA levels were then compared to those found in adipose tissue of ApN-KO mice (B). ApN-KO mice were of the same age and sex as ApN-Overex mice. Values were normalized to 18srRNA and presented as relative expression compared to respective WT mice. Results are means ± SEM for 6-7 mice per group. *P<0.05, **P<0.01, ***P<0.001 *vs.* respective WT.

In silico functional analysis of potential miRNA target genes

To get some insights into the potential roles of these 4 miRNAs, which were regulated by ApN *in vivo* (see Fig. 2A), we identified their predicted target genes using the algorithms miRanda. These algorithms generated 857 and 1062 unique targets for miR532-5p and miR883b-5p, respectively. There were no predicted targets for miR1983 and miR1934, as these 2 miRNAs are not yet included in the database.

We further annotated the biological function of target genes for miR532-5p and miR883b-5p using GENECODIS database. We focused on target genes implicated in inflammation/immune responses. There were no such targets for miR532-5p. By contrast, for miR883b-5p, there were 4 predicted target genes: Toll-like receptor 3 (TLR3), Toll-like receptor 6 (TLR6), TANK-binding kinase 1 (Tbk1), LPS-binding protein (LBP), which were enriched for the Toll-like receptor (TLR) signalling pathway (KEGG) and innate immune responses (GO) (P=0.004) (supplementary Fig. 1). LBP aids in lipopolysaccharides (LPS) binding to TLR4. Seven other predicted target genes for miR883b-5p were also enriched for the vascular endothelial growth factor (VEGF) signalling pathway (KEGG, P=0.006) and 10 others for the chemokine signalling pathway (KEGG, P=0.01). Based on these computational data, we hypothesized that miR883b-5p was likely to be involved in immune/inflammation processes. We therefore focused on this miRNA and further investigated its role in AT inflammation. In preliminary experiments performed in WT mice, we did not find any differences in the expression of this miRNA between the different fat depots (inguinal vs. epididymal) and between the different fractions (adipocytes vs. SVC) of subcutaneous fat (data not shown)

Direct upregulation of miR883b-5p by ApN in vitro

Because of the lack of effect of ApN deficiency on miR883b-5p in ApN-KO mice, we examined whether ApN directly regulates miR883b-5p expression in 3T3-F442A cells. In line with the *in vivo* results obtained in ApN-Overex mice, ApN treatment for 24h up-regulated miR883b-5p expression *in vitro* in either in preadipocytes or differentiated adipocytes by ~70- and 60%, respectively (Fig. 3).

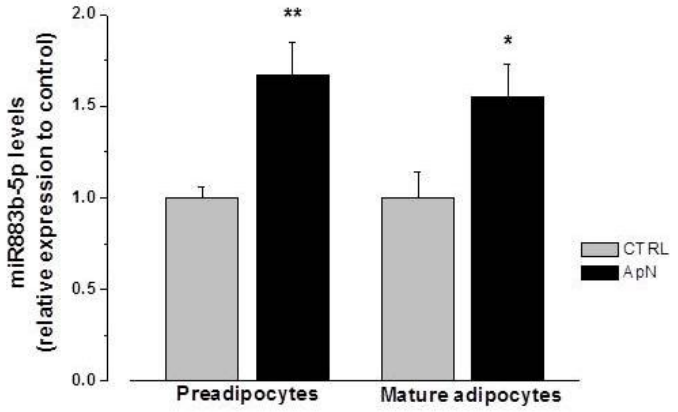

Fig.3. Direct upregulation of miR883b-5p expression by ApN *in vitro*. 3T3-F442A preadipocytes and differentiated adipocytes (day 9) were treated or not with 5µg/ml ApN for 24h. Expression of miR883b-5p was quantified by RT-qPCR. Values were normalized to 18srRNA and presented as relative expression compared to control conditions (CTRL; i.e. without ApN). Results are means ± SEM for 4 repeated experiments. *P<0.05, **P<0.01 *vs.* CTRL.

Involvement of target genes of miR883b-5p in the Toll-like receptor pathway in vitro

The four target genes of miR883b-5p, which were predicted by computational analysis as belonging to the TLR pathway, were further validated *in vitro*. To this end, we used a gain-of or a loss-of function approach in 3T3F442A (pre)-adipocytes transfected for 48 h with miR883b-5p mimic or inhibitor (Anti-miR), respectively.

Overexpression of miR883b-5p in preadipocytes down-regulated Tbk1 and LBP gene expression by 20 and 40%, respectively (Fig. 4A). Accordingly, LBP protein levels secreted into the culture medium were also reduced (- ~30%; Fig. 4B). However, using miR883b-5p mimic had no such effects on mature adipocytes (data not shown), likely because endogenous miR883b-5p levels rose spontaneously from day 7 to day 9 of differentiation (supplementary Fig. 2), which coincided with the time of transfection.

As expected, blockade of miR883b-5p induced an opposite effect to the mimic and up-regulated LBP mRNA levels in preadipocytes (Fig. 4C). The blockade of miR883b-5p also induced a 50% rise of LBP mRNA in mature adipocytes (Fig. 4D).

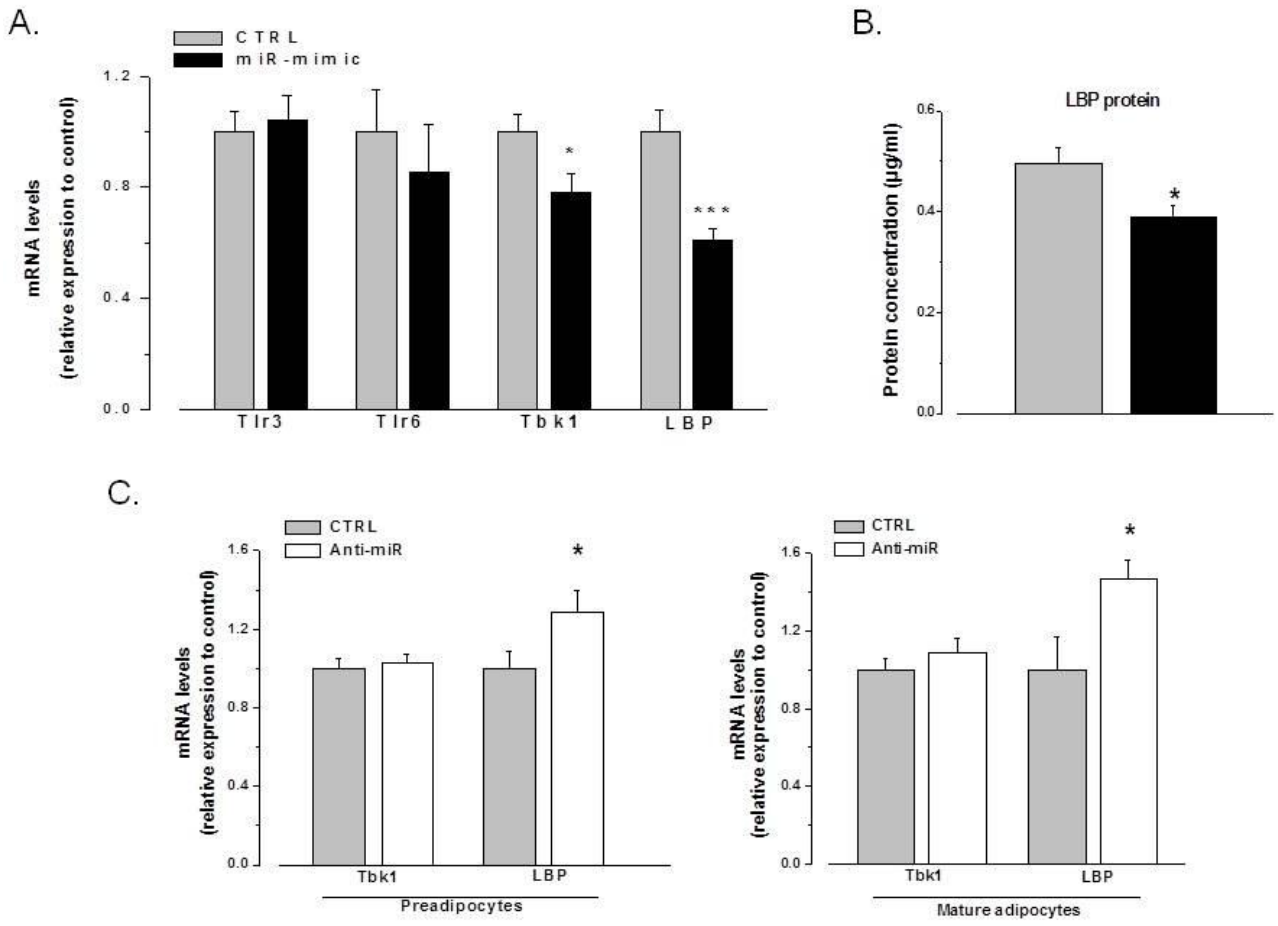

Fig.4. Identification of the predicted target genes of miR883b-5p *in vitro*. 3T3-F442A preadipocytes were transfected with miR883b-5p mimic or a negative control (CTRL). 48h post-transfection, mRNA levels of Toll-like receptor 3 (Tlr3), Toll-like receptor 6 (Tlr6), TANK-binding kinase 1 (Tbk1) and LPS-binding protein (LBP) were quantified by RT-qPCR (A) and protein level of LBP secreted into the medium was measured by ELISA (B). Pre- or differentiated adipocytes (day 7) were transfected with Anti-miR883b-5p or a negative control (CTRL). 48h post-transfection, mRNA levels of Tbk1 and LBP, which were shown to be modified by miR-mimic in panels A-B, were quantified by RT-qPCR (C, D). RT-qPCR data were normalized to cyclophilin levels and presented as relative expression compared to CTRL. Results are means ± SEM for 4-5 repeated experiments. *P<0.05, ***P<0.001 *vs.* respective CTRL.

miR883b-5p as a mediator of the anti-inflammatory action of ApN on downstream adipokines in vitro

To determine whether miR883b-5p was involved in the anti-inflammatory effects of ApN, we blocked endogenous miR883b-5p in (pre)-adipocytes challenged by TNF-α. We chose to challenge the cells by TNF-α rather than a direct exposure to LPS because TNF-α, which is released by adipocytes in response to LPS (21), induces the production of several pro-inflammatory adipokines in a very consistent (i.e. not serotype-dependent) manner (15). Next, we measured the inflammatory adipokines, previously identified as down-regulated by ApN in WAT *ex vivo* (6): 5 pro-inflammatory cytokines (IL-6, TNF-α, IL-12p70, IL-17B, IL-

21), 4 hematopoietic growth factors (TPO, GMCSF, GCSF, VEGFR1) and 2 chemokines (RANTES, ICAM-1).

This experiment was first conducted in preadipocytes (Fig. 5). TNF-α largely stimulated gene expression of most adipokines including its own expression (Fig. 5 and data not shown). ApN pre-treatment attenuated the stimulation of 4 adipokines, while Anti-miR reversed this preventive effect for 3 of them (IL-6, TNF-α and GCSF; Fig. 5). This indicates that this effect of ApN on these adipokines is mediated by miR883b-5p. It is of note that Anti-miR *per se,* used alone or in combination with TNF-α*,* did not affect either basal or TNF-α-induced expression of adipokines except for IL-6 (Fig. 5). For this cytokine, Anti-miR *per se* facilitated the simulation produced by TNF-α ($P<0.05$, TNF+Anti-miR *vs.* TNF; Fig. 5).

We next investigated whether miR883b-5p was also a mediator of the anti-inflammatory effects of ApN in differentiated adipocytes. Qualitatively similar results were obtained in mature adipocytes (Fig. 6) except for GCSF: its mRNA abundance stimulated by TNF-α was not prevented by ApN (data not shown).

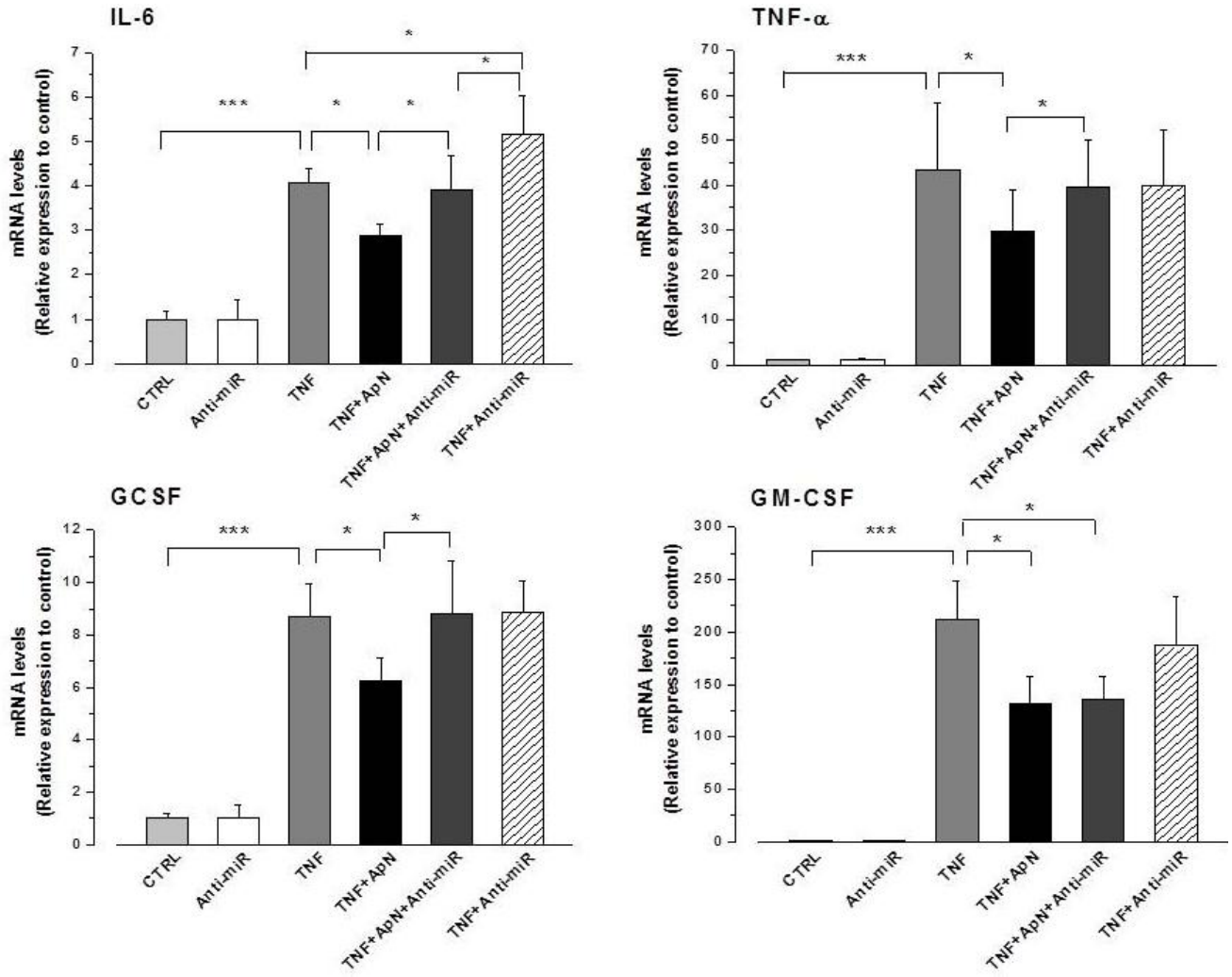

Fig.5. miR883b-5p as a mediator of the anti-inflammatory action of ApN on downstream adipokines in preadipocytes. 3T3-F442A preadipocytes were transfected with Anti-miR883b-5p or a negative control (CTRL). 24 h after transfection, the medium was renewed, then cells were treated with or without 10 µg/ml ApN for 24 h, while being or not challenged with 50 ng/ml TNF-α for the last 18 h. mRNA of target adipokines were measured by RT-qPCR. Values were normalized to cyclophilin levels and presented as relative expression compared to CTRL. Results are means ± SEM for 4 repeated experiments. ***P<0.001 *vs.* CTRL, *****P<0.05 *vs.* the indicated condition.

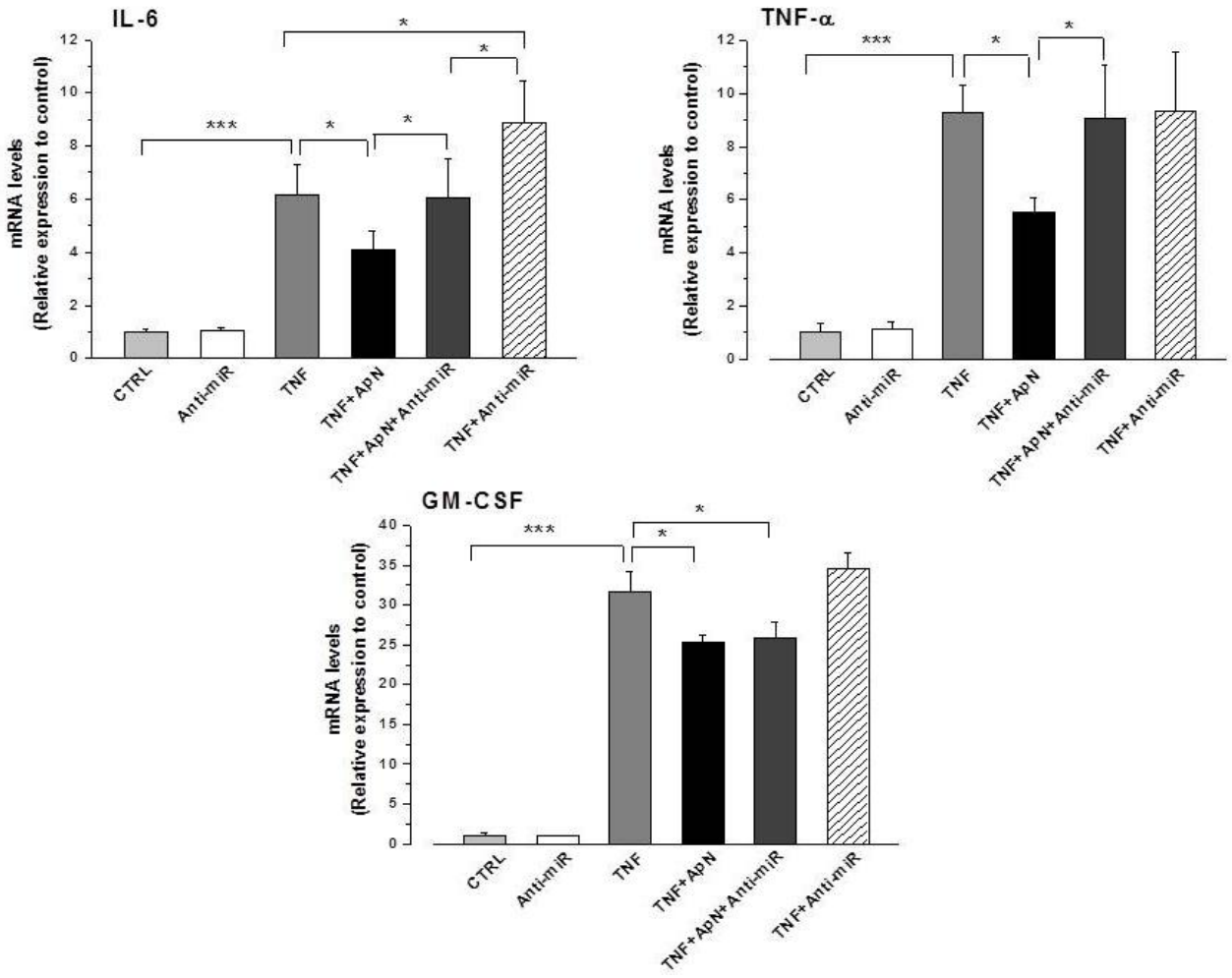

Fig.6. miR883b-5p as a mediator of the anti-inflammatory action of ApN on downstream adipokines in differentiated adipocytes. 3T3-F442A differentiated adipocytes (day 7) were transfected with Anti-miR miR883b-5p or a negative control (CTRL). 24 h after transfection, the medium was renewed, then cells were treated with or without 10 µg/ml ApN for 24 h, while being or not challenged with 50 ng/ml TNF-α for the last 18 h. mRNA of target adipokines were measured by RT-qPCR. Values were normalized to cyclophilin levels and presented as relative expression compared to CTRL. Results are means ± SEM for 4 repeated experiments. ***P<0.001 *vs.* CTRL, *****P<0.05 *vs.* the indicated conditions.

In vivo effects of miR883b-5p inhibition on de novo formed fat tissue

We next studied whether miR883b-5p plays a role *in vivo* on *de novo* fat pad formation and inflammatory state. 3T3-F442A preadipocytes were transfected with a plasmid expressing Anti-miR883b-5p or a scrambled sequence (CTRL), then injected subcutaneously into the back of nude mice. *De novo* tissue was formed from *in vivo* differentiation of these transfected cells. We chose to block miR883b-5p rather than overexpress it in the light of the evolved expression of miR883b-5p during differentiation (see supplementary Fig. 2) and because of potential oversaturation of endogenous small RNA pathways (21).

Four weeks after transplantation, mice of the Anti-miR group exhibited no significant differences in body weight, blood glucose and *de novo* fat pad weight when compared to those of the control group (data not shown). Histological examination shows similar presence of normal adipocytes in the center of *de novo* formed tissues from both groups (Fig. 7A) with comparable adipocyte size (522.4 ± 81.9µm^2 for anti-miR *vs*. 584.7 ± 78.0µm^2 for controls, n=4 mice/group). As expected, *de novo* tissue formed from Anti-miR expressing cells showed much higher levels of LBP mRNA and protein concentrations than controls (Fig. 7B, C). Mice of the Anti-miR group also exhibited elevated plasma LBP levels, suggesting that locally generated LBP by *de novo* tissue was secreted into systemic circulation (~ +60%, P<0.01 *vs.* CTRL, Fig. 7D). Moreover, *de novo* tissue of the Anti-miR group displayed higher inflammatory state than the control group as shown by a 70% increase of IL-6 mRNA (Fig. 7E) and a trend towards increased TNF-α mRNA (+40%, P=0.08 *vs.* CTRL, Fig. 7E).

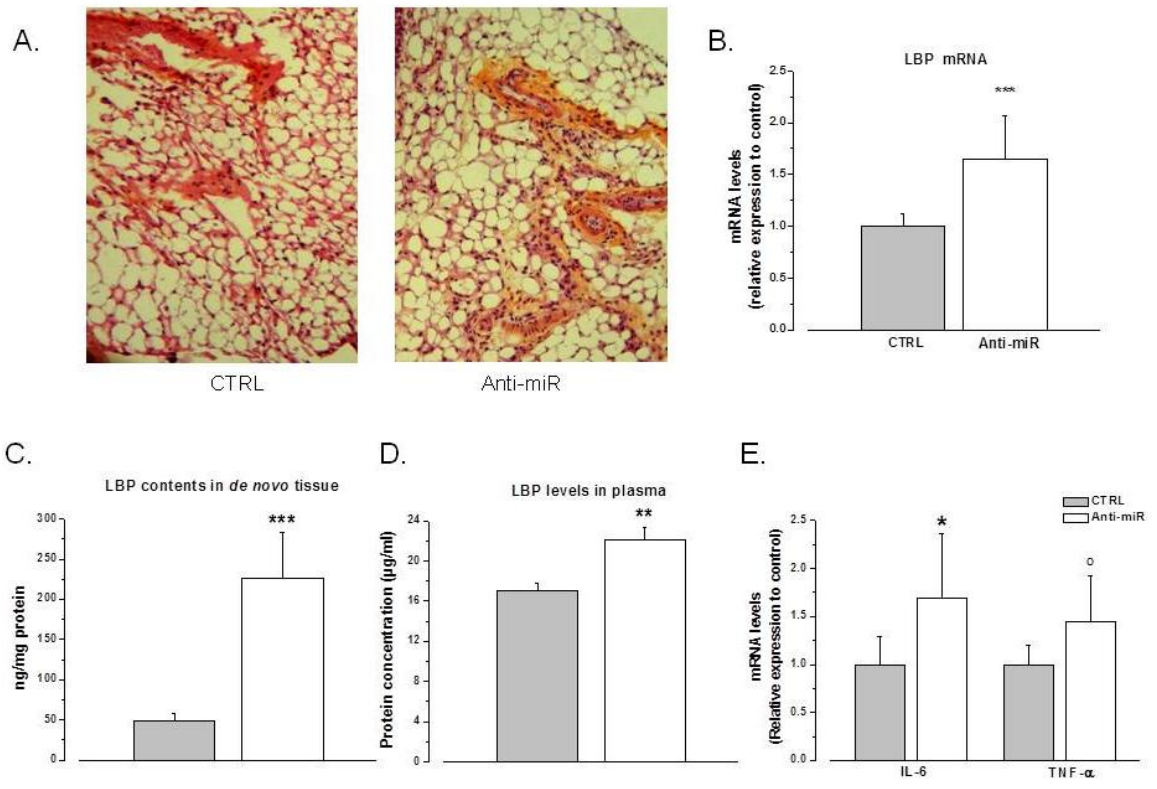

Fig.7. *In vivo* effects of miR883b-5p inhibition on *de novo* formed fat tissue. 3T3-F442A preadipocytes were transfected with a plasmid expressing Anti-miR883b-5p or a scrambled sequence (CTRL). Transfected cells were injected subcutaneously into the back of nude mice, which were sacrificed 4 weeks later. HES staining shows the presence of normal adipocytes in the center of *de novo* formed tissue (magnification X 100) (A). Increase of LBP mRNA (B) and protein levels (C) in *de novo* tissue, and of plasma LBP (D) in mice injected with preadipocytes expressing Anti-miR883b-5p. Up-regulation of IL-6 and TNF-α mRNA levels in *de novo* tissue of the same mice (E). mRNA levels were measured by RT-qPCR, normalized to 18srRNA levels and presented as relative expression compared to CTRL mice. Tissue or plasma LBP protein levels were measured by ELISA. Tissue LBP concentrations were next expressed per mg tissue protein (ng/mg protein). Results are means ± SEM for 4-5 mice per group. *P<0.05, **P<0.01, ***P<0.001 *vs.* CTRL; °P= 0.08 *vs.* CTRL.

Downregulation of miR883b-5p expression in human obesity

Finally, we examined the expression of miR883b-5p in omental AT, known to play a central role in the pathogenesis of the metabolic syndrome. We compared the expression of miR883b-5p between severely obese subjects associated with well-known AT inflammation (15; 22) and nonobese age- and sex-matched (control) subjects. As expected, ApN mRNA was decreased in obese subjects. Expression of miR883b-5p was downregulated as well (Fig. 8).

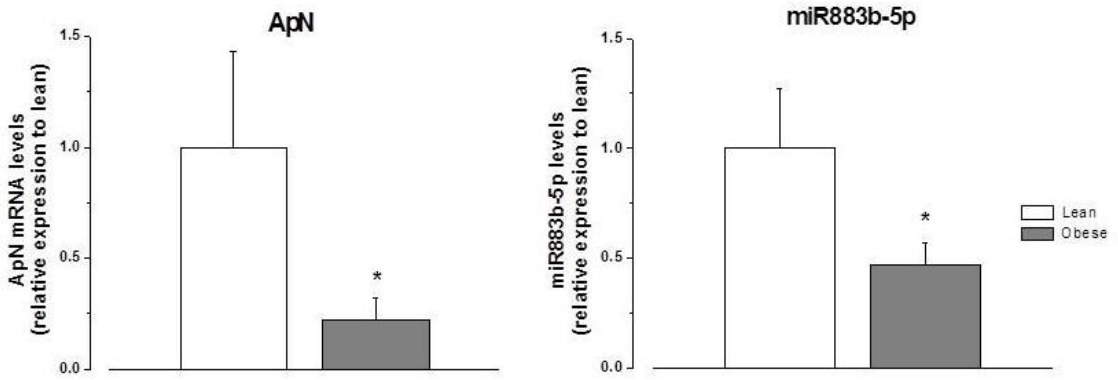

Fig.8. Downregulation of miR883b-5p in omental adipose tissue from obese subjects. Omental adipose tissue was sampled from lean and obese age- and sex-matched subjects undergoing elective abdominal surgery after an overnight fast. Expression of adiponectin (ApN) and miR883b-5p was quantified by RT-qCR, and normalized to the levels of 18srRNA and RNU6B respectively. Values are presented as relative expression compared to lean subjects. Results are means ± SEM for 6 subjects per group. *P<0.05 *vs.* lean.

DISCUSSION

We have shown in our transgenic mice overexpressing ApN specifically in white AT that ApN regulates *in vivo* the secretion of downstream adipokines, decreasing those with pro-inflammatory properties while enhancing those with anti-inflammatory effects. ApN induces therefore *in vivo* a shift of the immune balance in adipocytes toward a less inflammatory phenotype (6). Yet, the mechanisms underlying this shift are not fully elucidated. The aim of the present work was to explore whether miRNAs regulated by ApN may represent novel mechanisms for controlling AT inflammation.

The studies performed so far merely reported relationships between ApN parameters and some adipose tissue miRNAs. In human AT, subcutaneous miR-95 expression was positively related to serum ApN concentrations, while visceral miR 181a was negatively related to this parameter (23). In retroperitoneal fat of mice, miR143 was positively related to ApN gene expression, while miR221 and 222 were negatively related to mRNA level of this adipokine (24). Our study shows a direct regulation of miRNAs by ApN in AT both *in vivo* and *in vitro*.

Thanks to miRNA profiling in AT of ApN-Overex mice and subsequent RT-qPCR validation, we identified 4 novel miRNAs regulated by ApN. Such regulation is likely to result from the overexpression of ApN in both adipocytes and SVC (which contain macrophages), although the overall expression of ApN was much higher in adipocytes than in SVC (6), in line with the fact that the aP2 promoter is much more abundantly (~10.000-fold) expressed in adipocytes (26). Expression of two of these miRNAs (miR1983 and miR1934) showed a reverse pattern in the ApN-lacking condition, implying that ApN was a specific regulator of these changes. Remarkably, the expression of miR1934 fell to almost undetectable levels in the absence of ApN, indicating that ApN was a pre-requisite for its expression. However, expression of the other 2 miRNAs (miR532-5p and miR883b-5p) was not affected in ApN-KO mice. Yet, this does not exclude a role for ApN in their regulation, since the two models of mice are not exactly the opposite of each other. In ApN-Overex mice, the overexpression is moderate and primarily targeted to white AT. In ApN-KO mice, there is a complete and generalized lack of ApN and some compensatory mechanisms may operate and mask the expected repercussions especially on AT, in agreement with our previous data (6). In line with this view, we further showed that one of these miRNAs (miR883b-5p), which

was predicted by computational analysis to play a key role in inflammation, turned out to be directly up-regulated by ApN in 3T3-F442A cell lines.

The biological function of these novel miRNAs has never been described. Thanks to the available computational databases, we got some preliminary clues about their functions. However, the 2 miRNAs, which were inversely regulated in ApN-Overex and ApN-KO mice, were not yet included in the present databases. Further studies are thus warranted to unravel their functions. Regarding the other 2 miRNAs, the predicted target genes for one of them (miR883b-5p) turned out to belong to the Toll-like receptor signalling pathway as well as to the VEGF and chemokine signalling pathways. Activation of these pathways has been implicated in the development of low-grade inflammation in AT (25-28). In particular, activation of TLR4 signalling gives rise to the stimulation of intracellular pro-inflammatory molecules such as Nuclear Factor-κB (NF-κB) and Extracellular Signal-Regulated kinases (ERK1/2) in adipocytes (27;28). This is in keeping with the reduced activity of NF-κB and ERK1/2 observed in AT of ApN-Overex mice (6). Altogether these data led us to raise the hypothesis that miR883b-5p was likely to be involved in immune/inflammation responses of AT.

We next validated *in vitro* the target genes of miR883b-5p belonging to the TLR pathway, which were predicted by computational analysis. LBP was identified as one specific target of miR883b-5p in adipocytes. LBP is an acute-phase protein, which amplifies host responses to LPS and which is synthesized in the liver (29) and, as shown more recently, in 3T3-L1 adipocytes co-cultured with macrophages (30). Herein, we confirmed that adipocytes are a novel source of LBP. The role of LBP is to aid LPS to dock at the LPS receptor complex composed of TLR4 receptors, thereby triggering a downstream cascade leading to up-regulation of proinflammatory cytokines (29) in adipocytes (26) and other cells. This facilitating role of LBP on LPS action may thus be relevant to the context of the metabolic syndrome. Animal studies (31) and human evidence (32) have indeed suggested that subclinical endotoxemia, characterized by low to moderately and chronically elevated LPS, may be involved in the pathogenesis of metabolic disorders. Moreover, elevated circulating LBP has recently been associated with obesity, metabolic syndrome, and type 2 diabetes in apparently healthy Chinese (33).

Afterwards, we investigated whether miR883b-5p was necessary for the anti-inflammatory action of ApN on downstream adipokine secretion. We thus measured the pro-

inflammatory adipokines, previously identified as down-regulated by ApN in AT (6), after miR833b-5p blockade. Our data indicated that miR883b-5p is a mediator of the anti-inflammatory effects of ApN on TNF-α-induced IL-6, TNF-α and GCSF gene expression in 3T3-F442A cells. Our results are concordant with those of a recent study reporting that another miRNA (miR-146b-5p) mediated the anti-inflammatory action of globular ApN in THP-1 monocytes (34). In our work, the mediating role of miR883b-5p did not result from direct targeting of the mRNAs of the adipokines mentioned above for two reasons. First, miR883b-5p blockade *per se* did not affect basal gene expression of these adipokines (see Fig. 5; first two columns of each panel). Second, computational analysis indicates that the 3'UTRs of most cytokines lack miRNA binding sites (35). miR883b-5p may therefore probably target some intermediate machinery components, which are necessary for the regulatory action of ApN. Some of these intermediate components, whose expression was enhanced by miR883b-5p blockade, may probably be involved in the amplified response of IL-6 to TNF-α in the presence of Anti-miR (Fig. 5, 6; column 3 *vs.* 6 in each first panel).

In vivo experiments with transplanted 3T3-F442A preadipocytes recapitulated the *in vitro* data. The d*e novo* tissue formed from *in vivo* differentiation of preadipocytes transfected with anti-miR883b-5p exhibited greater LBP production and secretion, which resulted in higher circulating levels. This *de novo* formed tissue also displayed higher levels of pro-inflammatory cytokines like IL-6 or TNF-α.

Taken together our data suggest that ApN upregulates miR883b-5p, which in turn donwregulates LBP and possibly other targets belonging to inflammatory pathways (like the VEGF or chemokine pathways mentioned above). This ultimately leads to decreased production of downstream pro-inflammatory adipokines, thereby relieving AT inflammation (see supplementary Fig. 3). It is tempting to extend those data to humans *in vivo* as we showed that miR883b-5p expression together with that of ApN was actually down-regulated in omental AT of obese subjects.

In conclusion, we have identified several miRNAs regulated by ApN in AT *in vivo*. One of these, miR883b-5p, which was up-regulated by ApN repressed LBP production and turned out to be a major mediator of the anti-inflammatory action of ApN in adipocytes. Novel miRNAs regulated by ApN may open new therapeutic perspectives for controlling AT inflammation and related metabolic disorders.

ACKNOWLEDGMENTS

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DUALITY OF INTEREST

The authors have nothing to disclose.

CONTRIBUTION STATEMENT

Pr. Sonia M. Brichard is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

Q.G researched data, wrote manuscript, contributed to discussion, reviewed/edited manuscript. J.G researched data. L.N researched data, reviewed/edited manuscript. I.S reviewed/edited manuscript. S.B contributed discussion, reviewed/edited manuscript.

Reference List

1. **de Rooij SR, Nijpels G, Nilsson PM, Nolan JJ, Gabriel R, Bobbioni-Harsch E, Mingrone G and Dekker JM**. Low-grade chronic inflammation in the relationship between insulin sensitivity and cardiovascular disease (RISC) population: associations with insulin resistance and cardiometabolic risk profile. *Diabetes Care* 32: 1295-1301, 2009.

2. **Maury E and Brichard SM**. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol Cell Endocrinol* 314: 1-16, 2010.

3. **Ouchi N and Walsh K**. Adiponectin as an anti-inflammatory factor. *Clin Chim Acta* 380: 24-30, 2007.

4. **Chedid P, Hurtado-Nedelec M, Marion-Gaber B, Bournier O, Hayem G, Gougerot-Pocidalo MA, Frystyk J, Flyvbjerg A, El BJ and Marie JC**. Adiponectin and its globular fragment differentially modulate the oxidative burst of primary human phagocytes. *Am J Pathol* 180: 682-692, 2012.

5. **Bauche IB, El Mkadem SA, Pottier AM, Senou M, Many MC, Rezsohazy R, Penicaud L, Maeda N, Funahashi T and Brichard SM**. Overexpression of adiponectin targeted to adipose tissue in transgenic mice: impaired adipocyte differentiation. *Endocrinology* 148: 1539-1549, 2007.

6. **Ge Q, Ryken L, Noel L, Maury E and Brichard SM**. Adipokines identified as new downstream targets for adiponectin: lessons from adiponectin-overexpressing or -deficient mice. *Am J Physiol Endocrinol Metab* 301: E326-E335, 2011.

7. **Bartel DP**. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297, 2004.

8. **Guo H, Ingolia NT, Weissman JS and Bartel DP**. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* 466: 835-840, 2010.

9. **Heneghan HM, Miller N and Kerin MJ**. Role of microRNAs in obesity and the metabolic syndrome. *Obes Rev* 11: 354-361, 2010.

10. **Hulsmans M, De KD and Holvoet P**. MicroRNAs regulating oxidative stress and inflammation in relation to obesity and atherosclerosis. *FASEB J* 25: 2515-2527, 2011.

11. **Alexander R, Lodish H and Sun L**. MicroRNAs in adipogenesis and as therapeutic targets for obesity. *Expert Opin Ther Targets* 15: 623-636, 2011.

12. **Strum JC, Johnson JH, Ward J, Xie H, Feild J, Hester A, Alford A and Waters KM**. MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. *Mol Endocrinol* 23: 1876-1884, 2009.

13. **Zhuang G, Meng C, Guo X, Cheruku PS, Shi L, Xu H, Li H, Wang G, Evans AR, Safe S, Wu C and Zhou B**. A Novel Regulator of Macrophage Activation: miR-223 in Obesity Associated Adipose Tissue Inflammation. *Circulation* 2012.

14. **Lijnen HR, Christiaens V, Scroyen I, Voros G, Tjwa M, Carmeliet P and Collen D**. Impaired adipose tissue development in mice with inactivation of placental growth factor function. *Diabetes* 55: 2698-2704, 2006.

15. **Maury E, Noel L, Detry R and Brichard SM**. In vitro hyperresponsiveness to tumor necrosis factor-alpha contributes to adipokine dysregulation in omental adipocytes of obese subjects. *J Clin Endocrinol Metab* 94: 1393-1400, 2009.

16. **Nogales-Cadenas R, Carmona-Saez P, Vazquez M, Vicente C, Yang X, Tirado F, Carazo JM and Pascual-Montano A**. GeneCodis: interpreting gene lists through enrichment analysis and integration of diverse biological information. *Nucleic Acids Res* 37: W317-W322, 2009.

17. **Carmona-Saez P, Chagoyen M, Tirado F, Carazo JM and Pascual-Montano A**. GENECODIS: a web-based tool for finding significant concurrent annotations in gene lists. *Genome Biol* 8: R3, 2007.

18. **Gilson H, Schakman O, Kalista S, Lause P, Tsuchida K and Thissen JP**. Follistatin induces muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin and activin. *Am J Physiol Endocrinol Metab* 297: E157-E164, 2009.

19. **Hochberg Y and Benjamini Y**. More powerful procedures for multiple significance testing. *Stat Med* 9: 811-818, 1990.

20. **McGregor RA and Choi MS**. microRNAs in the regulation of adipogenesis and obesity. *Curr Mol Med* 11: 304-316, 2011.

21. **Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F and Kay MA**. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 441: 537-541, 2006.

22. **Aron-Wisnewsky J, Tordjman J, Poitou C, Darakhshan F, Hugol D, Basdevant A, Aissat A, Guerre-Millo M and Clement K**. Human adipose tissue macrophages: m1 and m2 cell surface markers in subcutaneous and omental depots and after weight loss. *J Clin Endocrinol Metab* 94: 4619-4623, 2009.

23. **Kloting N, Berthold S, Kovacs P, Schon MR, Fasshauer M, Ruschke K, Stumvoll M and Bluher M**. MicroRNA expression in human omental and subcutaneous adipose tissue. *PLoS One* 4: e4699, 2009.

24. **Parra P, Serra F and Palou A**. Expression of adipose microRNAs is sensitive to dietary conjugated linoleic acid treatment in mice. *PLoS One* 5: e13005, 2010.

25. **Kopp A, Buechler C, Neumeier M, Weigert J, Aslanidis C, Scholmerich J and Schaffler A**. Innate immunity and adipocyte function: ligand-specific activation of multiple Toll-like receptors modulates cytokine, adipokine, and chemokine secretion in adipocytes. *Obesity (Silver Spring)* 17: 648-656, 2009.

26. **Kopp A, Buechler C, Bala M, Neumeier M, Scholmerich J and Schaffler A**. Toll-like receptor ligands cause proinflammatory and prodiabetic activation of adipocytes via phosphorylation of extracellular signal-regulated kinase and c-Jun N-terminal kinase but not interferon regulatory factor-3. *Endocrinology* 151: 1097-1108, 2010.

27. **Villaret A, Galitzky J, Decaunes P, Esteve D, Marques MA, Sengenes C, Chiotasso P, Tchkonia T, Lafontan M, Kirkland JL and Bouloumie A**. Adipose tissue endothelial cells from obese human subjects: differences among depots in angiogenic, metabolic, and inflammatory gene expression and cellular senescence. *Diabetes* 59: 2755-2763, 2010.

28. **Surmi BK and Hasty AH**. The role of chemokines in recruitment of immune cells to the artery wall and adipose tissue. *Vascul Pharmacol* 52: 27-36, 2010.

29. **Palsson-McDermott EM and O'Neill LA**. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 113: 153-162, 2004.

30. **Nakarai H, Yamashita A, Nagayasu S, Iwashita M, Kumamoto S, Ohyama H, Hata M, Soga Y, Kushiyama A, Asano T, Abiko Y and Nishimura F**. Adipocyte-macrophage interaction may mediate LPS-induced low-grade inflammation: potential link with metabolic complications. *Innate Immun* 18: 164-170, 2012.

31. **Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L,**

Delzenne NM, Alessi MC and Burcelin R. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56: 1761-1772, 2007.

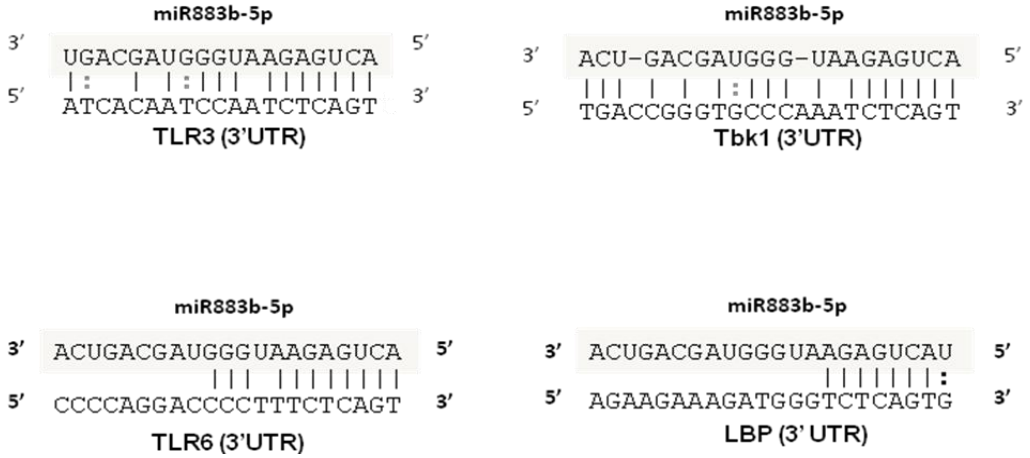
32. **Creely SJ, McTernan PG, Kusminski CM, Fisher M, Da Silva NF, Khanolkar M, Evans M, Harte AL and Kumar S**. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am J Physiol Endocrinol Metab* 292: E740-E747, 2007.

33. **Sun L, Yu Z, Ye X, Zou S, Li H, Yu D, Wu H, Chen Y, Dore J, Clement K, Hu FB and Lin X**. A marker of endotoxemia is associated with obesity and related metabolic disorders in apparently healthy Chinese. *Diabetes Care* 33: 1925-1932, 2010.

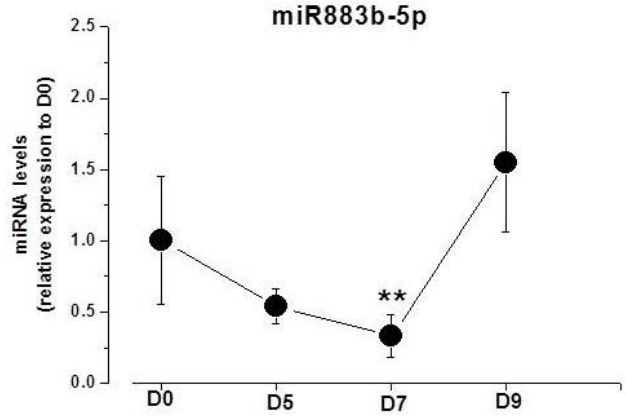
34. **Hulsmans M, Van DE, Mathieu C and Holvoet P**. Decrease of miR-146b-5p in monocytes during obesity is associated with loss of the anti-inflammatory but not insulin signaling action of adiponectin. *PLoS One* 7: e32794, 2012.

35. **Asirvatham AJ, Magner WJ and Tomasi TB**. miRNA regulation of cytokine genes. *Cytokine* 45: 58-69, 2009.

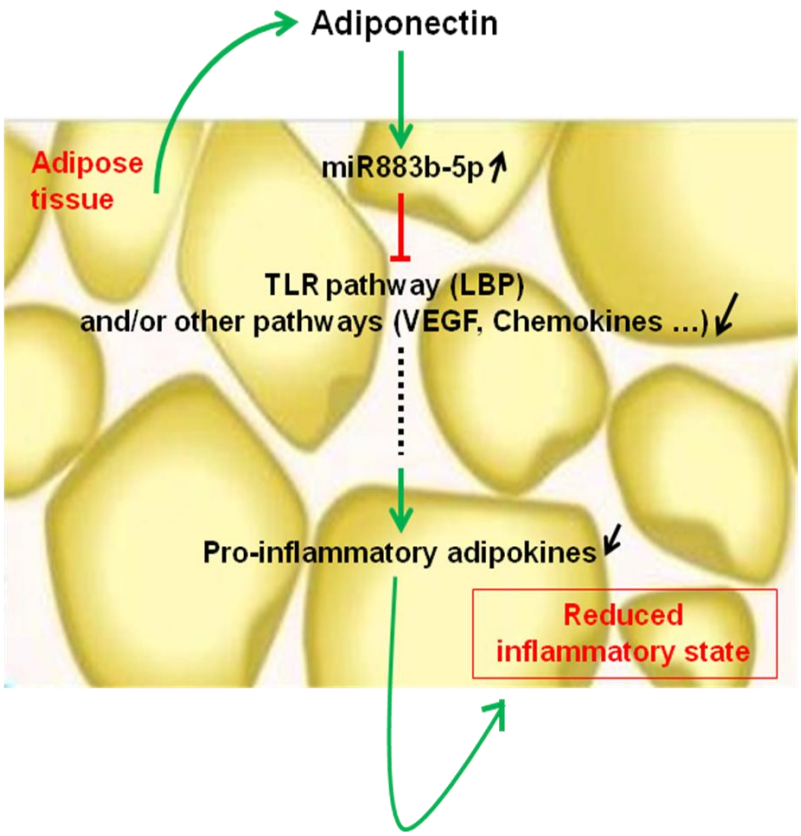
Supplementary Figures



Supplementary Fig.1. Predicted base complementarities of mature miR883b-5p to TLR3, Tbk1, TLR6 and LBP. The base complementarities of mature miR883b-5p to 3'UTR binding sites of TLR3, TLR6, Tbk1 and LBP are predicted by MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm).



Supplementary Fig.2. Expression of miR883b-5p during adipocyte differentiation 3T3-F442A preadipocytes were induced to differentiate in vitro from day 0 (D0). Cells were collected at D0, D5, D7 and D9 of differentiation, respectively. Expression of miR883b-5p was quantified by RT-qPCR. Values were normalized to 18s rRNA and presented as relative expression compared to D0. Results are means ± SEM for 3 repeated experiments. **P<0.01 *vs.* D0.



Supplementary Fig.3. Scheme illustrating for miR883b-5p-mediated anti-inflammatory action of ApN in adipose tissue. ApN upregulates miR883b-5p, which in turn downregulates LBP, a component of the Toll-like receptor (TLR) pathway, and possibly other targets belonging to other inflammatory pathways (like the VEGF or Chemokine pathways…). This ultimately leads to decreased production of downstream pro-inflammatory adipokines, thereby relieving adipocyte inflammation. Green arrows indicate induction or activation; blunted red arrows indicate inhibition and the dashed line potential effects.

Chapter 4: General Discussion and Conclusion

I. General discussion

1. Main questions and findings

Adipose tissue (AT) has been recognized as an active secretory organ, which releases a number of bioactive peptides, collectively named adipokines. Adipokines may exert endocrine action on distant organs or autocrine/paracrine action on AT. Virtually all known adipokines are dysregulated in obesity, type 2 diabetes and metabolic syndrome, leading to overproduction of deleterious adipokines and hyposecretion of defensive ones, such as ApN (4). Such a dysregulation triggers the development of a low-grade pro-inflammatory state, which is considered to build the common soil for the development of obesity-linked disorders. Thus, resetting the immunological balance in obese AT is a fundamental therapeutic strategy for the metabolic syndrome.

Although ApN has emerged as a master regulator of immune/inflammatory homeostasis in several organs such as liver, colon, cardiac or skeletal muscles, the effects of ApN on AT inflammation have been poorly investigated. Prior to my thesis, only few studies have tackled this question *in vitro*. It was shown that ApN attenuated LPS-induced IL-6 and TNF-α release in primary pig adipocytes (184). The globular domain of ApN also reduced the secretion of several cytokines as shown by protein array analysis of culture medium from human mammary adipocytes differentiated *in vitro* (247). However, these *in vitro* effects of ApN could not completely reflect the actual *in vivo* situation and merely focused on one cellular fraction of AT (i.e. the adipocyte fraction).

Moreover, the mechanisms underlying the anti-inflammatory effect of ApN in AT were poorly understood. ApN had been reported to inhibit LPS-stimulated activation of NF-κB, JNK and ERK1/2 (Extracellular Signal-Regulated kinases) (or related MAP kinase) in cultured porcine or human blood-derived macrophages (323; 324). However, only two studies showed that ApN inhibited LPS-stimulated pro-inflammatory adipokines (IL-6, TNF-α, MCP-1) via suppression of NF-kB in adipocytes (either primary pig adipocytes or 3T3-L1 cells) (184; 325). The involvement of other inflammatory signaling pathways (e.g. JNK, ERK1/2) was not documented. On the other hand, there were relationships between some miRNAs in AT and circulating or mRNA levels of ApN (294; 298). However, there was no report on a direct regulation of miRNAs by ApN in AT. Whether miRNAs regulated by ApN may contribute this anti-inflammatory action of this adipokine on AT was still unknown.

In my thesis, I have confirmed the auto/paracrine anti-inflammatory effects of ApN on AT secretory function. ApN *in vivo* regulated the secretion of downstream adipokines, decreasing those with pro-inflammatory properties, while enhancing those with anti-inflammatory effects. These changes in secretion were due to corresponding changes in mRNAs and occurred in both cellular fractions of AT. The adipokines regulated by ApN were classified into 4 categories: the pro-inflammatory cytokines, the chemokines, the hematopoietic growth factors and the anti-inflammatory factors. I concluded that ApN induced *in vivo* a shift of the immune balance in both adipocytes and SVCs toward a less inflammatory phenotype (Figure 19) (326).

Next, I elucidated the potential mechanisms contributing to this shift induced by ApN. I confirmed *in vivo* that ApN suppressed the activation of NF-κB and ERK1/2, as well increased the expression and activity of AMPK in AT (326). I also identified several miRNAs regulated by ApN *in vivo*. One of them, miR883b-5p, which was up-regulated by ApN, repressed the production of the lipopolysaccharide (LPS)-binding protein (LBP), a facilitator of LPS action. miR883b-5p turned out to be an important mediator of the anti-inflammatory action of ApN in adipocytes (327) (Figure 19).

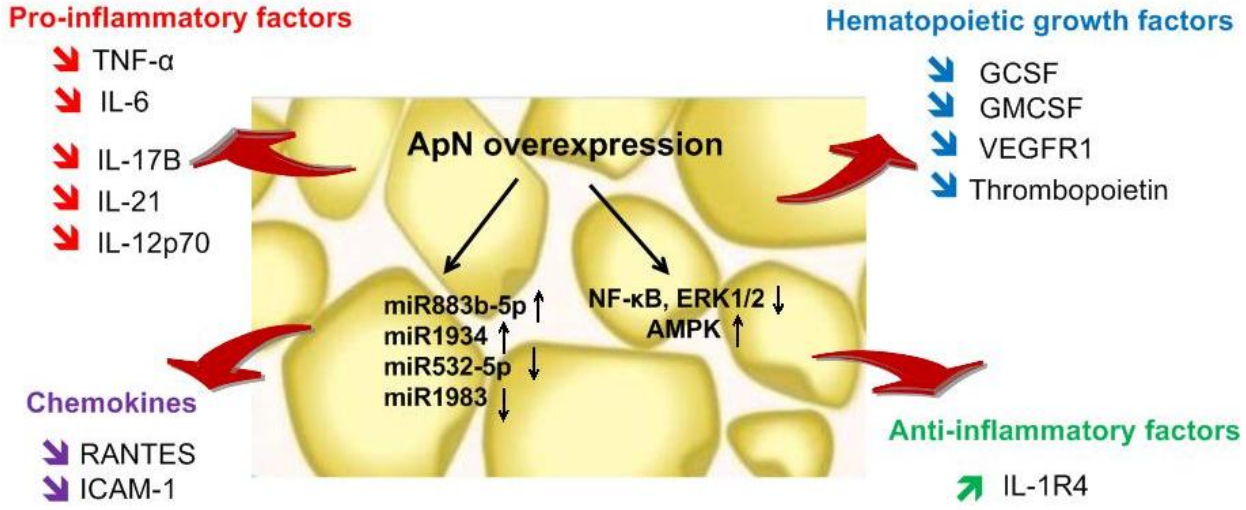


Figure 19: Main findings of my thesis. ApN regulates the secretion of downstream adipokines in AT, inducing a shift of the immune balance toward a less inflammatory phenotype. The altered activation of NF-κB, ERK1/2 and AMPK, as well the altered expression of miRNAs may contribute to the anti-inflammatory action of ApN in AT.

2. The *in vivo* effects of ApN were likely to be specific in ApN-Overex mice

To investigate the *in vivo* effects of ApN, I took advantage of the ApN-Overex mice. ApN-Overex mice are a unique transgenic mouse model generated in our lab (242), which exhibits a moderate over-expression of native full-length ApN targeted to white AT with no changes in the distribution of ApN multimers. Five-month-old ApN-Overex mice displayed reduced adiposity with improved insulin sensitivity and lipid profile when challenged by a high-sucrose diet (242). I have therefore studied ApN-Overex mice at a younger age, when they did not exhibit any overt changes in adiposity, insulin action and circulating lipids. This strategy was chosen to avoid the interference of confounding metabolic factors and to study the direct and specific effects of ApN on downstream adipokines and miRNAs.

3. A reverse profile of expression was observed for part of adipokines and miRNAs in ApN-KO mice

To further confirm the results obtained in ApN-Overex mice, I have also used ApN-knockout (ApN-KO) mice of the same age. When compared to ApN-Overex mice, a reverse profile of expression was observed for most adipokines and miRNAs in ApN-KO mice, implying that ApN was a specific regulator of these changes. However, some adipokines or miRNAs were not affected in ApN-KO mice. Yet, this does not exclude a role for ApN in their regulation, since the two models of mice are not exactly the opposite of each other. In ApN-Overex mice, the overexpression is moderate (i.e. within a physiological range) and primarily targeted to white AT. In ApN-KO mice, there is a complete and generalized lack of ApN and some compensatory mechanisms may operate and mask the expected repercussions especially on AT (e.g. these mice do not develop overt obesity) (38).

4. Both adipocyte and SVC fractions are sites of ApN action

I have investigated the effects of ApN in both adipocyte and SVC fractions, whereas previous studies merely focused in adipocytes. Although the SVC fraction accounts for only a small part of AT when compared to adipocytes (in % of total AT mass), it has been estimated that SVC and adipocytes exists in ratio of 1/2 in human subcutaneous tissue (328). The cells comprised in this SV fraction, mainly the macrophages and the endothelial cells are a primary

source of inflammatory mediators (329; 330). It has also been shown that both adipocytes and SVC may contribute to adipokine deregulation in human obesity (64; 331).

In our ApN-Overex mice, the transgene is placed under control of the aP2 promoter. aP2 is a lipid chaperone protein, which belongs to the group of fatty acid–binding proteins (FABPs) and which is much more abundantly (~10.000-fold) expressed in adipocytes than in macrophages (332). In line with this observation, in white AT of ApN-Overex mice, the overall overexpression of ApN was much higher in adipocytes than in SVC (326). Despite the weak overexpression of ApN in SVC, ApN regulated the adipokines from both cellular fractions and affected an even greater number of adipokines in the SV than in the adipocyte fraction. This may be explained by the fact that ApN secreted by the adipocytes may also influence the secretion of SVCs in paracrine manner. My data support therefore the idea that in addition to adipocytes, SVCs are also an important site for the anti-inflammatory action of ApN within AT. Furthermore, it would be more interesting to study deeply the paracrine effect of ApN by co-culturing adipocytes and SVC in the future works.

5. Potential mechanisms underlying the anti-inflammatory effects of ApN on adipose tissue

Obesity-induced AT inflammation is associated with altered signalling pathways. The intracellular signalling pathways underlying inflammatory changes in obesity mainly involve the activated IKKβ/NF-κB and the JNK systems as well as ERK pathway (333; 334). On the other hand, the activity of AMPK, which is a crucial mediator of ApN signalling in metabolically responsive tissues (183), has been found to be reduced in obese AT and to be associated with markers of inflammation (335). In my first study (326), I confirmed *in vivo* that the activity of NF-κB was indeed suppressed by ApN in AT. The activity of ERK1/2 was also suppressed, while the expression and activity of AMPK were enhanced by ApN. Hence, these signalling pathways restored by ApN were associated with- and were likely to contribute to- the protective effect of ApN against inflammation into AT. Furthermore, some studies have demonstrated that activation of AMPK suppressed NF-κB signalling pathway in several cell types including macrophages (336), endothelium (170) and cardiac myocytes (337). ApN has been shown to activate AMPK, which in turn suppressed NF-κB signalling in IL-18-stimulated endothelial cells (170) as well in hypertrophic neonatal rat ventricular myocytes (337). The enhanced expression and activity of AMPK could probably be a link

between ApN and the attenuation of downstream pro-inflammatory signalling pathways and adipokine production in AT (Figure 20).

In addition to altered signalling pathways, obesity-induced inflammation is also associated with deregulated expression of miRNAs. Xie *et al.* showed that the down-regulation of a set of miRNAs in adipocytes from obese mice was likely to result from the chronic inflammatory environment: these changes were mimicked by TNF-α treatment of adipocytes, indicating the involvement of miRNAs in adipocyte immune/inflammatory state (280). Very recently, miR-223 has been identified as a crucial regulator of macrophage polarization in mouse AT, its deficiency promoting AT inflammation (299). Before my work, whether miRNAs mediate the potent anti-inflammatory/ immunomodulatory action of ApN on AT was still unsettled; I have found that miR883b-5p, which was up-regulated by ApN, mediated the protective effects of ApN on pro-inflammatory adipokine induction after TNFα challenge (327). My results are concordant with those of a recent study reporting that another miRNA (miR-146b-5p) mediated the anti-inflammatory action of globular ApN in THP-1 monocytes (338). Based on my results, the mediating role of miR883b-5p did not result from direct targeting of the mRNAs of these pro-inflammatory adipokines for two reasons. First, miR883b-5p blockade per se did not affect basal gene expression of these adipokines. Second, computational analysis indicates that the 3'UTRs of most cytokines lack miRNA binding sites (271). miR883b-5p may therefore probably target some intermediate machinery components, which are necessary for the regulatory action of ApN (Figure 20). Computational analysis reveals indeed that miR883b-5p directly targets genes involved in TLR signalling pathway as well as in chemokine and VEGF pathways. Activation of all these pathways has been implicated in the development of low-grade inflammation in AT by interacting with other pro-inflammatory mediators, like NF-κB, and inducing the production of pro-inflammatory adipokines (133; 339-341).

Altogether, my study suggests that ApN may regulate downstream adipokines through activation of AMPK and suppression of NF-κB and ERK1/2 signalling. ApN may also up-regulate miR883b-5p expression, which in turn represses inflammatory mediators. Moreover, these two mechanisms may also interact, leading co-ordinately in less AT inflammation (Figure 20).

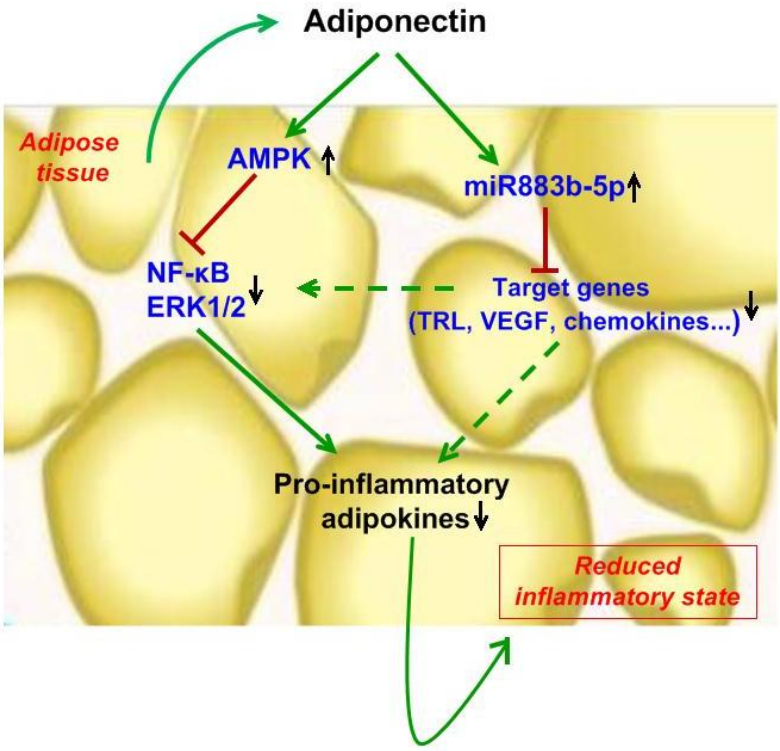


Figure 20: Scheme illustrating for the potential mechanisms underlying the anti-inflammatory effect of ApN on adipose tissue. The green arrows indicate induction or activation; blunted red arrows indicate inhibition and the dashed line potential effects.

6. Downstream adipokines regulated by ApN may be new therapeutic targets for the management of metabolic syndrome

I have identified several adipokines as newly secreted by AT and/or regulated by ApN (326). Besides decreasing TNF-α and IL-6 whose role in propagating inflammation and inducing insulin resistance is well known (4), ApN donwregulated (or prevented the stimulation of-) 3 other pro-inflammatory factors (i.e. 3 interleukins: IL12, IL17B, IL21) whose function on- or presence in- AT was still poorly documented. Yet, these 3 interleukins play a role in initiating and shaping immune-inflammatory responses (342-344). IL12 is also thought to contribute to the development of atherosclerosis and its circulating levels are increased in patients with type 2 diabetes (345). The chemokines, ICAM-1 and RANTES as well as the granulocyte (macrophage)-growth factors (GCSF and GM-CSF) are implicated in adipose tissue inflammation/immunity by recruiting monocytes/macrophages and T cells (69; 70; 112; 346). RANTES could also be implicated in type 2 diabetes development (59). Our lab previously showed that the megakaryocytic growth factor, thrombopoietin (TPO) was oversecreted by omental adipose tissue of obese subjects (64) and that its circulating levels were increased in obesity and negatively correlated with adiponectinemia (347). Herein, I extend those data by

demonstrating that TPO is unambiguously regulated in adipocytes by ApN excess or deficiency *in vivo*. Because high systemic TPO levels could participate in the pathogenesis of the acute coronary syndrome (71), it is tempting to speculate that suppressed TPO may further link ApN to cardiovascular protection. Like TPO, ApN specifically downregulated VEGFR1. This downregulation is of interest since inactivation of a VEGF homologue, which specifically binds VEGFR1 led to impaired adipose tissue development via reduced angiogenesis in obese rodents (67). It is of note that VEGF acts also as a pro-inflammatory cytokine (68). The only adipokine that was specifically upregulated by ApN in my work was IL-1R4, a member of the Toll-like 1 receptor family. Both soluble secreted form and total gene expression of IL-1R4 were enhanced by ApN. IL-1R4 may act as anti-inflammatory factor: it suppressed inflammatory responses induced by LPS both *in vitro* and *in vivo* (348), while IL-1R4 deficient mice produced elevated concentrations of pro-inflammatory cytokines and failed to develop endotoxin tolerance (349). Recently, a protective role for IL-1R4 has been proposed in atherosclerosis (350) and obesity as well (351). IL-1R4 protected obese mice against the development of AT inflammation and related metabolic disorders (351). Thus, several adipokines identified as downstream targets of ApN may be instrumental in further deciphering the pathogenesis of the metabolic syndrome.

7. miRNAs regulated by adiponectin may be new targets for controlling adipose inflammation

I next identified 4 novel miRNAs regulated by ApN in AT (327). Until my study, the biological function of these novel miRNAs had not yet been described. Thanks to the available computational databases, I got some preliminary clues about their functions. However, the 2 miRNAs, which were inversely regulated in ApN-Overex and ApN-KO mice, were not yet included in the present databases. Further studies are thus warranted to unravel their functions. Regarding the other 2 miRNAs, the predicted target genes for one of them (miR883b-5p) turned out to belong to the TLR signalling pathway as well as to the VEGF and chemokine signalling pathways. By *in vitro* and *in vivo* experiments, I validated LBP, a component of TLR signalling, as a specific target gene of miR883b-5p in adipocytes.

LBP is an acute-phase protein, which is synthesized in the liver (352) and, as shown more recently, in 3T3-L1 adipocytes co-cultured with macrophages (353). The role of LBP is to aid LPS to dock at the LPS receptor complex composed of TLR4, thereby triggering downstream cascades including NF-κB and JNK, which lead to up-regulation of pro-inflammatory cytokines (352) in adipocytes (339) and other cells (see Figure 4). Blood from LBP-knockout mice is markedly hyporesponsive to LPS, presumably as a result of the inability of LPS to rapidly associate with its receptors. This facilitating role of LBP on LPS action may be relevant to the context of the metabolic syndrome. Animal studies (144) and human evidence (135) have indeed suggested that subclinical endotoxemia, characterized by low to moderately and chronically elevated LPS may be involved in the pathogenesis of metabolic disorders. Moreover, elevated circulating LBP has been reported to associate with obesity, metabolic syndrome, and type 2 diabetes in apparently healthy Chinese (354). This study showed that plasma LBP was positively correlated with BMI, waist circumference, blood pressure, LDL cholesterol, triglycerides, glucose, insulin, and inflammatory markers (CRP and IL-6), while being negatively correlated with HDL cholesterol and HMW ApN (354). Finally, I observed an anti-inflammatory effect of miR883b-5p in AT as blockade of miR883b-5p exacerbated inflammation in adipocytes and *de novo* formed AT. This effect may presumably result from the repression of LBP and/or other target genes involved in inflammatory pathways (e.g. chemokines, VEGF). My preliminary data have shown that expression of miR883b-5p was markedly downregulated, together with that of ApN, in omental AT of obese subjects. Thus, miR883b-5p could serve as a novel therapeutic target in metabolic syndrome.

II. General conclusion

In my thesis, I have confirmed that ApN regulates *in vivo* the secretory profile of downstream adipokines, decreasing those with pro-inflammatory properties while up-regulating those with anti-inflammatory action. I have also identified several miRNAs regulated by ApN in AT *in vivo*, one of these, miR883b-5p, which was up-regulated by ApN, repressed the production of LBP and turned out to be a major mediator of the anti-inflammatory action of ApN in murine adipocytes. These newly identified downstream adipokines and miRNAs, may have therapeutic potential for the management of the metabolic syndrome.

Chapter 5: Perspectives

I. Background

I have recently identified several novel miRNAs, which are regulated by ApN in AT in vivo. The expression of miR532-5p and miR1983 was down-regulated, while that of miR883b-5p and miR1934 was up-regulated in AT of ApN-overexpressing mice. In this pioneer work, I mainly focused on miR883b-5p because this miRNA was already included in current database and identified by computational analysis as being involved in inflammatory pathways. I found that ApN upregulated miR883b-5p, which in turn down-regulated LBP, a LPS facilitator, and subsequently TLR-4 signaling both in vitro (in murine adipocyte line) and in vivo (in newly formed AT). This is of importance because subclinical endotoxemia, characterized by low to moderately and chronically elevated LPS, may be involved in the pathogenesis of the metabolic syndrome (144). I therefore hypothesized that miR883b-5p may act as a major mediator of the anti-inflammatory action of ApN on AT. Because the novel miRNAs regulated by ApN may open new therapeutic perspectives for controlling AT inflammation, I wish to further characterize their function in mice and humans.

II. Perspectives

The work will be conducted along three major lines

- To examine whether miR883b-5p is involved in other inflammatory pathways besides TLR4 signalling;
- To elucidate the function of the other miRNAs, which were specifically regulated by ApN *in vivo*
- To translate these data to human obesity

Planned work

1. To establish whether miR883b-5p is involved in multiple inflammatory pathways

I have identified by computational analysis 1062 unique targets for miR883b-5p using the algorithms miRanda (327). I have further annotated the biological function of these target genes using GENECODIS database (327). I have focused on target genes implicated in inflammation/immune responses and found 4 predicted target genes (including LBP), which were enriched for TLR signalling pathway and innate immune responses. These genes have

already been studied (327). However, there were also additional predicted target genes involved in other inflammatory pathways which have not been studied yet: 7 target genes, which were enriched for the VEGF signalling pathway and 10 others for the chemokine signalling pathway. Both pathways are strongly involved in inflammation (4; 68), VEGF being also involved in vasculature remodelling (68).

My hypothesis is that miR883b-5p may mediate its anti-inflammatory action, by down-regulating, not only TRL4 signalling, but also these additional pathways. I plan to study the target genes belonging to these pathways by using *in vitro* and *in vivo* protocols similar to those previously established to investigate TLR4.

***In vitro* functional studies** We will use a gain-of- or a loss-of- function approach in 3T3-F442A adipocytes transfected by miR883b-5p mimic or inhibitor, respectively (Qiagen). We will measure, in these transfected cells, the changes in mRNA levels of the relevant target genes (RT-qPCR) as well as in protein levels encoded by these genes (Western blot or ELISA).

***In vivo* functional studies** We will further examine whether miR883b-5p targets VEGF and chemokine pathways *in vivo* in *de novo* formed fat pad. We have already performed such experiments for studying TLR4 signaling. We have constructed a plasmid that stably expresses Anti-miR883b-5p or a scrambled sequence (CTRL). We have chosen to block miR883b-5p rather than to overexpress it, in the light of the potential oversaturation of endogenous small RNA pathways (321). As already described, 3T3-F442A preadipocytes will be transfected with the plasmid and grown to near confluence, then injected subcutaneously into the back of NUDE mice (327; 355). Alternatively, we could use human adipose-derived adult stem (ADAS) cells (356). Mice will be kept on a Western diet for 2-3 wks to induce development of adiposity and local inflammation. At the end of the experiments, the *de novo* formed adipose tissue, resulting from *in vivo* differentiation of implanted 3T3-F442A preadipocytes, will be weighted and processed for immuno-morphometry (size and density of adipocytes and blood vessels, macrophage number) as well as for expression of relevant target genes. In my previous study dealing with the repercussions of miR883b-5p silencing on TLR-4 signalling, I found no change in fat pad weight, adipocyte size and macrophage number, but marked increases of LBP and inflammatory markers (IL-6, TNF-α). In this new setup of experiments, we will study the expression of target genes belonging to VEGF and chemokine signalling pathways (RT-qPCR for mRNAs and Western blot/ELISA for the encoded

proteins). We thus hypothesize to disclose novel mechanisms underlying the anti-inflammatory properties of miR883b-5p and ApN *in vivo*.

Furthermore, I plan to generate transgenic mouse models, which overexpress or are lacking (KO) miR883b-5p and to challenge these mice with LPS.

2. To elucidate the function of other miRNAs specifically regulated by ApN in vivo

Two miRNAs (miR1934 and miR1983), which were modified in ApN-overexpressing mice showed a striking reverse pattern of expression in ApN-KO mice. One of these miRNAs (miR1934), which was up-regulated in ApN-overexpressing mice, was even undetectable in ApN-KO mice, suggesting that ApN was necessary for its expression (327). Alternatively, low expression of miR1934 could be due to a single nucleotide polymorphism (SNP) in the mature form of miR1934[1]. Although this hypothesis has to be checked, it seems rather unlikely because ApN-KO mice and their controls were on the same genetic background.

***In vitro* functional studies** As the two miRNAs mentioned above are not yet included in current database for target prediction, we cannot get preliminary clues about their function. We will therefore attempt to identify their target genes by using cDNA microarrays (Affimetrix®) in 3T3-F442A adipocytes transfected with the respective anti-miR (or miR mimic) or a scrambled oligonucleotide sequence. Genes, which are differentially expressed between the two groups (Anti-miR *vs.* control or miR mimic *vs*. control), will be functionally annotated by using GENECODIS database. Genes implicated in inflammatory/immune responses, cell differentiation or metabolism will be selected and quantified by RT-qPCR (mRNA levels) and Western blot/ELISA (encoded protein levels). Relevant genes will be further validated by a luciferase reporter system. To this end, a luciferase reporter construct containing the 3'-UTR of the relevant target gene and the mature sequence of the miRNA will be co-transfected in 3T3-F44A cells and the expected reduction of luciferase activity will be monitored (357).

***In vivo* functional studies** We will examine whether the target genes regulated by these two miRNAs (especially miR1934) *in vitro* are confirmed *in vivo*. We will therefore quantify the expression of target genes in *de novo* fat pad formed in NUDE mice from 3T3-F442A preadipocytes transfected with Anti-miR1934 plasmid or a control plasmid.

[1] http://www.ncbi.nlm.nih.gov/SNP/snp_ss.cgi?ss=ss37166983

3. To investigate whether these key miRNAs are relevant in human obesity

ApN is decreased in fat tissue and plasma of obese subjects (4). We will examine whether this impairment may induce a deregulation of the key miRNAs, described above, in AT of obese subjects.

***Ex vivo* studies** Paired omental and subcutaneous fat samples will be obtained from obese and from age- and sex-matched lean patients undergoing elective abdominal surgery (long-lasting collaboration with the Abdominal Surgery Unit, Saint-Luc University Hospital). Based on our past experience and on statistical power analysis, we will need to recruit 6-10 patients per group to demonstrate significant differences between the 2 groups (34; 64). We will examine whether these key miRNAs are modified together with the expected decrease of ApN in obese subjects and whether these changes are depot-specific. We will also investigate whether these changes are related to the inflammatory state of adipose tissue and to several anthropometric and circulating parameters (Body mass index, lipid profile, glucose homeostasis and insulin resistance estimated by the HOMA index (64).

***In vitro* studies** We will investigate in primary cultures of human adipocytes differentiated *in vitro* (34; 64) whether these key miRNAs are modified during the differentiation process and whether recombinant ApN may trigger a profile of miRNAs similar to that observed in ApN-overexpressing mice. By using a gain-of- or a loss-of- function approach similar to that described in 3T3-F442A, we will also study in human adipocytes transfected by relevant miR mimics or Anti-miRs whether those key miRNAs have similar target genes to those described in mice. Finally, we wish to establish the direct role of these key miRNAs on the anti-inflammatory properties of ApN in human AT. We will therefore test whether these miRNAs can modify the expression profile of pro-inflammatory adipokines previously identified as downstream targets of ApN (326). These experiments will be carried out in human adipocytes transfected as described above.

In conclusion, we hope (a) to characterize *in vivo* and *in vitro* the miRNAs regulated by one of the most important adipokines, ApN in mice and humans and (b) possibly open new therapeutic perspectives for controlling AT inflammation, a cornerstone of the metabolic syndrome.

General Reference List

1. **Tseng YH, Cypess AM and Kahn CR**. Cellular bioenergetics as a target for obesity therapy. *Nat Rev Drug Discov* 9: 465-482, 2010.

2. **Hotamisligil GS**. Inflammation and metabolic disorders. *Nature* 444: 860-867, 2006.

3. **Trujillo ME and Scherer PE**. Adipose tissue-derived factors: impact on health and disease. *Endocr Rev* 27: 762-778, 2006.

4. **Maury E and Brichard SM**. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol Cell Endocrinol* 314: 1-16, 2010.

5. **Cinti S**. The adipose organ. *Prostaglandins Leukot Essent Fatty Acids* 73: 9-15, 2005.

6. **Lafontan M and Berlan M**. Do regional differences in adipocyte biology provide new pathophysiological insights? *Trends Pharmacol Sci* 24: 276-283, 2003.

7. **Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM and Smith SC, Jr.** Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* 120: 1640-1645, 2009.

8. **Alberti KG, Zimmet P and Shaw J**. The metabolic syndrome--a new worldwide definition. *Lancet* 366: 1059-1062, 2005.

9. **Zimmet P, Magliano D, Matsuzawa Y, Alberti G and Shaw J**. The metabolic syndrome: a global public health problem and a new definition. *J Atheroscler Thromb* 12: 295-300, 2005.

10. **Lumeng CN and Saltiel AR**. Inflammatory links between obesity and metabolic disease. *J Clin Invest* 121: 2111-2117, 2011.

11. **Chen Y, Zhu J, Lum PY, Yang X, Pinto S, MacNeil DJ, Zhang C, Lamb J, Edwards S, Sieberts SK, Leonardson A, Castellini LW, Wang S, Champy MF, Zhang B, Emilsson V, Doss S, Ghazalpour A, Horvath S, Drake TA, Lusis AJ and Schadt EE**. Variations in DNA elucidate molecular networks that cause disease. *Nature* 452: 429-435, 2008.

12. **Emilsson V, Thorleifsson G, Zhang B, Leonardson AS, Zink F, Zhu J, Carlson S, Helgason A, Walters GB, Gunnarsdottir S, Mouy M, Steinthorsdottir V, Eiriksdottir GH, Bjornsdottir G, Reynisdottir I, Gudbjartsson D, Helgadottir A, Jonasdottir A, Jonasdottir A, Styrkarsdottir U, Gretarsdottir S, Magnusson KP, Stefansson H, Fossdal R, Kristjansson K, Gislason HG, Stefansson T, Leifsson BG, Thorsteinsdottir U, Lamb JR, Gulcher JR, Reitman ML, Kong A, Schadt EE and Stefansson K**. Genetics of gene expression and its effect on disease. *Nature* 452: 423-428, 2008.

13. **Varma V, Yao-Borengasser A, Rasouli N, Nolen GT, Phanavanh B, Starks T, Gurley C, Simpson P, McGehee RE, Jr., Kern PA and Peterson CA**. Muscle inflammatory response and insulin resistance: synergistic interaction between macrophages and fatty acids leads to impaired insulin action. *Am J Physiol Endocrinol Metab* 296: E1300-E1310, 2009.

14. **Huang W, Metlakunta A, Dedousis N, Zhang P, Sipula I, Dube JJ, Scott DK and O'Doherty RM**. Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. *Diabetes* 59: 347-357, 2010.

15. **Thaler JP, Yi CX, Schur EA, Guyenet SJ, Hwang BH, Dietrich MO, Zhao X, Sarruf DA, Izgur V, Maravilla KR, Nguyen HT, Fischer JD, Matsen ME, Wisse BE, Morton GJ, Horvath TL, Baskin DG, Tschop MH and Schwartz MW**. Obesity is associated with hypothalamic injury in rodents and humans. *J Clin Invest* 122: 153-162, 2012.

16. **Kang YM, Ma Y, Zheng JP, Elks C, Sriramula S, Yang ZM and Francis J**. Brain nuclear factor-kappa B activation contributes to neurohumoral excitation in angiotensin II-induced hypertension. *Cardiovasc Res* 82: 503-512, 2009.

17. **Calegari VC, Torsoni AS, Vanzela EC, Araujo EP, Morari J, Zoppi CC, Sbragia L, Boschero AC and Velloso LA**. Inflammation of the hypothalamus leads to defective pancreatic islet function. *J Biol Chem* 286: 12870-12880, 2011.

18. **Purkayastha S, Zhang H, Zhang G, Ahmed Z, Wang Y and Cai D**. Neural dysregulation of peripheral insulin action and blood pressure by brain endoplasmic reticulum stress. *Proc Natl Acad Sci U S A* 108: 2939-2944, 2011.

19. **Zambon A, Gervois P, Pauletto P, Fruchart JC and Staels B**. Modulation of hepatic inflammatory risk markers of cardiovascular diseases by PPAR-alpha activators: clinical and experimental evidence. *Arterioscler Thromb Vasc Biol* 26: 977-986, 2006.

20. **Stienstra R, Duval C, Muller M and Kersten S**. PPARs, Obesity, and Inflammation. *PPAR Res* 2007: 95974, 2007.

21. **Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG and Glass CK**. A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* 437: 759-763, 2005.

22. **Ialenti A, Grassia G, Di MP, Maffia P, Di RM and Ianaro A**. Mechanism of the anti-inflammatory effect of thiazolidinediones: relationship with the glucocorticoid pathway. *Mol Pharmacol* 67: 1620-1628, 2005.

23. **Ricote M, Li AC, Willson TM, Kelly CJ and Glass CK**. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 391: 79-82, 1998.

24. **Balk EM, Lau J, Goudas LC, Jordan HS, Kupelnick B, Kim LU and Karas RH**. Effects of statins on nonlipid serum markers associated with cardiovascular disease: a systematic review. *Ann Intern Med* 139: 670-682, 2003.

25. **Goldfine AB**. Statins: is it really time to reassess benefits and risks? *N Engl J Med* 366: 1752-1755, 2012.

26. **Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M and Shoelson SE**. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* 293: 1673-1677, 2001.

27. **Goldfine AB, Fonseca V, Jablonski KA, Pyle L, Staten MA and Shoelson SE**. The effects of salsalate on glycemic control in patients with type 2 diabetes: a randomized trial. *Ann Intern Med* 152: 346-357, 2010.

28. **Bernstein LE, Berry J, Kim S, Canavan B and Grinspoon SK**. Effects of etanercept in patients with the metabolic syndrome. *Arch Intern Med* 166: 902-908, 2006.

29. **Dominguez H, Storgaard H, Rask-Madsen C, Steffen HT, Ihlemann N, Baunbjerg ND, Spohr C, Kober L, Vaag A and Torp-Pedersen C**. Metabolic and vascular effects of tumor necrosis factor-alpha blockade with etanercept in obese patients with type 2 diabetes. *J Vasc Res* 42: 517-525, 2005.

30. **Farooqi IS, Matarese G, Lord GM, Keogh JM, Lawrence E, Agwu C, Sanna V, Jebb SA, Perna F, Fontana S, Lechler RI, DePaoli AM and O'Rahilly S**. Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *J Clin Invest* 110: 1093-1103, 2002.

31. **Filkova M, Haluzik M, Gay S and Senolt L**. The role of resistin as a regulator of inflammation: Implications for various human pathologies. *Clin Immunol* 133: 157-170, 2009.

32. **Dandona P, Weinstock R, Thusu K, Abdel-Rahman E, Aljada A and Wadden T**. Tumor necrosis factor-alpha in sera of obese patients: fall with weight loss. *J Clin Endocrinol Metab* 83: 2907-2910, 1998.

33. **Hotamisligil GS, Arner P, Atkinson RL and Spiegelman BM**. Differential regulation of the p80 tumor necrosis factor receptor in human obesity and insulin resistance. *Diabetes* 46: 451-455, 1997.

34. **Maury E, Noel L, Detry R and Brichard SM**. In vitro hyperresponsiveness to tumor necrosis factor-alpha contributes to adipokine dysregulation in omental adipocytes of obese subjects. *J Clin Endocrinol Metab* 94: 1393-1400, 2009.

35. **Uysal KT, Wiesbrock SM, Marino MW and Hotamisligil GS**. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 389: 610-614, 1997.

36. **Sabio G, Das M, Mora A, Zhang Z, Jun JY, Ko HJ, Barrett T, Kim JK and Davis RJ**. A stress signaling pathway in adipose tissue regulates hepatic insulin resistance. *Science* 322: 1539-1543, 2008.

37. **Shoelson SE, Lee J and Goldfine AB**. Inflammation and insulin resistance. *J Clin Invest* 116: 1793-1801, 2006.

38. **Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T and Matsuzawa Y**. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* 8: 731-737, 2002.

39. **Moller DE**. Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes. *Trends Endocrinol Metab* 11: 212-217, 2000.

40. **Balistreri CR, Caruso C and Candore G**. The role of adipose tissue and adipokines in obesity-related inflammatory diseases. *Mediators Inflamm* 2010: 802078, 2010.

41. **Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S and Coppack SW**. Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 82: 4196-4200, 1997.

42. **Starr ME, Evers BM and Saito H**. Age-associated increase in cytokine production during systemic inflammation: adipose tissue as a major source of IL-6. *J Gerontol A Biol Sci Med Sci* 64: 723-730, 2009.

43. **Bastard JP, Lagathu C, Caron M and Capeau J**. Point-counterpoint: Interleukin-6 does/does not have a beneficial role in insulin sensitivity and glucose homeostasis. *J Appl Physiol* 102: 821-822, 2007.

44. **Calabro P, Chang DW, Willerson JT and Yeh ET**. Release of C-reactive protein in response to inflammatory cytokines by human adipocytes: linking obesity to vascular inflammation. *J Am Coll Cardiol* 46: 1112-1113, 2005.

45. **van HG, Steensberg A, Sacchetti M, Fischer C, Keller C, Schjerling P, Hiscock N, Moller K, Saltin B, Febbraio MA and Pedersen BK**. Interleukin-6 stimulates lipolysis and fat oxidation in humans. *J Clin Endocrinol Metab* 88: 3005-3010, 2003.

46. **Matsuki T, Horai R, Sudo K and Iwakura Y**. IL-1 plays an important role in lipid metabolism by regulating insulin levels under physiological conditions. *J Exp Med* 198: 877-888, 2003.

47. **Lagathu C, Yvan-Charvet L, Bastard JP, Maachi M, Quignard-Boulange A, Capeau J and Caron M**. Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes. *Diabetologia* 49: 2162-2173, 2006.

48. **Spranger J, Kroke A, Mohlig M, Hoffmann K, Bergmann MM, Ristow M, Boeing H and Pfeiffer AF**. Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes* 52: 812-817, 2003.

49. **Suzawa M, Takada I, Yanagisawa J, Ohtake F, Ogawa S, Yamauchi T, Kadowaki T, Takeuchi Y, Shibuya H, Gotoh Y, Matsumoto K and Kato S**. Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. *Nat Cell Biol* 5: 224-230, 2003.

50. **Sartipy P and Loskutoff DJ**. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A* 100: 7265-7270, 2003.

51. **Bruun JM, Lihn AS, Pedersen SB and Richelsen B**. Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. *J Clin Endocrinol Metab* 90: 2282-2289, 2005.

52. **Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K and Kasuga M**. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* 116: 1494-1505, 2006.

53. **Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL and Ferrante AW, Jr.** Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112: 1796-1808, 2003.

54. **Simeoni E, Hoffmann MM, Winkelmann BR, Ruiz J, Fleury S, Boehm BO, Marz W and Vassalli G**. Association between the A-2518G polymorphism in the monocyte chemoattractant protein-1 gene and insulin resistance and Type 2 diabetes mellitus. *Diabetologia* 47: 1574-1580, 2004.

55. **Martinovic I, Abegunewardene N, Seul M, Vosseler M, Horstick G, Buerke M, Darius H and Lindemann S**. Elevated monocyte chemoattractant protein-1 serum levels in patients at risk for coronary artery disease. *Circ J* 69: 1484-1489, 2005.

56. **Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P and Rollins BJ**. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* 2: 275-281, 1998.

57. **Veillard NR, Kwak B, Pelli G, Mulhaupt F, James RW, Proudfoot AE and Mach F**. Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. *Circ Res* 94: 253-261, 2004.

58. **Boger CA, Fischereder M, Deinzer M, Aslanidis C, Schmitz G, Stubanus M, Banas B, Kruger B, Riegger GA and Kramer BK**. RANTES gene polymorphisms predict all-cause and cardiac mortality in type 2 diabetes mellitus hemodialysis patients. *Atherosclerosis* 183: 121-129, 2005.

59. **Herder C, Haastert B, Muller-Scholze S, Koenig W, Thorand B, Holle R, Wichmann HE, Scherbaum WA, Martin S and Kolb H**. Association of systemic chemokine concentrations with impaired glucose tolerance and type 2 diabetes: results from the Cooperative Health Research in the Region of Augsburg Survey S4 (KORA S4). *Diabetes* 54 Suppl 2: S11-S17, 2005.

60. **Straczkowski M, Dzienis-Straczkowska S, Stepien A, Kowalska I, Szelachowska M and Kinalska I**. Plasma interleukin-8 concentrations are increased in obese subjects and related to fat mass and tumor necrosis factor-alpha system. *J Clin Endocrinol Metab* 87: 4602-4606, 2002.

61. **Cancello R and Clement K**. Is obesity an inflammatory illness? Role of low-grade inflammation and macrophage infiltration in human white adipose tissue. *BJOG* 113: 1141-1147, 2006.

62. **Boisvert WA, Santiago R, Curtiss LK and Terkeltaub RA**. A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J Clin Invest* 101: 353-363, 1998.

63. **Cao Y**. Angiogenesis modulates adipogenesis and obesity. *J Clin Invest* 117: 2362-2368, 2007.

64. **Maury E, Ehala-Aleksejev K, Guiot Y, Detry R, Vandenhooft A and Brichard SM**. Adipokines oversecreted by omental adipose tissue in human obesity. *Am J Physiol Endocrinol Metab* 293: E656-E665, 2007.

65. **Hausman GJ and Richardson RL**. Adipose tissue angiogenesis. *J Anim Sci* 82: 925-934, 2004.

66. **Zhang QX, Magovern CJ, Mack CA, Budenbender KT, Ko W and Rosengart TK**. Vascular endothelial growth factor is the major angiogenic factor in omentum: mechanism of the omentum-mediated angiogenesis. *J Surg Res* 67: 147-154, 1997.

67. **Lijnen HR, Christiaens V, Scroyen I, Voros G, Tjwa M, Carmeliet P and Collen D**. Impaired adipose tissue development in mice with inactivation of placental growth factor function. *Diabetes* 55: 2698-2704, 2006.

68. **Takahashi H and Shibuya M**. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. *Clin Sci (Lond)* 109: 227-241, 2005.

69. **Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, Bouillot JL, Bouloumie A, Barbatelli G, Cinti S, Svensson PA, Barsh GS, Zucker JD, Basdevant A, Langin D and Clement K**. Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* 54: 2277-2286, 2005.

70. **Doran AC, Meller N, Cutchins A, Deliri H, Slayton RP, Oldham SN, Kim JB, Keller SR and McNamara CA**. The helix-loop-helix factors Id3 and E47 are novel regulators of adiponectin. *Circ Res* 103: 624-634, 2008.

71. **Senaran H, Ileri M, Altinbas A, Kosar A, Yetkin E, Ozturk M, Karaaslan Y and Kirazli S**. Thrombopoietin and mean platelet volume in coronary artery disease. *Clin Cardiol* 24: 405-408, 2001.

72. **Straczkowski M, Kowalska I, Nikolajuk A, Krukowska A and Gorska M**. Plasma interleukin-10 concentration is positively related to insulin sensitivity in young healthy individuals. *Diabetes Care* 28: 2036-2037, 2005.

73. **Lumeng CN, Bodzin JL and Saltiel AR**. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 117: 175-184, 2007.

74. **Juge-Aubry CE, Somm E, Pernin A, Alizadeh N, Giusti V, Dayer JM and Meier CA**. Adipose tissue is a regulated source of interleukin-10. *Cytokine* 29: 270-274, 2005.

75. **Shapiro L and Scherer PE**. The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. *Curr Biol* 8: 335-338, 1998.

76. **Kishore U, Gaboriaud C, Waters P, Shrive AK, Greenhough TJ, Reid KB, Sim RB and Arlaud GJ**. C1q and tumor necrosis factor superfamily: modularity and versatility. *Trends Immunol* 25: 551-561, 2004.

77. **Weigert J, Neumeier M, Schaffler A, Fleck M, Scholmerich J, Schutz C and Buechler C**. The adiponectin paralog CORS-26 has anti-inflammatory properties and is produced by human monocytic cells. *FEBS Lett* 579: 5565-5570, 2005.

78. **Schaffler A, Scholmerich J and Salzberger B**. Adipose tissue as an immunological organ: Toll-like receptors, C1q/TNFs and CTRPs. *Trends Immunol* 28: 393-399, 2007.

79. **Yang RZ, Lee MJ, Hu H, Pray J, Wu HB, Hansen BC, Shuldiner AR, Fried SK, McLenithan JC and Gong DW**. Identification of omentin as a novel depot-specific adipokine in human adipose tissue: possible role in modulating insulin action. *Am J Physiol Endocrinol Metab* 290: E1253-E1261, 2006.

80. **Souza Batista CM, Yang RZ, Lee MJ, Glynn NM, Yu DZ, Pray J, Ndubuizu K, Patil S, Schwartz A, Kligman M, Fried SK, Gong DW, Shuldiner AR, Pollin TI and McLenithan JC**. Omentin plasma levels and gene expression are decreased in obesity. *Diabetes* 56: 1655-1661, 2007.

81. **Tan BK, Adya R, Farhatullah S, Lewandowski KC, O'Hare P, Lehnert H and Randeva HS**. Omentin-1, a novel adipokine, is decreased in overweight insulin-resistant women with polycystic ovary syndrome: ex vivo and in vivo regulation of omentin-1 by insulin and glucose. *Diabetes* 57: 801-808, 2008.

82. **Yamawaki H, Tsubaki N, Mukohda M, Okada M and Hara Y**. Omentin, a novel adipokine, induces vasodilation in rat isolated blood vessels. *Biochem Biophys Res Commun* 393: 668-672, 2010.

83. **Tan BK, Adya R and Randeva HS**. Omentin: a novel link between inflammation, diabesity, and cardiovascular disease. *Trends Cardiovasc Med* 20: 143-148, 2010.

84. **Ahima RS**. Revisiting leptin's role in obesity and weight loss. *J Clin Invest* 118: 2380-2383, 2008.

85. **Buettner C, Muse ED, Cheng A, Chen L, Scherer T, Pocai A, Su K, Cheng B, Li X, Harvey-White J, Schwartz GJ, Kunos G, Rossetti L and Buettner C**. Leptin controls adipose tissue lipogenesis via central, STAT3-independent mechanisms. *Nat Med* 14: 667-675, 2008.

86. **Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D and Kahn BB**. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 415: 339-343, 2002.

87. **Mantzoros CS, Magkos F, Brinkoetter M, Sienkiewicz E, Dardeno TA, Kim SY, Hamnvik OP and Koniaris A**. Leptin in human physiology and pathophysiology. *Am J Physiol Endocrinol Metab* 301: E567-E584, 2011.

88. **Fernandez-Riejos P, Najib S, Santos-Alvarez J, Martin-Romero C, Perez-Perez A, Gonzalez-Yanes C and Sanchez-Margalet V**. Role of leptin in the activation of immune cells. *Mediators Inflamm* 2010: 568343, 2010.

89. **Schaffler A, Muller-Ladner U, Scholmerich J and Buchler C**. Role of adipose tissue as an inflammatory organ in human diseases. *Endocr Rev* 27: 449-467, 2006.

90. **Kielar D, Clark JS, Ciechanowicz A, Kurzawski G, Sulikowski T and Naruszewicz M**. Leptin receptor isoforms expressed in human adipose tissue. *Metabolism* 47: 844-847, 1998.

91. **Sethi JK, Xu H, Uysal KT, Wiesbrock SM, Scheja L and Hotamisligil GS**. Characterisation of receptor-specific TNFalpha functions in adipocyte cell lines lacking type 1 and 2 TNF receptors. *FEBS Lett* 469: 77-82, 2000.

92. **Path G, Bornstein SR, Gurniak M, Chrousos GP, Scherbaum WA and Hauner H**. Human breast adipocytes express interleukin-6 (IL-6) and its receptor system: increased IL-6 production by beta-adrenergic activation and effects of IL-6 on adipocyte function. *J Clin Endocrinol Metab* 86: 2281-2288, 2001.

93. **Rega G, Kaun C, Weiss TW, Demyanets S, Zorn G, Kastl SP, Steiner S, Seidinger D, Kopp CW, Frey M, Roehle R, Maurer G, Huber K and Wojta J**. Inflammatory cytokines interleukin-6 and oncostatin m induce plasminogen activator inhibitor-1 in human adipose tissue. *Circulation* 111: 1938-1945, 2005.

94. **Bruun JM, Lihn AS, Verdich C, Pedersen SB, Toubro S, Astrup A and Richelsen B**. Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. *Am J Physiol Endocrinol Metab* 285: E527-E533, 2003.

95. **White UA and Stephens JM**. The gp130 receptor cytokine family: regulators of adipocyte development and function. *Curr Pharm Des* 17: 340-346, 2011.

96. **Tam J, Duda DG, Perentes JY, Quadri RS, Fukumura D and Jain RK**. Blockade of VEGFR2 and not VEGFR1 can limit diet-induced fat tissue expansion: role of local versus bone marrow-derived endothelial cells. *PLoS One* 4: e4974, 2009.

97. **Karastergiou K and Mohamed-Ali V**. The autocrine and paracrine roles of adipokines. *Mol Cell Endocrinol* 318: 69-78, 2010.

98. **Bourlier V, Zakaroff-Girard A, Miranville A, De BS, Maumus M, Sengenes C, Galitzky J, Lafontan M, Karpe F, Frayn KN and Bouloumie A**. Remodeling phenotype of human subcutaneous adipose tissue macrophages. *Circulation* 117: 806-815, 2008.

99. **Faust IM, Johnson PR, Stern JS and Hirsch J**. Diet-induced adipocyte number increase in adult rats: a new model of obesity. *Am J Physiol* 235: E279-E286, 1978.

100. **Henegar C, Tordjman J, Achard V, Lacasa D, Cremer I, Guerre-Millo M, Poitou C, Basdevant A, Stich V, Viguerie N, Langin D, Bedossa P, Zucker JD and Clement K**. Adipose tissue transcriptomic signature highlights the pathological relevance of extracellular matrix in human obesity. *Genome Biol* 9: R14, 2008.

101. **Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hoffstedt J, Naslund E, Britton T, Concha H, Hassan M, Ryden M, Frisen J and Arner P**. Dynamics of fat cell turnover in humans. *Nature* 453: 783-787, 2008.

102. **Jiao P and Xu H**. Adipose inflammation: cause or consequence of obesity-related insulin resistance. *Diabetes Metab Syndr Obes* 1: 25-31, 2008.

103. **Skurk T, Alberti-Huber C, Herder C and Hauner H**. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* 92: 1023-1033, 2007.

104. **Jernas M, Palming J, Sjoholm K, Jennische E, Svensson PA, Gabrielsson BG, Levin M, Sjogren A, Rudemo M, Lystig TC, Carlsson B, Carlsson LM and Lonn M**. Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. *FASEB J* 20: 1540-1542, 2006.

105. **Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS and Obin MS**. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 46: 2347-2355, 2005.

106. **Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, DeFuria J, Jick Z, Greenberg AS and Obin MS**. Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes* 56: 2910-2918, 2007.

107. **Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA and Chen H**. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112: 1821-1830, 2003.

108. **Gordon S**. The macrophage: past, present and future. *Eur J Immunol* 37 Suppl 1: S9-17, 2007.

109. **Lumeng CN, DelProposto JB, Westcott DJ and Saltiel AR**. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. *Diabetes* 57: 3239-3246, 2008.

110. **Zeyda M and Stulnig TM**. Adipose tissue macrophages. *Immunol Lett* 112: 61-67, 2007.

111. **Chatzigeorgiou A, Karalis KP, Bornstein SR and Chavakis T**. Lymphocytes in obesity-related adipose tissue inflammation. *Diabetologia* 55: 2583-2592, 2012.

112. **Sengenes C, Miranville A, Lolmede K, Curat CA and Bouloumie A**. The role of endothelial cells in inflamed adipose tissue. *J Intern Med* 262: 415-421, 2007.

113. **Ferri C, Desideri G, Valenti M, Bellini C, Pasin M, Santucci A and De MG**. Early upregulation of endothelial adhesion molecules in obese hypertensive men. *Hypertension* 34: 568-573, 1999.

114. **Brakenhielm E, Cao R, Gao B, Angelin B, Cannon B, Parini P and Cao Y**. Angiogenesis inhibitor, TNP-470, prevents diet-induced and genetic obesity in mice. *Circ Res* 94: 1579-1588, 2004.

115. **Rupnick MA, Panigrahy D, Zhang CY, Dallabrida SM, Lowell BB, Langer R and Folkman MJ**. Adipose tissue mass can be regulated through the vasculature. *Proc Natl Acad Sci U S A* 99: 10730-10735, 2002.

116. **Alessi MC, Bastelica D, Morange P, Berthet B, Leduc I, Verdier M, Geel O and Juhan-Vague I**. Plasminogen activator inhibitor 1, transforming growth factor-beta1, and BMI are closely associated in human adipose tissue during morbid obesity. *Diabetes* 49: 1374-1380, 2000.

117. **Chavey C, Mari B, Monthouel MN, Bonnafous S, Anglard P, Van OE and Tartare-Deckert S**. Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. *J Biol Chem* 278: 11888-11896, 2003.

118. **Maquoi E, Munaut C, Colige A, Collen D and Lijnen HR**. Modulation of adipose tissue expression of murine matrix metalloproteinases and their tissue inhibitors with obesity. *Diabetes* 51: 1093-1101, 2002.

119. **Xiao Y, Junfeng H, Tianhong L, Lu W, Shulin C, Yu Z, Xiaohua L, Weixia J, Sheng Z, Yanyun G, Guo L and Min L**. Cathepsin K in adipocyte differentiation and its potential role in the pathogenesis of obesity. *J Clin Endocrinol Metab* 91: 4520-4527, 2006.

120. **Naour N, Rouault C, Fellahi S, Lavoie ME, Poitou C, Keophiphath M, Eberle D, Shoelson S, Rizkalla S, Bastard JP, Rabasa-Lhoret R, Clement K and Guerre-Millo M**. Cathepsins in human obesity: changes in energy balance predominantly affect cathepsin s in adipose tissue and in circulation. *J Clin Endocrinol Metab* 95: 1861-1868, 2010.

121. **Taleb S, Cancello R, Clement K and Lacasa D**. Cathepsin s promotes human preadipocyte differentiation: possible involvement of fibronectin degradation. *Endocrinology* 147: 4950-4959, 2006.

122. **Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, Furukawa S, Tochino Y, Komuro R, Matsuda M and Shimomura I**. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* 56: 901-911, 2007.

123. **Kabon B, Nagele A, Reddy D, Eagon C, Fleshman JW, Sessler DI and Kurz A**. Obesity decreases perioperative tissue oxygenation. *Anesthesiology* 100: 274-280, 2004.

124. **Virtanen KA, Lonnroth P, Parkkola R, Peltoniemi P, Asola M, Viljanen T, Tolvanen T, Knuuti J, Ronnemaa T, Huupponen R and Nuutila P**. Glucose uptake and perfusion in subcutaneous and visceral adipose tissue during insulin stimulation in nonobese and obese humans. *J Clin Endocrinol Metab* 87: 3902-3910, 2002.

125. **Ye J, Gao Z, Yin J and He Q**. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am J Physiol Endocrinol Metab* 293: E1118-E1128, 2007.

126. **Wang B, Wood IS and Trayhurn P**. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. *Pflugers Arch* 455: 479-492, 2007.

127. **Lolmede K, Durand de SF, V, Galitzky J, Lafontan M and Bouloumie A**. Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. *Int J Obes Relat Metab Disord* 27: 1187-1195, 2003.

128. **Halberg N, Khan T, Trujillo ME, Wernstedt-Asterholm I, Attie AD, Sherwani S, Wang ZV, Landskroner-Eiger S, Dineen S, Magalang UJ, Brekken RA and Scherer PE**. Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. *Mol Cell Biol* 29: 4467-4483, 2009.

129. **Zhang K and Kaufman RJ**. From endoplasmic-reticulum stress to the inflammatory response. *Nature* 454: 455-462, 2008.

130. **Tu BP and Weissman JS**. Oxidative protein folding in eukaryotes: mechanisms and consequences. *J Cell Biol* 164: 341-346, 2004.

131. **Haynes CM, Titus EA and Cooper AA**. Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. *Mol Cell* 15: 767-776, 2004.

132. **Schaeffler A, Gross P, Buettner R, Bollheimer C, Buechler C, Neumeier M, Kopp A, Schoelmerich J and Falk W**. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity. *Immunology* 126: 233-245, 2009.

133. **Kopp A, Buechler C, Neumeier M, Weigert J, Aslanidis C, Scholmerich J and Schaffler A**. Innate immunity and adipocyte function: ligand-specific activation of multiple Toll-like receptors modulates cytokine, adipokine, and chemokine secretion in adipocytes. *Obesity (Silver Spring)* 17: 648-656, 2009.

134. **Vitseva OI, Tanriverdi K, Tchkonia TT, Kirkland JL, McDonnell ME, Apovian CM, Freedman J and Gokce N**. Inducible Toll-like receptor and NF-kappaB regulatory pathway expression in human adipose tissue. *Obesity (Silver Spring)* 16: 932-937, 2008.

135. **Creely SJ, McTernan PG, Kusminski CM, Fisher M, Da Silva NF, Khanolkar M, Evans M, Harte AL and Kumar S**. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am J Physiol Endocrinol Metab* 292: E740-E747, 2007.

136. **Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H and Flier JS**. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* 116: 3015-3025, 2006.

137. **Davis JE, Gabler NK, Walker-Daniels J and Spurlock ME**. Tlr-4 deficiency selectively protects against obesity induced by diets high in saturated fat. *Obesity (Silver Spring)* 16: 1248-1255, 2008.

138. **Song MJ, Kim KH, Yoon JM and Kim JB**. Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. *Biochem Biophys Res Commun* 346: 739-745, 2006.

139. **Suganami T, Mieda T, Itoh M, Shimoda Y, Kamei Y and Ogawa Y**. Attenuation of obesity-induced adipose tissue inflammation in C3H/HeJ mice carrying a Toll-like receptor 4 mutation. *Biochem Biophys Res Commun* 354: 45-49, 2007.

140. **Ajuwon KM and Spurlock ME**. Palmitate activates the NF-kappaB transcription factor and induces IL-6 and TNFalpha expression in 3T3-L1 adipocytes. *J Nutr* 135: 1841-1846, 2005.

141. **Medzhitov R**. Origin and physiological roles of inflammation. *Nature* 454: 428-435, 2008.

142. **Kopp A, Buechler C, Bala M, Neumeier M, Scholmerich J and Schaffler A**. Toll-like receptor ligands cause proinflammatory and prodiabetic activation of adipocytes via phosphorylation of extracellular signal-regulated kinase and c-Jun N-terminal kinase but not interferon regulatory factor-3. *Endocrinology* 151: 1097-1108, 2010.

143. **Prieur X, Roszer T and Ricote M**. Lipotoxicity in macrophages: evidence from diseases associated with the metabolic syndrome. *Biochim Biophys Acta* 1801: 327-337, 2010.

144. **Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC and Burcelin R**. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56: 1761-1772, 2007.

145. **Monte SV, Caruana JA, Ghanim H, Sia CL, Korzeniewski K, Schentag JJ and Dandona P**. Reduction in endotoxemia, oxidative and inflammatory stress, and insulin resistance after Roux-en-Y gastric bypass surgery in patients with morbid obesity and type 2 diabetes mellitus. *Surgery* 151: 587-593, 2012.

146. **Ghoshal S, Witta J, Zhong J, de VW and Eckhardt E**. Chylomicrons promote intestinal absorption of lipopolysaccharides. *J Lipid Res* 50: 90-97, 2009.

147. **Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM and Burcelin R**. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 57: 1470-1481, 2008.

148. **Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck A, Lambert DM, Muccioli GG and Delzenne NM**. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut* 58: 1091-1103, 2009.

149. **Muccioli GG, Naslain D, Backhed F, Reigstad CS, Lambert DM, Delzenne NM and Cani PD**. The endocannabinoid system links gut microbiota to adipogenesis. *Mol Syst Biol* 6: 392, 2010.

150. **Wang Y, Xu A, Knight C, Xu LY and Cooper GJ**. Hydroxylation and glycosylation of the four conserved lysine residues in the collagenous domain of adiponectin. Potential role in the modulation of its insulin-sensitizing activity. *J Biol Chem* 277: 19521-19529, 2002.

151. **Schaffler A and Scholmerich J**. Innate immunity and adipose tissue biology. *Trends Immunol* 31: 228-235, 2010.

152. **Zu L, He J, Jiang H, Xu C, Pu S and Xu G**. Bacterial endotoxin stimulates adipose lipolysis via toll-like receptor 4 and extracellular signal-regulated kinase pathway. *J Biol Chem* 284: 5915-5926, 2009.

153. **Finlay BB and Hancock RE**. Can innate immunity be enhanced to treat microbial infections? *Nat Rev Microbiol* 2: 497-504, 2004.

154. **Kadowaki T and Yamauchi T**. Adiponectin and adiponectin receptors. *Endocr Rev* 26: 439-451, 2005.

155. **Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, Vasseur F, Froguel P, Kimura S, Nagai R and Kadowaki T**. Impaired multimerization of human adiponectin mutants associated with diabetes. Molecular structure and multimer formation of adiponectin. *J Biol Chem* 278: 40352-40363, 2003.

156. **Xu A, Chan KW, Hoo RL, Wang Y, Tan KC, Zhang J, Chen B, Lam MC, Tse C, Cooper GJ and Lam KS**. Testosterone selectively reduces the high molecular weight form of adiponectin by inhibiting its secretion from adipocytes. *J Biol Chem* 280: 18073-18080, 2005.

157. **Wang Y, Lam KS, Yau MH and Xu A**. Post-translational modifications of adiponectin: mechanisms and functional implications. *Biochem J* 409: 623-633, 2008.

158. **Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE and Lodish HF**. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci U S A* 98: 2005-2010, 2001.

159. **Badman MK and Flier JS**. The adipocyte as an active participant in energy balance and metabolism. *Gastroenterology* 132: 2103-2115, 2007.

160. **Jortay J, Senou M, Abou-Samra M, Noel L, Robert A, Many MC and Brichard SM**. Adiponectin and skeletal muscle: pathophysiological implications in metabolic stress. *Am J Pathol* 181: 245-256, 2012.

161. **Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R and Kadowaki T**. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423: 762-769, 2003.

162. **Yamauchi T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M, Okada-Iwabu M, Kawamoto S, Kubota N, Kubota T, Ito Y, Kamon J, Tsuchida A, Kumagai K, Kozono H, Hada Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Awazawa M, Takamoto I, Froguel P, Hara K, Tobe K, Nagai R, Ueki K and Kadowaki T**. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. *Nat Med* 13: 332-339, 2007.

163. **Bjursell M, Ahnmark A, Bohlooly Y, William-Olsson L, Rhedin M, Peng XR, Ploj K, Gerdin AK, Arnerup G, Elmgren A, Berg AL, Oscarsson J and Linden D**. Opposing effects of adiponectin receptors 1 and 2 on energy metabolism. *Diabetes* 56: 583-593, 2007.

164. **Liu Y, Michael MD, Kash S, Bensch WR, Monia BP, Murray SF, Otto KA, Syed SK, Bhanot S, Sloop KW, Sullivan JM and Reifel-Miller A**. Deficiency of adiponectin receptor 2 reduces diet-induced insulin resistance but promotes type 2 diabetes. *Endocrinology* 148: 683-692, 2007.

165. **Capeau J**. The story of adiponectin and its receptors AdipoR1 and R2: to follow. *J Hepatol* 47: 736-738, 2007.

166. **Rasmussen MS, Lihn AS, Pedersen SB, Bruun JM, Rasmussen M and Richelsen B**. Adiponectin receptors in human adipose tissue: effects of obesity, weight loss, and fat depots. *Obesity (Silver Spring)* 14: 28-35, 2006.

167. **Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS and Lodish HF**. T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. *Proc Natl Acad Sci U S A* 101: 10308-10313, 2004.

168. **Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ, Fang Q, Christ-Roberts CY, Hong JY, Kim RY, Liu F and Dong LQ**. APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. *Nat Cell Biol* 8: 516-523, 2006.

169. **Kobayashi H, Ouchi N, Kihara S, Walsh K, Kumada M, Abe Y, Funahashi T and Matsuzawa Y**. Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin. *Circ Res* 94: e27-e31, 2004.

170. **Chandrasekar B, Boylston WH, Venkatachalam K, Webster NJ, Prabhu SD and Valente AJ**. Adiponectin blocks interleukin-18-mediated endothelial cell death via APPL1-dependent AMP-activated protein kinase (AMPK) activation and IKK/NF-kappaB/PTEN suppression. *J Biol Chem* 283: 24889-24898, 2008.

171. **Zhou L, Deepa SS, Etzler JC, Ryu J, Mao X, Fang Q, Liu DD, Torres JM, Jia W, Lechleiter JD, Liu F and Dong LQ**. Adiponectin activates AMP-activated protein kinase in muscle cells via APPL1/LKB1-dependent and phospholipase C/Ca2+/Ca2+/calmodulin-dependent protein kinase kinase-dependent pathways. *J Biol Chem* 284: 22426-22435, 2009.

172. **Cleasby ME, Lau Q, Polkinghorne E, Patel SA, Leslie SJ, Turner N, Cooney GJ, Xu A and Kraegen EW**. The adaptor protein APPL1 increases glycogen accumulation in rat skeletal muscle through activation of the PI3-kinase signalling pathway. *J Endocrinol* 210: 81-92, 2011.

173. **Fang X, Palanivel R, Cresser J, Schram K, Ganguly R, Thong FS, Tuinei J, Xu A, Abel ED and Sweeney G**. An APPL1-AMPK signaling axis mediates beneficial metabolic effects of adiponectin in the heart. *Am J Physiol Endocrinol Metab* 299: E721-E729, 2010.

174. **Park M, Youn B, Zheng XL, Wu D, Xu A and Sweeney G**. Globular adiponectin, acting via AdipoR1/APPL1, protects H9c2 cells from hypoxia/reoxygenation-induced apoptosis. *PLoS One* 6: e19143, 2011.

175. **Saito T, Jones CC, Huang S, Czech MP and Pilch PF**. The interaction of Akt with APPL1 is required for insulin-stimulated Glut4 translocation. *J Biol Chem* 282: 32280-32287, 2007.

176. **Heiker JT, Kosel D and Beck-Sickinger AG**. Molecular mechanisms of signal transduction via adiponectin and adiponectin receptors. *Biol Chem* 391: 1005-1018, 2010.

177. **Ouchi N, Kobayashi H, Kihara S, Kumada M, Sato K, Inoue T, Funahashi T and Walsh K**. Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. *J Biol Chem* 279: 1304-1309, 2004.

178. **Chen H, Montagnani M, Funahashi T, Shimomura I and Quon MJ**. Adiponectin stimulates production of nitric oxide in vascular endothelial cells. *J Biol Chem* 278: 45021-45026, 2003.

179. **Ouchi N and Walsh K**. Adiponectin as an anti-inflammatory factor. *Clin Chim Acta* 380: 24-30, 2007.

180. **Ouedraogo R, Wu X, Xu SQ, Fuchsel L, Motoshima H, Mahadev K, Hough K, Scalia R and Goldstein BJ**. Adiponectin suppression of high-glucose-induced reactive oxygen species in vascular endothelial cells: evidence for involvement of a cAMP signaling pathway. *Diabetes* 55: 1840-1846, 2006.

181. **Shibata R, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N and Walsh K**. Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms. *Nat Med* 11: 1096-1103, 2005.

182. **Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T and Matsuzawa Y**. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* 103: 1057-1063, 2001.

183. **Wu X, Motoshima H, Mahadev K, Stalker TJ, Scalia R and Goldstein BJ**. Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. *Diabetes* 52: 1355-1363, 2003.

184. **Ajuwon KM and Spurlock ME**. Adiponectin inhibits LPS-induced NF-kappaB activation and IL-6 production and increases PPARgamma2 expression in adipocytes. *Am J Physiol Regul Integr Comp Physiol* 288: R1220-R1225, 2005.

185. **Das K, Lin Y, Widen E, Zhang Y and Scherer PE**. Chromosomal localization, expression pattern, and promoter analysis of the mouse gene encoding adipocyte-specific secretory protein Acrp30. *Biochem Biophys Res Commun* 280: 1120-1129, 2001.

186. **Zhou L, Liu M, Zhang J, Chen H, Dong LQ and Liu F**. DsbA-L alleviates endoplasmic reticulum stress-induced adiponectin downregulation. *Diabetes* 59: 2809-2816, 2010.

187. **Liu M and Liu F**. Transcriptional and post-translational regulation of adiponectin. *Biochem J* 425: 41-52, 2010.

188. **Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, Makishima M and Shimomura I**. Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. *Diabetes* 52: 1655-1663, 2003.

189. **Qiao L and Shao J**. SIRT1 regulates adiponectin gene expression through Foxo1-C/enhancer-binding protein alpha transcriptional complex. *J Biol Chem* 281: 39915-39924, 2006.

190. **Park BH, Qiang L and Farmer SR**. Phosphorylation of C/EBPbeta at a consensus extracellular signal-regulated kinase/glycogen synthase kinase 3 site is required for the induction of adiponectin gene expression during the differentiation of mouse fibroblasts into adipocytes. *Mol Cell Biol* 24: 8671-8680, 2004.

191. **Kita A, Yamasaki H, Kuwahara H, Moriuchi A, Fukushima K, Kobayashi M, Fukushima T, Takahashi R, Abiru N, Uotani S, Kawasaki E and Eguchi K**. Identification of the promoter region required for human adiponectin gene transcription: Association with CCAAT/enhancer binding protein-beta and tumor necrosis factor-alpha. *Biochem Biophys Res Commun* 331: 484-490, 2005.

192. **Qi L, Saberi M, Zmuda E, Wang Y, Altarejos J, Zhang X, Dentin R, Hedrick S, Bandyopadhyay G, Hai T, Olefsky J and Montminy M**. Adipocyte CREB promotes insulin resistance in obesity. *Cell Metab* 9: 277-286, 2009.

193. **Kim HB, Kong M, Kim TM, Suh YH, Kim WH, Lim JH, Song JH and Jung MH**. NFATc4 and ATF3 negatively regulate adiponectin gene expression in 3T3-L1 adipocytes. *Diabetes* 55: 1342-1352, 2006.

194. **Ikeda K, Maegawa H, Ugi S, Tao Y, Nishio Y, Tsukada S, Maeda S and Kashiwagi A**. Transcription factor activating enhancer-binding protein-2beta. A negative regulator of adiponectin gene expression. *J Biol Chem* 281: 31245-31253, 2006.

195. **Zappala G and Rechler MM**. IGFBP-3, hypoxia and TNF-alpha inhibit adiponectin transcription. *Biochem Biophys Res Commun* 382: 785-789, 2009.

196. **Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J and Paschke R**. Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 301: 1045-1050, 2003.

197. **Richards AA, Stephens T, Charlton HK, Jones A, Macdonald GA, Prins JB and Whitehead JP**. Adiponectin multimerization is dependent on conserved lysines in the collagenous domain: evidence for regulation of multimerization by alterations in posttranslational modifications. *Mol Endocrinol* 20: 1673-1687, 2006.

198. **Qiang L, Wang H and Farmer SR**. Adiponectin secretion is regulated by SIRT1 and the endoplasmic reticulum oxidoreductase Ero1-L alpha. *Mol Cell Biol* 27: 4698-4707, 2007.

199. **Wang ZV, Schraw TD, Kim JY, Khan T, Rajala MW, Follenzi A and Scherer PE**. Secretion of the adipocyte-specific secretory protein adiponectin critically depends on thiol-mediated protein retention. *Mol Cell Biol* 27: 3716-3731, 2007.

200. **Liu M, Zhou L, Xu A, Lam KS, Wetzel MD, Xiang R, Zhang J, Xin X, Dong LQ and Liu F**. A disulfide-bond A oxidoreductase-like protein (DsbA-L) regulates adiponectin multimerization. *Proc Natl Acad Sci U S A* 105: 18302-18307, 2008.

201. **Koh EH, Park JY, Park HS, Jeon MJ, Ryu JW, Kim M, Kim SY, Kim MS, Kim SW, Park IS, Youn JH and Lee KU**. Essential role of mitochondrial function in adiponectin synthesis in adipocytes. *Diabetes* 56: 2973-2981, 2007.

202. **Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M and Shimomura I**. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 114: 1752-1761, 2004.

203. **Soares AF, Guichardant M, Cozzone D, Bernoud-Hubac N, Bouzaidi-Tiali N, Lagarde M and Geloen A**. Effects of oxidative stress on adiponectin secretion and lactate production in 3T3-L1 adipocytes. *Free Radic Biol Med* 38: 882-889, 2005.

204. **Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J and Paschke R**. Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 301: 1045-1050, 2003.

205. **Lim JY, Kim WH and Park SI**. GO6976 prevents TNF-alpha-induced suppression of adiponectin expression in 3T3-L1 adipocytes: putative involvement of protein kinase C. *FEBS Lett* 582: 3473-3478, 2008.

206. **Mohlig M, Wegewitz U, Osterhoff M, Isken F, Ristow M, Pfeiffer AF and Spranger J**. Insulin decreases human adiponectin plasma levels. *Horm Metab Res* 34: 655-658, 2002.

207. **Dadson K, Liu Y and Sweeney G**. Adiponectin action: a combination of endocrine and autocrine/paracrine effects. *Front Endocrinol (Lausanne)* 2: 62, 2011.

208. **Kubota N, Terauchi Y, Kubota T, Kumagai H, Itoh S, Satoh H, Yano W, Ogata H, Tokuyama K, Takamoto I, Mineyama T, Ishikawa M, Moroi M, Sugi K, Yamauchi T, Ueki K, Tobe K, Noda T, Nagai R and Kadowaki T**. Pioglitazone ameliorates insulin resistance and diabetes by both adiponectin-dependent and -independent pathways. *J Biol Chem* 281: 8748-8755, 2006.

209. **Liu M and Liu F**. Up- and down-regulation of adiponectin expression and multimerization: Mechanisms and therapeutic implication. *Biochimie* 2012.

210. **Adamia N, Virsaladze D, Charkviani N, Skhirtladze M and Khutsishvili M**. Effect of metformin therapy on plasma adiponectin and leptin levels in obese and insulin resistant postmenopausal females with type 2 diabetes. *Georgian Med News* 52-55, 2007.

211. **Zulian A, Cancello R, Girola A, Gilardini L, Alberti L, Croci M, Micheletto G, Danelli P and Invitti C**. In vitro and in vivo effects of metformin on human adipose tissue adiponectin. *Obes Facts* 4: 27-33, 2011.

212. **Maynadier M, Basile I and Gary-Bobo M**. Adiponectin normalization: a clue to the anti-metabolic syndrome action of rimonabant. *Drug Discov Today* 14: 192-197, 2009.

213. **Ge Q, Maury E, Rycken L, Gerard J, Noel L, Detry R, Navez B and Brichard SM**. Endocannabinoids regulate adipokine production and the immune balance of omental adipose tissue in human obesity. *Int J Obes (Lond)* 2012.

214. **Li FY, Cheng KK, Lam KS, Vanhoutte PM and Xu A**. Cross-talk between adipose tissue and vasculature: role of adiponectin. *Acta Physiol (Oxf)* 203: 167-180, 2011.

215. **Stefan N, Vozarova B, Funahashi T, Matsuzawa Y, Weyer C, Lindsay RS, Youngren JF, Havel PJ, Pratley RE, Bogardus C and Tataranni PA**. Plasma adiponectin concentration is associated with skeletal muscle insulin receptor tyrosine phosphorylation, and low plasma concentration precedes a decrease in whole-body insulin sensitivity in humans. *Diabetes* 51: 1884-1888, 2002.

216. **Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K and Tobe K**. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest* 116: 1784-1792, 2006.

217. **Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P and Kadowaki T**. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7: 941-946, 2001.

218. **Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, Hioki K, Uchida S, Ito Y, Takakuwa K, Matsui J, Takata M, Eto K, Terauchi Y, Komeda K, Tsunoda M, Murakami K, Ohnishi Y, Naitoh T, Yamamura K, Ueyama Y, Froguel P, Kimura S, Nagai R and Kadowaki T**. Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. *J Biol Chem* 278: 2461-2468, 2003.

219. **Combs TP, Pajvani UB, Berg AH, Lin Y, Jelicks LA, Laplante M, Nawrocki AR, Rajala MW, Parlow AF, Cheeseboro L, Ding YY, Russell RG, Lindemann D, Hartley A, Baker GR, Obici S, Deshaies Y, Ludgate M, Rossetti L and Scherer PE**. A transgenic mouse with a deletion in the collagenous domain of adiponectin displays elevated circulating adiponectin and improved insulin sensitivity. *Endocrinology* 145: 367-383, 2004.

220. **Combs TP, Berg AH, Obici S, Scherer PE and Rossetti L**. Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. *J Clin Invest* 108: 1875-1881, 2001.

221. **Machado M and Cortez-Pinto H**. Non-alcoholic steatohepatitis and metabolic syndrome. *Curr Opin Clin Nutr Metab Care* 9: 637-642, 2006.

222. **Wang Y, Zhou M, Lam KS and Xu A**. Protective roles of adiponectin in obesity-related fatty liver diseases: mechanisms and therapeutic implications. *Arq Bras Endocrinol Metabol* 53: 201-212, 2009.

223. **Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T and Matsuzawa Y**. Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 100: 2473-2476, 1999.

224. **Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, Arita Y, Okamoto Y, Shimomura I, Hiraoka H, Nakamura T, Funahashi T and Matsuzawa Y**. Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler Thromb Vasc Biol* 23: 85-89, 2003.

225. **Nakamura Y, Shimada K, Fukuda D, Shimada Y, Ehara S, Hirose M, Kataoka T, Kamimori K, Shimodozono S, Kobayashi Y, Yoshiyama M, Takeuchi K and Yoshikawa J**. Implications of plasma concentrations of adiponectin in patients with coronary artery disease. *Heart* 90: 528-533, 2004.

226. **Adamczak M, Wiecek A, Funahashi T, Chudek J, Kokot F and Matsuzawa Y**. Decreased plasma adiponectin concentration in patients with essential hypertension. *Am J Hypertens* 16: 72-75, 2003.

227. **Iwashima Y, Katsuya T, Ishikawa K, Ouchi N, Ohishi M, Sugimoto K, Fu Y, Motone M, Yamamoto K, Matsuo A, Ohashi K, Kihara S, Funahashi T, Rakugi H, Matsuzawa Y and Ogihara T**. Hypoadiponectinemia is an independent risk factor for hypertension. *Hypertension* 43: 1318-1323, 2004.

228. **Beauloye V, Zech F, Tran HT, Clapuyt P, Maes M and Brichard SM**. Determinants of early atherosclerosis in obese children and adolescents. *J Clin Endocrinol Metab* 92: 3025-3032, 2007.

229. **Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T and Matsuzawa Y**. Adiponectin, an

adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation* 102: 1296-1301, 2000.

230. **Kobashi C, Urakaze M, Kishida M, Kibayashi E, Kobayashi H, Kihara S, Funahashi T, Takata M, Temaru R, Sato A, Yamazaki K, Nakamura N and Kobayashi M**. Adiponectin inhibits endothelial synthesis of interleukin-8. *Circ Res* 97: 1245-1252, 2005.

231. **Ouedraogo R, Gong Y, Berzins B, Wu X, Mahadev K, Hough K, Chan L, Goldstein BJ and Scalia R**. Adiponectin deficiency increases leukocyte-endothelium interactions via upregulation of endothelial cell adhesion molecules in vivo. *J Clin Invest* 117: 1718-1726, 2007.

232. **Arita Y, Kihara S, Ouchi N, Maeda K, Kuriyama H, Okamoto Y, Kumada M, Hotta K, Nishida M, Takahashi M, Nakamura T, Shimomura I, Muraguchi M, Ohmoto Y, Funahashi T and Matsuzawa Y**. Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. *Circulation* 105: 2893-2898, 2002.

233. **Motoshima H, Wu X, Mahadev K and Goldstein BJ**. Adiponectin suppresses proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL. *Biochem Biophys Res Commun* 315: 264-271, 2004.

234. **Shibata R, Ouchi N, Ito M, Kihara S, Shiojima I, Pimentel DR, Kumada M, Sato K, Schiekofer S, Ohashi K, Funahashi T, Colucci WS and Walsh K**. Adiponectin-mediated modulation of hypertrophic signals in the heart. *Nat Med* 10: 1384-1389, 2004.

235. **Liao Y, Takashima S, Maeda N, Ouchi N, Komamura K, Shimomura I, Hori M, Matsuzawa Y, Funahashi T and Kitakaze M**. Exacerbation of heart failure in adiponectin-deficient mice due to impaired regulation of AMPK and glucose metabolism. *Cardiovasc Res* 67: 705-713, 2005.

236. **Ouchi N, Ohishi M, Kihara S, Funahashi T, Nakamura T, Nagaretani H, Kumada M, Ohashi K, Okamoto Y, Nishizawa H, Kishida K, Maeda N, Nagasawa A, Kobayashi H, Hiraoka H, Komai N, Kaibe M, Rakugi H, Ogihara T and Matsuzawa Y**. Association of hypoadiponectinemia with impaired vasoreactivity. *Hypertension* 42: 231-234, 2003.

237. **Ohashi K, Kihara S, Ouchi N, Kumada M, Fujita K, Hiuge A, Hibuse T, Ryo M, Nishizawa H, Maeda N, Maeda K, Shibata R, Walsh K, Funahashi T and Shimomura I**. Adiponectin replenishment ameliorates obesity-related hypertension. *Hypertension* 47: 1108-1116, 2006.

238. **Tanida M, Shen J, Horii Y, Matsuda M, Kihara S, Funahashi T, Shimomura I, Sawai H, Fukuda Y, Matsuzawa Y and Nagai K**. Effects of adiponectin on the renal sympathetic nerve activity and blood pressure in rats. *Exp Biol Med (Maywood)* 232: 390-397, 2007.

239. **Spranger J, Kroke A, Mohlig M, Bergmann MM, Ristow M, Boeing H and Pfeiffer AF**. Adiponectin and protection against type 2 diabetes mellitus. *Lancet* 361: 226-228, 2003.

240. **Liu H, Chen S, Zhang S, Xiao C, Ren Y, Tian H and Li X**. Adiponectin gene variation -4522C/T is associated with type 2 diabetic obesity and insulin resistance in Chinese. *J Genet Genomics* 34: 877-884, 2007.

241. **Berg AH, Combs TP and Scherer PE**. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends Endocrinol Metab* 13: 84-89, 2002.

242. **Bauche IB, El Mkadem SA, Pottier AM, Senou M, Many MC, Rezsohazy R, Penicaud L, Maeda N, Funahashi T and Brichard SM**. Overexpression of adiponectin targeted to adipose tissue in transgenic mice: impaired adipocyte differentiation. *Endocrinology* 148: 1539-1549, 2007.

243. **Ahima RS and Lazar MA**. Adipokines and the peripheral and neural control of energy balance. *Mol Endocrinol* 22: 1023-1031, 2008.

244. **Engeli S, Feldpausch M, Gorzelniak K, Hartwig F, Heintze U, Janke J, Mohlig M, Pfeiffer AF, Luft FC and Sharma AM**. Association between adiponectin and mediators of inflammation in obese women. *Diabetes* 52: 942-947, 2003.

245. **Mantzoros CS, Li T, Manson JE, Meigs JB and Hu FB**. Circulating adiponectin levels are associated with better glycemic control, more favorable lipid profile, and reduced inflammation in women with type 2 diabetes. *J Clin Endocrinol Metab* 90: 4542-4548, 2005.

246. **Krakoff J, Funahashi T, Stehouwer CD, Schalkwijk CG, Tanaka S, Matsuzawa Y, Kobes S, Tataranni PA, Hanson RL, Knowler WC and Lindsay RS**. Inflammatory markers, adiponectin, and risk of type 2 diabetes in the Pima Indian. *Diabetes Care* 26: 1745-1751, 2003.

247. **Dietze-Schroeder D, Sell H, Uhlig M, Koenen M and Eckel J**. Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 54: 2003-2011, 2005.

248. **Barb D, Williams CJ, Neuwirth AK and Mantzoros CS**. Adiponectin in relation to malignancies: a review of existing basic research and clinical evidence. *Am J Clin Nutr* 86: s858-s866, 2007.

249. **Otabe S, Yuan X, Fukutani T, Wada N, Hashinaga T, Nakayama H, Hirota N, Kojima M and Yamada K**. Overexpression of human adiponectin in transgenic mice results in suppression of fat accumulation and prevention of premature death by high-calorie diet. *Am J Physiol Endocrinol Metab* 293: E210-E218, 2007.

250. **Lewis BP, Burge CB and Bartel DP**. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120: 15-20, 2005.

251. **Bartel DP**. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297, 2004.

252. **Winter J, Jung S, Keller S, Gregory RI and Diederichs S**. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol* 11: 228-234, 2009.

253. **Huntzinger E and Izaurralde E**. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet* 12: 99-110, 2011.

254. **Hendrickson DG, Hogan DJ, McCullough HL, Myers JW, Herschlag D, Ferrell JE and Brown PO**. Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA. *PLoS Biol* 7: e1000238, 2009.

255. **Guo H, Ingolia NT, Weissman JS and Bartel DP**. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* 466: 835-840, 2010.

256. **Min H and Yoon S**. Got target? Computational methods for microRNA target prediction and their extension. *Exp Mol Med* 42: 233-244, 2010.

257. **Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP and Burge CB**. Prediction of mammalian microRNA targets. *Cell* 115: 787-798, 2003.

258. **Grun D, Wang YL, Langenberger D, Gunsalus KC and Rajewsky N**. microRNA target predictions across seven Drosophila species and comparison to mammalian targets. *PLoS Comput Biol* 1: e13, 2005.

259. **Enright AJ, John B, Gaul U, Tuschl T, Sander C and Marks DS**. MicroRNA targets in Drosophila. *Genome Biol* 5: R1, 2003.

260. **John B, Enright AJ, Aravin A, Tuschl T, Sander C and Marks DS**. Human MicroRNA targets. *PLoS Biol* 2: e363, 2004.

261. **O'Carroll D, Mecklenbrauker I, Das PP, Santana A, Koenig U, Enright AJ, Miska EA and Tarakhovsky A**. A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway. *Genes Dev* 21: 1999-2004, 2007.

262. **Xiao C, Srinivasan L, Calado DP, Patterson HC, Zhang B, Wang J, Henderson JM, Kutok JL and Rajewsky K**. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat Immunol* 9: 405-414, 2008.

263. **Zhou B, Wang S, Mayr C, Bartel DP and Lodish HF**. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. *Proc Natl Acad Sci U S A* 104: 7080-7085, 2007.

264. **Cobb BS, Nesterova TB, Thompson E, Hertweck A, O'Connor E, Godwin J, Wilson CB, Brockdorff N, Fisher AG, Smale ST and Merkenschlager M**. T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J Exp Med* 201: 1367-1373, 2005.

265. **Chen CZ, Li L, Lodish HF and Bartel DP**. MicroRNAs modulate hematopoietic lineage differentiation. *Science* 303: 83-86, 2004.

266. **Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, Braich R, Manoharan M, Soutschek J, Skare P, Klein LO, Davis MM and Chen CZ**. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* 129: 147-161, 2007.

267. **Chong MM, Rasmussen JP, Rudensky AY and Littman DR**. The RNAseIII enzyme Drosha is critical in T cells for preventing lethal inflammatory disease. *J Exp Med* 205: 2005-2017, 2008.

268. **Jones KL, Roche M, Gantier MP, Begum NA, Honjo T, Caradonna S, Williams BR and Mak J**. X4 and R5 HIV-1 have distinct post-entry requirements for uracil DNA glycosylase during infection of primary cells. *J Biol Chem* 285: 18603-18614, 2010.

269. **Asirvatham AJ, Gregorie CJ, Hu Z, Magner WJ and Tomasi TB**. MicroRNA targets in immune genes and the Dicer/Argonaute and ARE machinery components. *Mol Immunol* 45: 1995-2006, 2008.

270. **Bakheet T, Frevel M, Williams BR, Greer W and Khabar KS**. ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucleic Acids Res* 29: 246-254, 2001.

271. **Asirvatham AJ, Magner WJ and Tomasi TB**. miRNA regulation of cytokine genes. *Cytokine* 45: 58-69, 2009.

272. **Lefterova MI and Lazar MA**. New developments in adipogenesis. *Trends Endocrinol Metab* 20: 107-114, 2009.

273. **Rosen ED and Spiegelman BM**. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* 444: 847-853, 2006.

274. **Xu P, Vernooy SY, Guo M and Hay BA**. The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. *Curr Biol* 13: 790-795, 2003.

275. **Mudhasani R, Imbalzano AN and Jones SN**. An essential role for Dicer in adipocyte differentiation. *J Cell Biochem* 110: 812-816, 2010.

276. **Mudhasani R, Puri V, Hoover K, Czech MP, Imbalzano AN and Jones SN**. Dicer is required for the formation of white but not brown adipose tissue. *J Cell Physiol* 226: 1399-1406, 2011.

277. **Ortega FJ, Moreno-Navarrete JM, Pardo G, Sabater M, Hummel M, Ferrer A, Rodriguez-Hermosa JI, Ruiz B, Ricart W, Peral B and Fernandez-Real JM**. MiRNA expression profile of human subcutaneous adipose and during adipocyte differentiation. *PLoS One* 5: e9022, 2010.

278. **Alexander R, Lodish H and Sun L**. MicroRNAs in adipogenesis and as therapeutic targets for obesity. *Expert Opin Ther Targets* 15: 623-636, 2011.

279. **Esau C, Kang X, Peralta E, Hanson E, Marcusson EG, Ravichandran LV, Sun Y, Koo S, Perera RJ, Jain R, Dean NM, Freier SM, Bennett CF, Lollo B and Griffey R**. MicroRNA-143 regulates adipocyte differentiation. *J Biol Chem* 279: 52361-52365, 2004.

280. **Xie H, Lim B and Lodish HF**. MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. *Diabetes* 58: 1050-1057, 2009.

281. **Qin L, Chen Y, Niu Y, Chen W, Wang Q, Xiao S, Li A, Xie Y, Li J, Zhao X, He Z and Mo D**. A deep investigation into the adipogenesis mechanism: profile of microRNAs regulating adipogenesis by modulating the canonical Wnt/beta-catenin signaling pathway. *BMC Genomics* 11: 320, 2010.

282. **Kim YJ, Hwang SJ, Bae YC and Jung JS**. MiR-21 regulates adipogenic differentiation through the modulation of TGF-beta signaling in mesenchymal stem cells derived from human adipose tissue. *Stem Cells* 27: 3093-3102, 2009.

283. **Kinoshita M, Ono K, Horie T, Nagao K, Nishi H, Kuwabara Y, Takanabe-Mori R, Hasegawa K, Kita T and Kimura T**. Regulation of adipocyte differentiation by activation of serotonin (5-HT) receptors 5-HT2AR and 5-HT2CR and involvement of microRNA-448-mediated repression of KLF5. *Mol Endocrinol* 24: 1978-1987, 2010.

284. **Lin Q, Gao Z, Alarcon RM, Ye J and Yun Z**. A role of miR-27 in the regulation of adipogenesis. *FEBS J* 276: 2348-2358, 2009.

285. **Kim SY, Kim AY, Lee HW, Son YH, Lee GY, Lee JW, Lee YS and Kim JB**. miR-27a is a negative regulator of adipocyte differentiation via suppressing PPARgamma expression. *Biochem Biophys Res Commun* 392: 323-328, 2010.

286. **Karbiener M, Fischer C, Nowitsch S, Opriessnig P, Papak C, Ailhaud G, Dani C, Amri EZ and Scheideler M**. microRNA miR-27b impairs human adipocyte differentiation and targets PPARgamma. *Biochem Biophys Res Commun* 390: 247-251, 2009.

287. **Tang YF, Zhang Y, Li XY, Li C, Tian W and Liu L**. Expression of miR-31, miR-125b-5p, and miR-326 in the adipogenic differentiation process of adipose-derived stem cells. *OMICS* 13: 331-336, 2009.

288. **Sun T, Fu M, Bookout AL, Kliewer SA and Mangelsdorf DJ**. MicroRNA let-7 regulates 3T3-L1 adipogenesis. *Mol Endocrinol* 23: 925-931, 2009.

289. **Zhou X, Benson KF, Ashar HR and Chada K**. Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C. *Nature* 376: 771-774, 1995.

290. **McGregor RA and Choi MS**. microRNAs in the regulation of adipogenesis and obesity. *Curr Mol Med* 11: 304-316, 2011.

291. **Wang T, Li M, Guan J, Li P, Wang H, Guo Y, Shuai S and Li X**. MicroRNAs miR-27a and miR-143 Regulate Porcine Adipocyte Lipid Metabolism. *Int J Mol Sci* 12: 7950-7959, 2011.

292. **Trajkovski M, Hausser J, Soutschek J, Bhat B, Akin A, Zavolan M, Heim MH and Stoffel M**. MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature* 474: 649-653, 2011.

293. **He A, Zhu L, Gupta N, Chang Y and Fang F**. Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes. *Mol Endocrinol* 21: 2785-2794, 2007.

294. **Kloting N, Berthold S, Kovacs P, Schon MR, Fasshauer M, Ruschke K, Stumvoll M and Bluher M**. MicroRNA expression in human omental and subcutaneous adipose tissue. *PLoS One* 4: e4699, 2009.

295. **Rantalainen M, Herrera BM, Nicholson G, Bowden R, Wills QF, Min JL, Neville MJ, Barrett A, Allen M, Rayner NW, Fleckner J, McCarthy MI, Zondervan KT, Karpe F, Holmes CC and Lindgren CM**. MicroRNA expression in abdominal and gluteal adipose tissue is associated with mRNA expression levels and partly genetically driven. *PLoS One* 6: e27338, 2011.

296. **Heneghan HM, Miller N and Kerin MJ**. Role of microRNAs in obesity and the metabolic syndrome. *Obes Rev* 11: 354-361, 2010.

297. **Chartoumpekis DV, Zaravinos A, Ziros PG, Iskrenova RP, Psyrogiannis AI, Kyriazopoulou VE and Habeos IG**. Differential Expression of MicroRNAs in Adipose Tissue after Long-Term High-Fat Diet-Induced Obesity in Mice. *PLoS One* 7: e34872, 2012.

298. **Parra P, Serra F and Palou A**. Expression of adipose microRNAs is sensitive to dietary conjugated linoleic acid treatment in mice. *PLoS One* 5: e13005, 2010.

299. **Zhuang G, Meng C, Guo X, Cheruku PS, Shi L, Xu H, Li H, Wang G, Evans AR, Safe S, Wu C and Zhou B**. A Novel Regulator of Macrophage Activation: miR-223 in Obesity Associated Adipose Tissue Inflammation. *Circulation* 2012.

300. **Krutzfeldt J and Stoffel M**. MicroRNAs: a new class of regulatory genes affecting metabolism. *Cell Metab* 4: 9-12, 2006.

301. **Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R, Subramaniam A, Propp S, Lollo BA, Freier S, Bennett CF, Bhanot S and Monia BP**. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* 3: 87-98, 2006.

302. **Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M and Stoffel M**. Silencing of microRNAs in vivo with 'antagomirs'. *Nature* 438: 685-689, 2005.

303. **Rayner KJ, Esau CC, Hussain FN, McDaniel AL, Marshall SM, van Gils JM, Ray TD, Sheedy FJ, Goedeke L, Liu X, Khatsenko OG, Kaimal V, Lees CJ, Fernandez-Hernando C, Fisher EA, Temel RE and Moore KJ**. Inhibition of miR-33a/b in non-human primates raises plasma HDL and lowers VLDL triglycerides. *Nature* 478: 404-407, 2011.

304. **Guay C, Roggli E, Nesca V, Jacovetti C and Regazzi R**. Diabetes mellitus, a microRNA-related disease? *Transl Res* 157: 253-264, 2011.

305. **Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P and Stoffel M**. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* 432: 226-230, 2004.

306. **Plaisance V, Abderrahmani A, Perret-Menoud V, Jacquemin P, Lemaigre F and Regazzi R**. MicroRNA-9 controls the expression of Granuphilin/Slp4 and the secretory response of insulin-producing cells. *J Biol Chem* 281: 26932-26942, 2006.

307. **Lovis P, Gattesco S and Regazzi R**. Regulation of the expression of components of the exocytotic machinery of insulin-secreting cells by microRNAs. *Biol Chem* 389: 305-312, 2008.

308. **Tang X, Muniappan L, Tang G and Ozcan S**. Identification of glucose-regulated miRNAs from pancreatic {beta} cells reveals a role for miR-30d in insulin transcription. *RNA* 15: 287-293, 2009.

309. **Cao L, Lin EJ, Cahill MC, Wang C, Liu X and During MJ**. Molecular therapy of obesity and diabetes by a physiological autoregulatory approach. *Nat Med* 15: 447-454, 2009.

310. **Mellios N, Huang HS, Grigorenko A, Rogaev E and Akbarian S**. A set of differentially expressed miRNAs, including miR-30a-5p, act as post-transcriptional inhibitors of BDNF in prefrontal cortex. *Hum Mol Genet* 17: 3030-3042, 2008.

311. **Lugli G, Larson J, Martone ME, Jones Y and Smalheiser NR**. Dicer and eIF2c are enriched at postsynaptic densities in adult mouse brain and are modified by neuronal activity in a calpain-dependent manner. *J Neurochem* 94: 896-905, 2005.

312. **Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Zhang Y, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Wang J, Zen K, Zhang J and Zhang CY**. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 18: 997-1006, 2008.

313. **Heneghan HM, Miller N, Lowery AJ, Sweeney KJ and Kerin MJ**. MicroRNAs as Novel Biomarkers for Breast Cancer. *J Oncol* 2009: 950201, 2009.

314. **Heneghan HM, Miller N, Kelly R, Newell J and Kerin MJ**. Systemic miRNA-195 differentiates breast cancer from other malignancies and is a potential biomarker for detecting noninvasive and early stage disease. *Oncologist* 15: 673-682, 2010.

315. **Heneghan HM, Miller N, McAnena OJ, O'Brien T and Kerin MJ**. Differential miRNA expression in omental adipose tissue and in the circulation of obese patients identifies novel metabolic biomarkers. *J Clin Endocrinol Metab* 96: E846-E850, 2011.

316. **McDermott AM, Heneghan HM, Miller N and Kerin MJ**. The therapeutic potential of microRNAs: disease modulators and drug targets. *Pharm Res* 28: 3016-3029, 2011.

317. **Weiler J, Hunziker J and Hall J**. Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease? *Gene Ther* 13: 496-502, 2006.

318. **Colin A, Faideau M, Dufour N, Auregan G, Hassig R, Andrieu T, Brouillet E, Hantraye P, Bonvento G and Deglon N**. Engineered lentiviral vector targeting astrocytes in vivo. *Glia* 57: 667-679, 2009.

319. **Brown BD, Venneri MA, Zingale A, Sergi SL and Naldini L**. Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. *Nat Med* 12: 585-591, 2006.

320. **Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S, Lindholm M, Hedtjarn M, Hansen HF, Berger U, Gullans S, Kearney P, Sarnow P, Straarup EM and Kauppinen S**. LNA-mediated microRNA silencing in non-human primates. *Nature* 452: 896-899, 2008.

321. **Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F and Kay MA**. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 441: 537-541, 2006.

322. **Sibley CR, Seow Y and Wood MJ**. Novel RNA-based strategies for therapeutic gene silencing. *Mol Ther* 18: 466-476, 2010.

323. **Wulster-Radcliffe MC, Ajuwon KM, Wang J, Christian JA and Spurlock ME**. Adiponectin differentially regulates cytokines in porcine macrophages. *Biochem Biophys Res Commun* 316: 924-929, 2004.

324. **Folco EJ, Rocha VZ, Lopez-Ilasaca M and Libby P**. Adiponectin inhibits pro-inflammatory signaling in human macrophages independent of interleukin-10. *J Biol Chem* 284: 25569-25575, 2009.

325. **Zoico E, Garbin U, Olioso D, Mazzali G, Fratta Pasini AM, Di F, V, Sepe A, Cominacini L and Zamboni M**. The effects of adiponectin on interleukin-6 and MCP-1 secretion in lipopolysaccharide-treated 3T3-L1 adipocytes: role of the NF-kappaB pathway. *Int J Mol Med* 24: 847-851, 2009.

326. **Ge Q, Ryken L, Noel L, Maury E and Brichard SM**. Adipokines identified as new downstream targets for adiponectin: lessons from adiponectin-overexpressing or -deficient mice. *Am J Physiol Endocrinol Metab* 301: E326-E335, 2011.

327. **Ge Q, Gerard J, Noel L, Scroyen I and Brichard SM**. MicroRNAs Regulated by Adiponectin as Novel Targets for Controlling Adipose Tissue Inflammation. *Endocrinology* 2012.

328. **O'Brien SN, Mantzke KA, Kilgore MW and Price TM**. Relationship between adipose stromal-vascular cells and adipocytes in human adipose tissue. *Anal Quant Cytol Histol* 18: 137-143, 1996.

329. **Krishnaswamy G, Kelley J, Yerra L, Smith JK and Chi DS**. Human endothelium as a source of multifunctional cytokines: molecular regulation and possible role in human disease. *J Interferon Cytokine Res* 19: 91-104, 1999.

330. **Nair S, Lee YH, Rousseau E, Cam M, Tataranni PA, Baier LJ, Bogardus C and Permana PA**. Increased expression of inflammation-related genes in cultured preadipocytes/stromal vascular cells from obese compared with non-obese Pima Indians. *Diabetologia* 48: 1784-1788, 2005.

331. **Fain JN, Cheema P, Tichansky DS and Madan AK**. The inflammatory response seen when human omental adipose tissue explants are incubated in primary culture is not dependent upon albumin and is primarily in the nonfat cells. *J Inflamm (Lond)* 7: 4, 2010.

332. **Furuhashi M, Fucho R, Gorgun CZ, Tuncman G, Cao H and Hotamisligil GS**. Adipocyte/macrophage fatty acid-binding proteins contribute to metabolic deterioration through actions in both macrophages and adipocytes in mice. *J Clin Invest* 118: 2640-2650, 2008.

333. **Shoelson SE, Herrero L and Naaz A**. Obesity, inflammation, and insulin resistance. *Gastroenterology* 132: 2169-2180, 2007.

334. **Ito A, Suganami T, Miyamoto Y, Yoshimasa Y, Takeya M, Kamei Y and Ogawa Y**. Role of MAPK phosphatase-1 in the induction of monocyte chemoattractant protein-1 during the course of adipocyte hypertrophy. *J Biol Chem* 282: 25445-25452, 2007.

335. **Gauthier MS, O'Brien EL, Bigornia S, Mott M, Cacicedo JM, Xu XJ, Gokce N, Apovian C and Ruderman N**. Decreased AMP-activated protein kinase activity is associated with increased inflammation in visceral adipose tissue and with whole-body insulin resistance in morbidly obese humans. *Biochem Biophys Res Commun* 404: 382-387, 2011.

336. **Yang Z, Kahn BB, Shi H and Xue BZ**. Macrophage alpha1 AMP-activated protein kinase (alpha1AMPK) antagonizes fatty acid-induced inflammation through SIRT1. *J Biol Chem* 285: 19051-19059, 2010.

337. **Wang C, Li L, Zhang ZG, Fan D, Zhu Y and Wu LL**. Globular adiponectin inhibits angiotensin II-induced nuclear factor kappaB activation through AMP-activated protein kinase in cardiac hypertrophy. *J Cell Physiol* 222: 149-155, 2010.

338. **Hulsmans M, Van DE, Mathieu C and Holvoet P**. Decrease of miR-146b-5p in monocytes during obesity is associated with loss of the anti-inflammatory but not insulin signaling action of adiponectin. *PLoS One* 7: e32794, 2012.

339. **Kopp A, Buechler C, Bala M, Neumeier M, Scholmerich J and Schaffler A**. Toll-like receptor ligands cause proinflammatory and prodiabetic activation of adipocytes via phosphorylation of extracellular signal-regulated kinase and c-Jun N-terminal kinase but not interferon regulatory factor-3. *Endocrinology* 151: 1097-1108, 2010.

340. **Villaret A, Galitzky J, Decaunes P, Esteve D, Marques MA, Sengenes C, Chiotasso P, Tchkonia T, Lafontan M, Kirkland JL and Bouloumie A**. Adipose tissue endothelial cells from obese human subjects: differences among depots in angiogenic, metabolic, and inflammatory gene expression and cellular senescence. *Diabetes* 59: 2755-2763, 2010.

341. **Surmi BK and Hasty AH**. The role of chemokines in recruitment of immune cells to the artery wall and adipose tissue. *Vascul Pharmacol* 52: 27-36, 2010.

342. **Brombacher F, Kastelein RA and Alber G**. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol* 24: 207-212, 2003.

343. **Yamaguchi Y, Fujio K, Shoda H, Okamoto A, Tsuno NH, Takahashi K and Yamamoto K**. IL-17B and IL-17C are associated with TNF-alpha production and contribute to the exacerbation of inflammatory arthritis. *J Immunol* 179: 7128-7136, 2007.

344. **Monteleone G, Pallone F and Macdonald TT**. Interleukin-21 as a new therapeutic target for immune-mediated diseases. *Trends Pharmacol Sci* 30: 441-447, 2009.

345. **Wegner M, Winiarska H, Bobkiewicz-Kozlowska T and Dworacka M**. IL-12 serum levels in patients with type 2 diabetes treated with sulphonylureas. *Cytokine* 42: 312-316, 2008.

346. **Keophiphath M, Rouault C, Divoux A, Clement K and Lacasa D**. CCL5 promotes macrophage recruitment and survival in human adipose tissue. *Arterioscler Thromb Vasc Biol* 30: 39-45, 2010.

347. **Maury E, Brichard SM, Pataky Z, Carpentier A, Golay A and Bobbioni-Harsch E**. Effect of obesity on growth-related oncogene factor-alpha, thrombopoietin, and tissue inhibitor metalloproteinase-1 serum levels. *Obesity (Silver Spring)* 18: 1503-1509, 2010.

348. **Sweet MJ, Leung BP, Kang D, Sogaard M, Schulz K, Trajkovic V, Campbell CC, Xu D and Liew FY**. A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. *J Immunol* 166: 6633-6639, 2001.

349. **Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, O'Neill LA and Liew FY**. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat Immunol* 5: 373-379, 2004.

350. **Miller AM, Xu D, Asquith DL, Denby L, Li Y, Sattar N, Baker AH, McInnes IB and Liew FY**. IL-33 reduces the development of atherosclerosis. *J Exp Med* 205: 339-346, 2008.

351. **Miller AM, Asquith DL, Hueber AJ, Anderson LA, Holmes WM, McKenzie AN, Xu D, Sattar N, McInnes IB and Liew FY**. Interleukin-33 induces protective effects in adipose tissue inflammation during obesity in mice. *Circ Res* 107: 650-658, 2010.

352. **Palsson-McDermott EM and O'Neill LA**. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 113: 153-162, 2004.

353. **Nakarai H, Yamashita A, Nagayasu S, Iwashita M, Kumamoto S, Ohyama H, Hata M, Soga Y, Kushiyama A, Asano T, Abiko Y and Nishimura F**. Adipocyte-macrophage interaction may mediate LPS-induced low-grade inflammation: potential link with metabolic complications. *Innate Immun* 18: 164-170, 2012.

354. **Sun L, Yu Z, Ye X, Zou S, Li H, Yu D, Wu H, Chen Y, Dore J, Clement K, Hu FB and Lin X**. A marker of endotoxemia is associated with obesity and related metabolic disorders in apparently healthy Chinese. *Diabetes Care* 33: 1925-1932, 2010.

355. **Scroyen I, Cosemans L and Lijnen HR**. Effect of tissue inhibitor of matrix metalloproteinases-1 on in vitro and in vivo adipocyte differentiation. *Thromb Res* 124: 578-583, 2009.

356. **Gimble J and Guilak F**. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* 5: 362-369, 2003.

357. **Strum JC, Johnson JH, Ward J, Xie H, Feild J, Hester A, Alford A and Waters KM**. MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. *Mol Endocrinol* 23: 1876-1884, 2009.

Annex

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ORIGINAL ARTICLE

Endocannabinoids regulate adipokine production and the immune balance of omental adipose tissue in human obesity

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OBJECTIVES: (1) To investigate whether modulation of the cannabinoid type 1 receptor (CB1R) directly regulates the production of adiponectin (ApN) and other adipokines in omental adipose tissue (OAT) of obese subjects, (2) to establish in which cellular fraction of OAT the effects of CB1R blockade take place and (3) to unravel the underlying mechanisms.
SUBJECTS AND METHODS: OAT was obtained from 30 obese subjects (body mass index: $40.6 \pm 1.3\ kg\ m^{-2}$) undergoing abdominal surgery. Primary cultures of explants or of freshly isolated adipocytes or stromal-vascular cells (SVCs) were used.
RESULTS: In OAT explants, the CB1R blocker Rimonabant upregulated ApN gene expression. mRNA abundance of omentin that exhibits insulin-sensitizing properties was upregulated as well. Conversely, mRNA levels of two pro-inflammatory cytokines, macrophage inflammatory protein (MIP)-1β and interleukin (IL)-7 were downregulated. We next examined where these effects took place within OAT. CB1R expression was similar in both cellular fractions. In isolated mature adipocytes, blockade of CB1R reproduced the increase of ApN mRNA and the decrease of IL-7 mRNA, while inducing a rise of ApN secretion into the medium. In isolated SVC, gene expression of omentin, which is restricted to this fraction, was augmented, while that of MIP-1β was diminished. Finally, we deciphered the mechanisms leading to ApN regulation by the endocannabinoid system (ES). We first established that ApN regulation was actually mediated by the CB1R: ApN gene expression was upregulated by Rimonabant and downregulated by the CB1R agonist arachidonyl-2-chloroethylamide (ACEA). Upregulation of ApN by Rimonabant was unaltered by inhibiting cAMP production. However, downregulation of ApN by ACEA was fully reversed by an inhibitor of p38 mitogen-activated protein kinase (p38MAPK) and ACEA increased p38MAPK phosphorylation.
CONCLUSIONS: Blockade of CB1R attenuates the inflammatory state in both cellular fractions of OAT either by increasing ApN and omentin production or by decreasing mRNAs of MIP-1β and IL-7. ApN regulation by the ES partly involves p38MAPK.

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Keywords: endocannabinoid; rimonabant; adipokines; adipose tissue; adiponectin

INTRODUCTION

Obesity is locally characterized by adipose tissue inflammation and dysregulation of adipokine production with oversecretion of deleterious regulatory peptides and hyposecretion of defensive ones, like adiponectin (ApN).[1,2] Such a dysregulation triggers the development of a systemic low-grade pro-inflammatory state, which is considered to build the common soil for the development of obesity comorbidities and the metabolic syndrome.[1,3,4] Preferential accumulation of central-omental rather than subcutaneous fat appears to be a stronger risk factor for this adverse health profile.[1]

The endocannabinoid system (ES), consisting of the cannabinoid type 1 receptor (CB1R) and of endogenous lipid-derived ligands, modulates energy homeostasis through central orexigenic effects and peripheral metabolic effects on several organs including adipose tissue where energy storage is increased.[5,6] Obese subjects exhibit higher endocannabinoid levels in visceral fat and serum than lean controls[7,8] and also show higher endocannabinoid levels in visceral than in subcutaneous fat.[9] A strong activation of the ES might thus be considered as a driving force leading to—or associated with—central obesity.[10]

Treatment of obese patients with the selective CB1R blocker Rimonabant promoted significant decreases of body weight and waist circumference (a marker of abdominal adiposity) as well as improvements in cardiovascular and metabolic risk factors.[11] Parts of these improvements have been ascribed to a rise of circulating ApN levels. This rise was partly independent of weight loss and suggested specific improvement of adipose tissue function.[12]

In line with this observation, Rimonabant increased the expression of ApN both in adipose tissue of rodents treated *in vivo* and in murine 3T3-F442A adipocyte line *in vitro*.[13] However, the peripheral effects of cannabinoids have been poorly investigated in human adipose tissue. Few reports yielded controversial data on adipokine release by human fat cells.[14–16] These experiments were carried out on adipocytes differentiated *in vitro* from stromal precursors, which were isolated mainly from subcutaneous fat of non-obese subjects,[15,16] then submitted to pharmacological doses of adipogenic cocktails. However, omental rather than subcutaneous fat, as well as obesity are linked to the metabolic syndrome and abnormal endocannabinoid tone. Additionally, differentiation of fat cells within an *in-vivo* context together with preserved cell interactions may be a prerequisite for optimal adipokine gene expression.[17]

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Besides downregulation of ApN, we like others have shown that several adipokines are oversecreted by omental adipose tissue (OAT) of obese subjects and could contribute to metabolic and cardiovascular disorders as well.[18] Both adipocytes and stromal-vascular cells (SVCs; that is, non-fat cells) contribute to such adipokine deregulation in human obesity.[18] Yet, the potential direct role of the ES on the SVC fraction has never been addressed. Moreover, the mechanisms underlying a potential regulation of adipokines by the ES are still unexplored.

The aims of this work were (1) to assess whether modulation of CB1R directly regulates the production of ApN and other adipokines in OAT of obese subjects, (2) to establish in which cellular fraction of OAT the effects of CB1R blockade take place and (3) to unravel the underlying mechanisms.

SUBJECTS AND METHODS

Subjects

OAT was obtained from 30 obese subjects undergoing bariatric surgery (vertical banded gastroplasty) after an overnight fast (Table 1). Patients were not treated with hormones (for example, glucocorticoids; except for insulin for one of them), non-steroid anti-inflammatory drugs or any medications known to influence fat mass or metabolism (for example, thiazolidinediones, systemic and non-specific modulators of adrenergic receptors).

For each patient, blood was collected in the fasting state before surgery. As shown in Table 1, these patients were severely obese, had higher systolic blood pressure, fasting glycemia, low-density lipoprotein cholesterol and C-reactive protein levels than the recommended or normal values and women had lower high-density lipoprotein cholesterol concentrations. Eight patients were diagnosed as type 2 diabetic and were treated by diet and/or oral anti-diabetic agents or by insulin for one of them. Two-thirds of the patients met criteria of the metabolic syndrome as defined by a joint scientific statement.[19]

For each culture, explants or cells from only one subject were used. Due to the limited tissue availability, not all data could be generated from all patients.

The study protocol had the approval of the local Ethical Committee of the Cliniques Universitaires Saint-Luc.

Cell or adipose tissue culture

We used established protocols to study the production of adipokines by fragments or isolated cells from OAT.[18,20,21] Under those experimental conditions, neither tissue fractionation nor culturing *per se* did alter adipokine expression levels.[18]

Briefly, OAT biopsies were transported to the laboratory within 5–10 min after sampling.[20] Samples were fractionated into adipocytes and SVC by collagenase treatment, as reported.[18,20] This yields two high purity fractions.[18] Adipocytes (150 µl packed cells) and SVCs (150 µl per 3.6 g initial tissue) were cultured in MEM supplemented with 10% fetal calf serum and 1/500 (v/v) antibiotics (Primocin InvivoGen, Toulouse, France), as described.[18] Cells were allowed to stabilize for 1 h in this medium; next, the medium was renewed and cells were cultured for another 24 h with or without SR141716 (Rimonabant, a selective CB1R antagonist; Sanofi-Aventis, Diegem, Belgium) or a selective CB1R agonist ACEA (arachidonyl-2-chloroethylamide, Tocris Bioscience, Ellisville, MO, USA). In some experiments, adipocytes were cultured for 25 h with specific inhibitors of kinases (c-Jun NH_2-terminal protein kinase (JNK), SP600125; p38 mitogen-activated protein kinase (p38MAPK), SB203580; Extracellular Signal-Regulated kinases 1/2 (ERK1/2), PD 98059) or of adenylate cyclase (MDL-12,330A), before the addition of ACEA or SR141716 for the last 24 h. All inhibitors were from Calbiochem (La Jolla, CA, USA); the concentrations used were based on those recommended by the manufacturer or previously published in the literature.[21–24] Because SR141716, SP600125, SB203580 and PD 98059 were dissolved in dimethylsulfoxide and ACEA was dissolved in ethanol, dimethylsulfoxide or ethanol was used as vehicle for the control conditions. The final concentrations of dimethylsulfoxide or ethanol in medium were $<0.1\%$. In our first experiments, adipose tissue explants (2–3 mm^3 fragments, 400 mg tissue) were also cultured for 24 h, as described for cellular fractions.[20,25] After culturing, cells or explants were harvested and frozen at $-80\,^{\circ}C$. Aliquots from adipocyte culture medium were also saved and stored at $-20\,^{\circ}C$.

Table 1. Clinical and laboratory characteristics of the obese subjects

		Reference values	
		Cut-points for the MS[19]	*Normal laboratory values*
Clinical parameters			
Age (years)	43 ± 2		
Sex ratio (Men/Women)	11/19		
BMI (kg m^{-2})	40.6 ± 1.2	⩾30[a]	
Systolic blood pressure (mm Hg)	137 ± 3	⩾130 (or drug treatment) and/or	
Diastolic blood pressure (mm Hg)	84 ± 2	⩾85 (or drug treatment)	
Glucose homeostasis			
Glucose (mg dl^{-1})	110 ± 8	⩾100 (or drug treatment)	
Diabetes (%)	27%		
Lipids			
Cholesterol total (mg dl^{-1})	194 ± 8		<190
LDL cholesterol (mg dl^{-1})	124 ± 7		<115
HDL cholesterol (mg dl^{-1})	44 ± 2 (men)/ 46 ± 3 (women)	<40/<50	
Triglycerides (mg dl^{-1})	131 ± 12	⩽150	
Inflammation			
CRP (mg dl^{-1})	2.5 ± 0.5		<0.1
Metabolic syndrome (%)	67%		

Abbreviations: BMI, body mass index; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Clinical and laboratory parameters were obtained in the fasted state before surgery. Reference values take into account cut-points for the metabolic syndrome (MS)[19] or our normal laboratory values. [a]It is usually assumed that central obesity is present when the BMI is ⩾30 kg m^{-2} (Alberti *et al.*[19]). Values are means ± s.e.m. for 30 obese subjects.

RNA extraction and real-time quantitative PCR (RTQ-PCR)

Total RNA from cells or tissue was extracted by using TriPure Isolation Reagent (Roche Diagnostics, Vilvoorde, Belgium). In all, 0.2–2 µg of total RNA was reverse transcripted as described. RTQ-PCR primers were designed by using Primer Express Software (Applied Biosystems; see Table 2). In all, 4–40 ng of total RNA equivalents was amplified with iQSyber Green Supermix (Bio-Rad Laboratories, Nazareth, Belgium) containing 200 nM of each specific primer using iCycler iQ Real Time PCR detection System (Bio-Rad Laboratories). Briefly, the threshold cycles (CT) were measured in duplicate. ΔCT values were calculated in every sample for each gene of interest as follows: $CT_{\text{gene of interest}} - CT_{\text{reporter gene}}$ with TATA box-binding protein (TBP) as the reporter gene. Relative changes in the expression level of one specific gene (ΔΔCT) were calculated as ΔCt of the test group minus ΔCT of the reference group, and then presented as $2^{-\Delta\Delta CT}$ (Delaigle *et al.*[26]).

Determination of ApN concentration in medium by RIA

ApN concentrations were measured in medium from cultured adipocytes by using a commercially available RIA kit (Linco Research, Nuclilab, The Netherlands). This kit does not discriminate between the different forms of ApN. Samples from diluted medium (1:1) were run in duplicate.

Table 2. Human gene sequences used as forward and reverse primers for RTQ-PCR

Gene	*Forward primer*	*Reverse primer*	*Amplicon (bp)*
Adiponectin	GCAGAGATGGCACTCCTGGA	CCCTTCAGTTCCTGTCATTCC	101
CB1R	CCCTCTTGTGAAGGCACTGC	GCGGCCCTGTGAACACTG	101
GRO	GCCAGTGCTTGCAGACCCT	GGCTATGACTTCGGTTTGGG	101
IL-7	GCACTGGTGATTTTGATCTCCA	CAGGGCAGCTGGTTTTCTTC	101
Leptin	AAGCTGTGCCCATCCAAAAA	GGAGGAGACTGACTGCGTGTG	101
MIP-1β	GCTGCTTTTCTTACACCGCG	GGTTTGGAATACCACAGCTGG	101
Omentin	CCGGTGATCCCTGTGGTCTA	AACTGAACAAATCCCGCAGTG	101
RANTES	AGCCCTCGCTGTCATCCTC	GGGCAATGTAGGCAAAGCAG	101
TIMP-1	TGGCATCCTGTTGTTGCTGT	TGATGACGAGGTCGGAATTG	101
TNF-α	CTCTTCTGCCTGCTGCACTTT	GATGATCTGACTGCCTGGGC	101
TPO	ATGGAGGAGACCAAGGCACA	GATGAGAGGCAAGTGGGTCC	101
TBP	CCCCATGACTCCCATGACCC	ACGAAGTGCAATGGTCTTTAGGT	136

Abbreviations: CB1R, CB1 receptor; GRO, growth-related oncogen factor; IL, interleukin; MIP-1β, macrophage inflammatory protein-1β; TBP, TATA Box-binding protein; TIMPs, tissue inhibitor of metalloproteinases; TNF-α, tumor necrosis factor-α; TPO, thrombopoietin; RANTES, regulated upon activation normal T cells expressed and secreted; RTQ-PCR, real-time quantitative PCR.

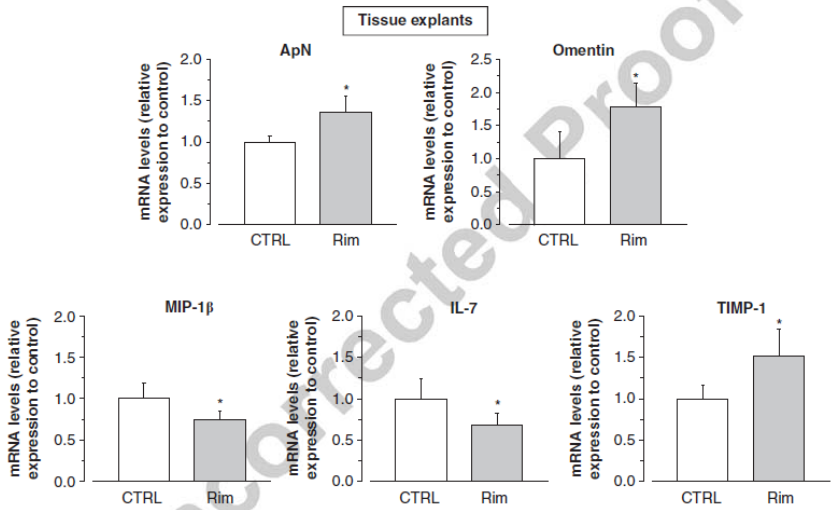

Figure 1. Gene expression of adipokines in explants of human OAT treated with Rimonabant. Explants of OAT form obese subjects were cultured with or without 10–100 nM Rimonabant (Rim) for 24 h. Adipokine mRNA levels were quantified by RTQ-PCR, normalized to the levels of TBP (used as reporter gene) and are presented as relative expression compared with control (untreated) explants. Results are mean ± s.e.m. for 8–12 obese subjects. *$P < 0.05$ vs control conditions.

cAMP immunoassay

Cellular cAMP concentrations were measured by a competitive ELISA kit (R&D Systems Europe Ltd., Abingdon, UK) according to manufacturer's instructions. cAMP levels were normalized to cellular protein levels as determined by the Bradford method.

Western blot

Adipocytes were homogenized in a lysis buffer (Cell Signaling Technology, BIOKÉ, Leiden, The Netherlands) supplemented with 1% protease inhibitor cocktail (Roche Diagnostics). In all, 20 μg proteins were dissolved in Laemmli buffer, subjected to SDS-PAGE under reducing and heat-denaturating conditions and then transferred onto PVDF membranes. The following antibodies were used for immunodetection: anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-phospho-SAPK/JNK (Thr183/Tyr185) and anti-phospho-p38MAPK (Thr180/Tyr182). Each antibody was used according to manufacturer's instructions (Cell Signaling Technology, BIOKÉ). Signals were revealed by enhanced Chemiluminescence. Band intensities were quantified by scanning densitometry (Gel-Doc2000, Bio-Rad Laboratories, UK Ltd), analyzed with Quantity One (Bio-Rad) and normalized to Actin (Sigma-Aldrich, Bornem, Belgium) band intensity.

Presentation of the results and statistical analysis

The results are presented as mean values ± s.e.m. for the indicated numbers of patients. Comparisons between different conditions within a same group were made using two-tailed paired Student's *t*-test (two conditions) or repeated analysis of variance (several conditions) followed by the Dunnett's or Newman–Keuls' test, when appropriate. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of CB1R blockade on adipokine gene expression in cultured explants

We first investigated the effects of blocking CB1R with Rimonabant on whole adipose tissue of obese patients. Rimonabant for 24 h upregulated ApN gene expression by ~40% in omental

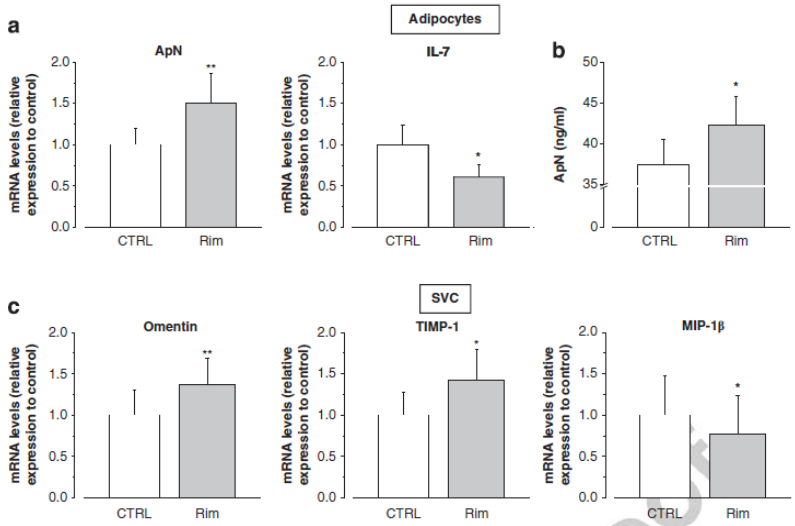

Figure 2. Adipokine production in human adipocytes and SVCs treated with Rimonabant. Mature adipocytes and SVC isolated from OAT of obese subjects were cultured with or without 100 nM Rimonabant (Rim) for 24 h. Adipokine mRNA levels (**a**, **c**) were quantified by RTQ-PCR, normalized to the levels of TBP and presented as relative expression compared with control cells. The concentration of ApN in medium (**b**) was measured by RIA and expressed as ng ml^{-1}. Results are mean values ± s.e.m. for 12–15 (mRNAs) or 18 (protein secretion) obese subjects. *$P<0.05$, ** $P<0.01$ vs control conditions.

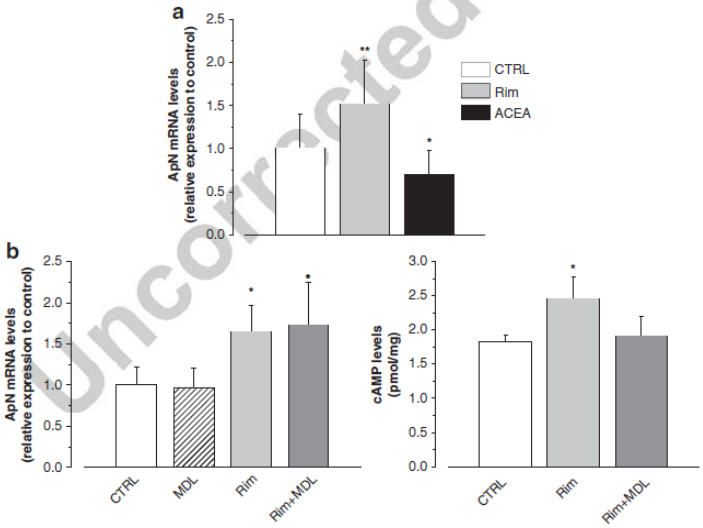

Figure 3. ApN regulation by CB1R modulation (**a**) is independent of cAMP levels (**b**, **c**). (**a**) CB1R modulation on ApN gene expression in adipocytes. Adipocytes were cultured with 100 nM Rimonabant (Rim) or with 1 μM ACEA for 24 h. ApN mRNA levels are presented as relative expression compared with control adipocytes. (**b**) Inhibition of cAMP production on Rimonabant-stimulated ApN gene expression in adipocytes. Adipocytes were cultured with or without MDL-12330A (20 μM) for 25 h, while Rim (100 nM) was added during the last 24 h. ApN mRNA levels are presented as relative expression compared with control adipocytes. (**c**) Increase of cAMP production by Rim and prevention by MDL. For cAMP measurement, adipocytes were cultured in serum-free medium (2% BSA) with or without 100 nM Rim for 10 min. In all, 20 μM MDL-12330A was added 1 h before Rim treatment. cAMP levels were measured by ELISA and normalized to adipocyte protein content. Results are mean values ± s.e.m. for seven (**a**), eight (**b**) and five (**c**) obese subjects. *$P<0.05$, **$P<0.01$ vs respective control conditions.

adipose explants (Figure 1). mRNA abundance of omentin which, like ApN, exhibits insulin-sensitizing properties and is decreased in obesity[27] was upregulated as well (approximately +78%; Figure 1). We further examined the effects of Rimonabant on several other adipokines that we have previously identified as overproduced by OAT in human obesity[18]: three chemokines (Growth-Related Oncogen factor, Regulated upon Activation Normal T cells Expressed and Secreted, macrophage

inflammatory protein (MIP)-1β), one interleukin (IL-7), one tissue inhibitor of metalloproteinases (TIMP-1) and one hematopoietic growth factor (thrombopoietin). Gene expression of MIP-1β and IL-7 was downregulated by Rimonabant (approximately −26% and approximately −32%, respectively; $P<0.05$) while TIMP-1 was upregulated (approximately +40%, $P<0.05$; Figure 1). mRNA levels of the other adipokines (Regulated upon Activation Normal T cells Expressed and Secreted, Growth-Related Oncogen factor and thrombopoietin) were not modified by CB1R blockade. Likewise, gene expression of leptin and tumor necrosis factor-α was not affected (data not shown, $n=7$–8 patients/group).

Effect of CB1R blockade on adipokines in mature adipocytes and SVC

We next examined where the effects of Rimonabant on adipokine production took place within OAT. It is of note that CB1R was similarly expressed in both cellular fractions of OAT (relative mRNA abundance: 1.0 ± 0.5 in adipocytes vs 1.1 ± 0.6 in SVC, $n=9$). In isolated mature adipocytes, blockade of CB1R reproduced the increase of ApN mRNA and the decrease of IL-7 mRNA while inducing a moderate but significant increase of ApN secretion into the medium (+20%; Figures 2a and b). In isolated SVC, gene expression of omentin, which is known to be restricted to this fraction[27] was augmented like that of TIMP-1, while MIP-1β mRNA was diminished (Figure 2c).

Mechanisms involved in ApN regulation by the ES

To decipher the mechanisms leading to ApN regulation by the ES, we investigated candidate signaling pathways in adipocytes.

We first established that ApN regulation by the ES was actually mediated by the CB1R. Indeed, while ApN gene expression was upregulated by the selective CB1R antagonist Rimonabant, its expression was downregulated by using a specific agonist of CB1R, ACEA (Figure 3a).

Next, we investigated post-CB1R signaling pathways. The classic post-receptor signal transduction pathways of CB1R activation include inhibition of cAMP production and stimulation of MAPKs, including p38MAPK, JNK and ERK1/2 in a variety of cell types.[28]

We thus investigated whether the stimulation of ApN gene by Rimonabant resulted from a rise of cAMP levels. The upregulation of ApN mRNA by Rimonabant was unaltered by using the adenylate cyclase inhibitor MDL-12330A (Figure 3b). Yet, this inhibitor was effective as it prevented the rise of cAMP levels caused by Rimonabant in adipocytes (Figure 3b). Taken together, these data suggest that cAMP may not be involved in ApN regulation by the ES. We next blocked components of MAP kinase signaling by using specific inhibitors of p38MAPK (SB203580), ERK1/2 (PD98059) and JNK (SP60025). These inhibitors were tested during stimulation of CB1R signaling. This strategy was chosen because basal phosphorylation of MAPK components was rather low and it thus appeared to be easier to disclose an increase (rather than a decrease) of phosphorylation levels. None of the

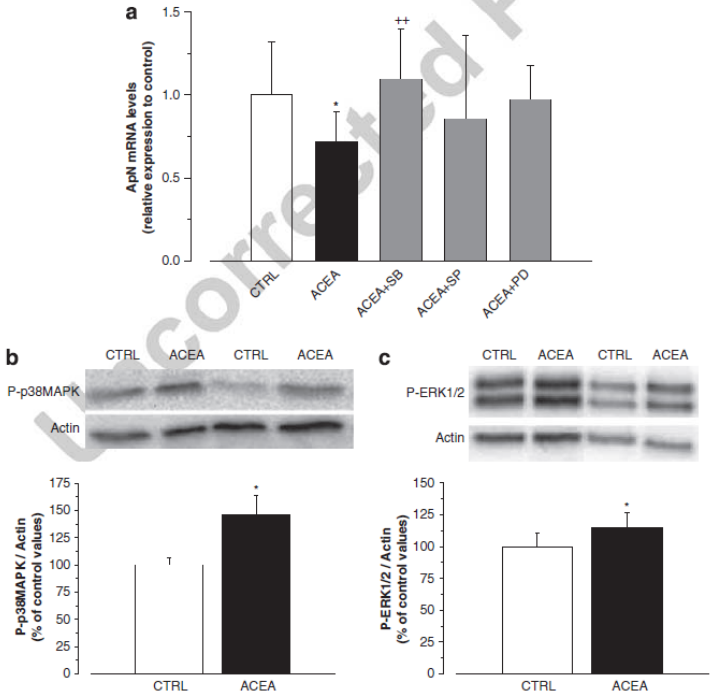

Figure 4. p38MAPK-mediated ApN regulation by the endocannabinoid system. (**a**) Blockade of different MAPK signaling pathways (p38MAPK, ERK1/2, JNK) on ApN gene expression in adipocytes treated by the CB1R agonist, ACEA. Adipocytes were cultured with or without p38MAPK, ERK1/2, JNK inhibitors (20 μM SB203580, 20 μM PD98059 and 10 μM SP60025, respectively) for 25 h in the presence of ACEA (1 μM), which was added during the last 24#amp;#x02009;h. ApN mRNA levels are presented as relative expression compared with control adipocytes. Results are mean values ± s.e.m. for seven obese subjects. *$P<0.05$ vs control, $^{++}P<0.01$ vs ACEA. (**b**, **c**) Phosphorylation of p38MAPK and ERK1/2 in response to CB1R stimulation was measured by western blot analysis. Adipocytes were cultured in serum-free medium (2% BSA) with or without 1 μM ACEA for 1 h. Representative blots are shown as inserts. p38MAPK and ERK1/2 phosphorylation was normalized to actin levels and these ratios were expressed as percentages of control values in untreated adipocytes. Results are mean values ± s.e.m. for four obese subjects. *$P<0.05$ vs control.

inhibitors used alone altered ApN gene expression (not shown). Downregulation of ApN expression by ACEA was fully reversed by the selective inhibitor of p38MAPK ($P<0.01$ for ACEA vs ACEA + SB), while the differences between ACEA alone vs ACEA + SP or ACEA + PD did not reach statistical significance (Figure 4a). This suggests that p38MAPK pathway could mediate CB1R-regulated ApN expression. Western blot analysis further showed that stimulation of CB1R with 1 μM ACEA did actually increase by ~50% p38MAPK phosphorylation ($P<0.05$; Figure 4b) and only slightly increase ERK1/2 phosphorylation in adipocytes (~15%, $P<0.05$; Figure 4c). JNK phosphorylation was unaffected (data not shown).

DISCUSSION

We show that CB1R modulation alters adipokine production both in explants and in each cellular fraction isolated from OAT of obese subjects. Previous work on human adipocytes differentiated *in vitro* (mainly from the subcutaneous depot and from non-obese subjects) yielded controversial data. On one hand, neither treatment with a CB1R agonist nor with a CB1R blocker affected ApN expression,[14] in line with the lack of relationship between human adipose tissue CB1R expression and ApN production.[29] On the other hand, a synthetic cannabinoid upregulated pro-inflammatory adipokines in human fat cells,[15] while the selective CB1R blocker Rimonabant restored to basal levels the secretion of ApN, which was downregulated by lipopolysaccharide co-treatment.[16] Herein, we unambiguously show that a selective CB1R agonist (ACEA) decreased ApN expression, while a selective CB1R blocker upregulated ApN and concurrently downregulated the expression of several pro-inflammatory adipokines in both cellular fractions of OAT. The fact that we were able to disclose an effect of CB1R blockade even in basal conditions may be explained by the already enhanced cannabinoid tone in omental fat of obese subjects. We further demonstrated that CB1R blockade alters the adipokine production by SVC as well. This is novel information as all the studies performed so far have focused on the adipocyte fraction. In line with our data, the mRNA abundance of CB1R was similar in both cellular fractions of human OAT. It should be mentioned that ACEA may have a weak affinity for CB2R[30] and that Rimonabant may be an agonist of the orphan G protein-coupled receptor GPR55.[31] However, action of these compounds thorough these alternate receptors is unlikely in our study for two reasons. First, such action requires much higher concentrations of each compound that those used herein.[32] Second, the inverse regulation of ApN by ACEA and Rimonabant strongly supports the concept that ApN production is actually CB1R regulated.

Besides ApN, other adipokines were regulated by CB1R blockade. We found that Rimonabant upregulated the expression of omentin and TIMP-1 in SVC, while downregulating that of MIP-1β and IL-7 in SVC and adipocytes, respectively. All these adipokines are over-produced in obesity[18] except for omentin, which is decreased like ApN.[33] Omentin is a novel adipokine preferentially produced by visceral adipose tissue, with a main contribution of SVC.[27] This adipokine exhibits insulin-like properties in human adipocytes,[27] inhibits vascular inflammation[34] and may exert cardiovascular protection.[35] The increase of omentin may thus represent a novel benefit of CB1R blockade in the context of the metabolic syndrome. TIMP-1 was also upregulated by CB1R blockade. Matrix metalloproteinases and their tissue inhibitors (TIMPs) may have a role in development of obesity by contributing to adipogenesis and extracellular matrix degradation. However, the exact role of TIMP-1 on fat mass development and adipogenesis is still unclear: some studies reported enhanced adipocyte differentiation and hypertrophy,[36,37] while others did not observe any effect[38] or reported impaired differentiation.[39] CB1R blockade also downregulated two adipokines: IL-7 and MIP-1β. IL-7 is a potent pro-inflammatory cytokine, known for its critical role in T-cell development and survival and its role in B-cell development.[40] MIP-1β is a chemokine, which is crucial in recruiting macrophages and other pro-inflammatory cells.[41] Although the function of these adipokines on adipose tissue is still poorly documented, both may have a role in initiating and shaping the immune-inflammatory responses in this tissue. The adipokines regulated by CB1R modulation may thus represent novel mechanisms involved in the peripheral metabolic, immune and cardiovascular effects of ES in human obesity.

Major mediators of CB1R involve inhibition of adenylyl cyclase and regulation of ion channels as well as phosphorylation of MAPKs, such as p38MAPK, ERK1/2 and JNK.[28] However, the mechanisms by which CB1R regulates ApN have never been studied in human adipocytes. There is only one report indicating that Rimonabant increased ApN expression and concurrently decreased ERK1/2 activity in a murine preadipocyte cell line.[42] We therefore examined whether cAMP and MAPKs were involved in the regulation of ApN in human mature adipocytes. CB1R blockade increased cAMP production, while CB1R stimulation increased the phosphorylation of p38MAPK and also possibly that of ERK1/2 without affecting JNK. cAMP is a well-known potent inhibitor (not a stimulator) of ApN production in human visceral adipose tissue.[43] This fact may be *a priori* difficult to reconcile with the observation that CB1R blockade increased both cAMP and ApN levels. However, the upregulation of ApN was unaltered by inhibiting cAMP production, ruling out a potential involvement of cAMP in this observation. Thus, CB1R blockade upregulated ApN in spite of rising cAMP. We also found that CB1R activation downregulated ApN gene expression and that this effect was reversed by specific inhibitors of p38MAPK. These data were confirmed by CB1R-induced phosphorylation of p38MAPK. Our finding is consonant the report that ACEA reduced mitochondrial biogenesis also through activation of the same component (that is, p38MAK) in mouse adipocytes.[44] Taken together, these data indicate that p38MAPK could mediate the effects of CB1R on ApN regulation.

In summary, the ES directly regulates the immune balance of OAT in human obesity. Specifically, blockade of CB1R attenuates the inflammatory state in both adipocytes and SVC either by increasing ApN and omentin production or by decreasing MIP-1β and IL-7. ApN regulation by the ES partly involves p38MAPK.

CONFLICT OF INTEREST

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REFERENCES

1 Matsuzawa Y. Therapy Insight: adipocytokines in metabolic syndrome and related cardiovascular disease. *Nat Clin Pract Cardiovasc Med* 2006; **3**: 35–42.
2 Guerre-Millo M. Adipose tissue and adipokines: for better or worse. *Diabetes Metab* 2004; **30**: 13–19.
3 Jernas M, Palming J, Sjoholm K, Jennische E, Svensson PA, Gabrielsson BG *et al*. Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. *FASEB J* 2006; **20**: 1540–1542.
4 Skurk T, Alberti-Huber C, Herder C, Hauner H. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* 2007; **92**: 1023–1033.
5 Scheen AJ. The endocannabinoid system: a promising target for the management of type 2 diabetes. *Curr Protein Pept Sci* 2009; **10**: 56–74.
6 Muccioli GG, Naslain D, Backhed F, Reigstad CS, Lambert DM, Delzenne NM *et al*. The endocannabinoid system links gut microbiota to adipogenesis. *Mol Syst Biol* 2010; **6**: 392.
7 Engeli S, Bohnke J, Feldpausch M, Gorzelniak K, Janke J, Batkai S *et al*. Activation of the peripheral endocannabinoid system in human obesity. *Diabetes* 2005; **54**: 2838–2843.
8 Bluher M, Engeli S, Kloting N, Berndt J, Fasshauer M, Batkai S *et al*. Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity. *Diabetes* 2006; **55**: 3053–3060.

9 Matias I, Gonthier MP, Orlando P, Martiadis V, De PL, Cervino C *et al.* Regulation, function, and dysregulation of endocannabinoids in models of adipose and beta-pancreatic cells and in obesity and hyperglycemia. *J Clin Endocrinol Metab* 2006; **91**: 3171–3180.
10 Bellocchio L, Cervino C, Pasquali R, Pagotto U. The endocannabinoid system and energy metabolism. *J Neuroendocrinology* 2008; **20**: 850–857.
11 Ruilope LM, Despres JP, Scheen A, Pi-Sunyer X, Mancia G, Zanchetti A *et al.* Effect of rimonabant on blood pressure in overweight/obese patients with/without co-morbidities: analysis of pooled RIO study results. *J Hypertens* 2008; **26**: 357–367.
12 Despres JP, Golay A, Sjostrom L. Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. *N Engl J Med* 2005; **353**: 2121–2134.
13 Bensaid M, Gary-Bobo M, Esclangon A, Maffrand JP, Le Fur G, Oury-Donat F *et al.* The cannabinoid CB1 receptor antagonist SR141716 increases Acrp30 mRNA expression in adipose tissue of obese fa/fa rats and in cultured adipocyte cells. *Mol Pharmacol* 2003; **63**: 908–914.
14 Pagano C, Pilon C, Calcagno A, Urbanet R, Rossato M, Milan G *et al.* The endogenous cannabinoid system stimulates glucose uptake in human fat cells via phosphatidylinositol 3-kinase and calcium-dependent mechanisms. *J Clin Endocrinol Metab* 2007; **92**: 4810–4819.
15 Gonzalez-Muniesa P, Bing C, Trayhurn P. Upregulation of the expression of inflammatory and angiogenic markers in human adipocytes by a synthetic cannabinoid, JTE-907. *Horm Metab Res* 2010; **42**: 710–717.
16 Murumalla R, Bencharif K, Gence L, Bhattacharya A, Tallet F, Gonthier MP *et al.* Effect of the Cannabinoid Receptor-1 antagonist SR141716A on human adipocyte inflammatory profile and differentiation. *J Inflamm (Lond)* 2011; **8**: 33.
17 Hwang CS, Loftus TM, Mandrup S, Lane MD. Adipocyte differentiation and leptin expression. *Annu Rev Cell Dev Biol* 1997; **13**: 231–259.
18 Maury E, Ehala-Aleksejev K, Guiot Y, Detry R, Vandenhooft A, Brichard SM. Adipokines oversecreted by omental adipose tissue in human obesity. *Am J Physiol Endocrinol Metab* 2007; **293**: E656–E665.
19 Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA *et al.* Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* 2009; **120**: 1640–1645.
20 Halleux CM, Declerck PJ, Tran SL, Detry R, Brichard SM. Hormonal control of plasminogen activator inhibitor-1 gene expression and production in human adipose tissue: stimulation by glucocorticoids and inhibition by catecholamines. *J Clin Endocrinol Metab* 1999; **84**: 4097–4105.
21 Maury E, Noel L, Detry R, Brichard SM. *In vitro* hyperresponsiveness to tumor necrosis factor-alpha contributes to adipokine dysregulation in omental adipocytes of obese subjects. *J Clin Endocrinol Metab* 2009; **94**: 1393–1400.
22 Hsu HF, Tsou TC, Chao HR, Shy CG, Kuo YT, Tsai FY *et al.* Effects of arecoline on adipogenesis, lipolysis, and glucose uptake of adipocytes-A possible role of betel-quid chewing in metabolic syndrome. *Toxicol Appl Pharmacol* 2010; **245**: 370–377.
23 Patel NG, Holder JC, Smith SA, Kumar S, Eggo MC. Differential regulation of lipogenesis and leptin production by independent signaling pathways and rosiglitazone during human adipocyte differentiation. *Diabetes* 2003; **52**: 43–50.
24 Xu A, Wong LC, Wang Y, Xu JY, Cooper GJ, Lam KS. Chronic treatment with growth hormone stimulates adiponectin gene expression in 3T3-L1 adipocytes. *FEBS Lett* 2004; **572**: 129–134.
25 Halleux CM, Servais I, Reul BA, Detry R, Brichard SM. Multihormonal control of ob gene expression and leptin secretion from cultured human visceral adipose tissue: increased responsiveness to glucocorticoids in obesity. *J Clin Endocrinol Metab* 1998; **83**: 902–910.
26 Delaigle AM, Jonas JC, Bauche IB, Cornu O, Brichard SM. Induction of adiponectin in skeletal muscle by inflammatory cytokines: *in vivo* and *in vitro* studies. *Endocrinology* 2004; **145**: 5589–5597.
27 Yang RZ, Lee MJ, Hu H, Pray J, Wu HB, Hansen BC *et al.* Identification of omentin as a novel depot-specific adipokine in human adipose tissue: possible role in modulating insulin action. *Am J Physiol Endocrinol Metab* 2006; **290**: E1253–E1261.
28 Turu G, Hunyady L. Signal transduction of the CB1 cannabinoid receptor. *J Mol Endocrinol* 2010; **44**: 75–85.
29 Lofgren P, Sjolin E, Wahlen K, Hoffstedt J. Human adipose tissue cannabinoid receptor 1 gene expression is not related to fat cell function or adiponectin level. *J Clin Endocrinol Metab* 2007; **92**: 1555–1559.
30 Hillard CJ, Manna S, Greenberg MJ, DiCamelli R, Ross RA, Stevenson LA *et al.* Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). *J Pharmacol Exp Ther* 1999; **289**: 1427–1433.
31 Moreno-Navarrete JM, Catalan V, Whyte L, Diaz-Arteaga A, Vazquez-Martinez R, Rotellar F *et al.* The L-alpha-lysophosphatidylinositol/GPR55 system and its potential role in human obesity. *Diabetes* 2012; **61**: 281–291.
32 Henstridge CM, Balenga NA, Schroder R, Kargl JK, Platzer W, Martini L *et al.* GPR55 ligands promote receptor coupling to multiple signalling pathways. *Br J Pharmacol* 2010; **160**: 604–614.
33 de Souza Batista CM, Yang RZ, Lee MJ, Glynn NM, Yu DZ, Pray J *et al.* Omentin plasma levels and gene expression are decreased in obesity. *Diabetes* 2007; **56**: 1655–1661.
34 Tan BK, Adya R, Farhatullah S, Chen J, Lehnert H, Randeva HS. Metformin treatment may increase omentin-1 levels in women with polycystic ovary syndrome. *Diabetes* 2010; **59**: 3023–3031.
35 Yamawaki H, Tsubaki N, Mukohda M, Okada M, Omentin Hara Y. a novel adipokine, induces vasodilation in rat isolated blood vessels. *Biochem Biophys Res Commun* 2010; **393**: 668–672.
36 Alexander CM, Selvarajan S, Mudgett J, Werb Z. Stromelysin-1 regulates adipogenesis during mammary gland involution. *J Cell Biol* 2001; **152**: 693–703.
37 Lijnen HR, Demeulemeester D, Van Hoef B, Collen D, Maquoi E. Deficiency of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) impairs nutritionally induced obesity in mice. *Thromb Haemost* 2003; **89**: 249–255.
38 Scroyen I, Cosemans L, Lijnen HR. Effect of tissue inhibitor of matrix metalloproteinases-1 on *in vitro* and *in vivo* adipocyte differentiation. *Thromb Res* 2009; **124**: 578–583.
39 Meissburger B, Stachorski L, Roder E, Rudofsky G, Wolfrum C. Tissue inhibitor of matrix metalloproteinase 1 (TIMP1) controls adipogenesis in obesity in mice and in humans. *Diabetologia* 2011; **54**: 1468–1479.
40 Hartgring SA, Bijlsma JW, Lafeber FP, van Roon JA. Interleukin-7 induced immunopathology in arthritis. *Ann Rheum Dis* 2006; **65**(Suppl 3): iii69–iii74.
41 Maurer M, von Stebut E. Macrophage inflammatory protein-1. *Int J Biochem Cell Biol* 2004; **36**: 1882–1886.
42 Gary-Bobo M, Elachouri G, Scatton B, Le Fur G, Oury-Donat F, Bensaid M. The cannabinoid CB1 receptor antagonist rimonabant (SR141716) inhibits cell proliferation and increases markers of adipocyte maturation in cultured mouse 3T3 F442A preadipocytes. *Mol Pharmacol* 2006; **69**: 471–478.
43 Delporte ML, Funahashi T, Takahashi M, Matsuzawa Y, Brichard SM. Pre- and post-translational negative effect of beta-adrenoceptor agonists on adiponectin secretion: *in vitro* and *in vivo* studies. *Biochem J* 2002; **367**(Part 3): 677–685.
44 Tedesco L, Valerio A, Dossena M, Cardile A, Ragni M, Pagano C *et al.* Cannabinoid receptor stimulation impairs mitochondrial biogenesis in mouse white adipose tissue, muscle, and liver: the role of eNOS, p38 MAPK, and AMPK pathways. *Diabetes* 2010; **59**: 2826–2836.