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PROTECTIVE EFFECTS OF CYANIDIN-3-*O*-GLUCOSIDE AGAINST LIPOTOXICITY IN HYPERTROPHIC ADIPOCYTES

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Abbreviations

AC	Anthocyanins
ACC	Acetyl-coenzyme A carboxylase
ACS	Acyl-CoA synthetase
AGAT	1-acyl-glycerol-3-phosphate acyltransferase
AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
C/EBPs	CAAT/Enhancer binding proteins
C3G	Cyanidin-3-*O*-glucoside
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and Amphetamine-regulated transcript
CCR-2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CD-1	Cluster of differentiation-1 protein
CIDE-A	Cell death activator
CK2	Cytokine receptor kinase 2
CNS	Central nervous system
COX	Cyclooxygenase
CPT-1	Carnitine palmitoyl transferase 1
CRP	C-reactive protein
CSF-1	Colony stimulating factor-1
DAG	Diacylglycerols
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ET-1	Endothelin-1
FABP4	Fatty acid-binding protein 4
FAS	Fatty acid synthase
FATP1	Fatty acid transport protein 1
FFA	Free fatty acids
FOXO1	Forkhead Box O1
FSP27	Fat-specific protein 27
GH	Growth hormone
GLUT-4	Glucose transporter type 4
HFD	High-fat diet
HO-1	Hemeoxygenase
HSL	Hormone-sensitive lipase
ICAM-1	Intercellular Adhesion Molecule-1
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IKK	IkB kinase
IL-6	Interleukin-6
INOS	Inducible nitric oxide synthase
IR	Insulin Receptor

IRE-1	Inositol-requiring enzyme 1
IRS-1	Insulin receptor substrate-1
JNK	C-Jun NH2-terminal kinase
JNK	c-Jun N-terminal kinase
LD	Lipid droplets
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCP-1/CCL2	Monocyte chemoattractant protein-1
NF-κb	Nuclear factor-κb
NO	Nitric oxide
NQO-1	Quinone reductase-oxide 1
NRF2	NF-E2-related factor-2
NST	Nonshivering thermogenesis
PA	Palmitic acid
PAI-1	Plasminogen activator inhibitor-1
PEPCK	Phosphoenolpyruvate carboxykinase
PI3K	Phosphatidylinositol-3-phosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKR	Protein kinase RNA-activated
PNS	Peripheral nervous system
PPAR-γ	Peroxisome proliferator-activated receptor γ
RBP4	Retinol-binding protein-4
ROS	Reactive oxygen species
SFRPs	Secreted frizzled-related proteins
SH2	Src homology 2 domain
SNS	Sympathetic nervous system
SOCS	Suppressor of cytokine signalling
SREBP-1c	Sterol regulatory element binding protein-1c
T2DM	Type 2 diabetes mellitus
TLR	Toll-Like Receptor
TNF-α	Tumor necrosis factor-α
TSH	Thyrostatic hormone
UCP-1	Uncoupling protein-1
UPR	Unfolded protein response
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factors
WAT	White adipose tissue
XBP-1	X-box binding protein 1
ZFP36	Zinc finger protein 36

Abstract

Obesity is a metabolic disorder of multifactorial origin correlated with an elevated morbidity and mortality rates. It predisposes to the metabolic syndrome and is characterized by excess adipose tissue, altered levels of circulating proinflammatory adipokines, imbalances of the adaptive immune system and local and systemic chronic inflammation. In particular, obesity is associated with a state of "chronic low-grade inflammation", which plays an important pathophysiological role in the development and progression of many chronic pathologies, such as insulin resistance, endothelial dysfunction and dyslipidemia. Furthermore lipotoxicity, common in the adipose tissue, contributes to exacerbate the problems associated with these pathological conditions. FFA, including palmitic acid (PA), are in fact considered among the main causes of the onset of inflammation and insulin resistance in the adipose tissue.

In recent years, epidemiological evidences have shown that anthocyanins, natural phenols commonly present in food and vegetables from Mediterranean Diet possesses not only a high antioxidant and anti-inflammatory activity, but also a marked anti-obesity and insulin sensitizing effect. Therefore, the aim of this work was to evaluate the potential beneficial effects of cyanidin-3-*O*-glucoside (C3G) in counteracting the inflammatory condition and the insulin resistance induced by high concentrations of PA at the adipose tissue level, through the use of an *in vitro* experimental models on murine (3T3-L1) and human (SGBS) adipocytes.
In all experiments fully differentiated 3T3-L1 and SGBS adipocytes were pretreated with different concentrations of C3G for 24 h and then exposed to high concentrations of PA for further 24 h in order to induce cellular hypertrophy. To evaluate the insulin resistance condition, cells were subsequently treated with insulin.
In particular, to characterize the effect of PA on the inflammatory process and the insulin resistance at molecular level and to demonstrate the protective effect of C3G in such conditions, for 3T3-L1 cells, we evaluated cellular signal pathways involved in adipogenesis (PPAR-γ pathway), inflammatory process (NF-kB pathway) and insulin resistance (IRS-1/PI3K/Akt pathway).
Instead, in order to confirm the effects on human SGBS cells we assessed the mRNA levels of the main cytokines modulated by NF-kB (TNF-α, IL-6, IL-8, and MCP-1), and GLUT-1, GLUT-4, hexokinase and adiponectin as markers of insulin sensitivity.

Data reported in this thesis demonstrate that C3G ameliorates inflammation and insulin resistance conditions induced by PA, thus suggesting new potential roles for this natural compound in the prevention and treatment of pathological conditions linked to obesity.

Keywords: Lipotoxicity, Free fatty acids, Anthocyanin, Cyanidin-3-*O*-glucoside, Inflammation, Insulin resistance.

PART 1: BACKGROUND

CHAPTER 1

Obesity, Inflammation and Insulin resistance

1.1 Introduction

Obesity is a chronic multifactorial disease, characterized by an excessive accumulation of body fat, which can lead to adverse health effects, resulting in a reduction of life expectancy. It is determined in most cases by improper life styles: among the predisposing conditions there are hypercaloric feeding and reduced energy expenditure, due to inadequate physical activity (Makki *et al.*, 2013).

In the last decades, obesity has become one of the major public health problems worldwide, mainly because its prevalence is in constant and worrying increase both in western countries and in low-middle income classes. It is turned to be a major risk factor for many chronic diseases, such as type 2 diabetes mellitus (T2DM), cardiovascular and tumoral diseases, resulting in a reduced quality of life, high morbidity and mortality rates. It has been estimated that 44% of cases of T2DM, 23% of ischemic heart disease and up to 41% of some cancers, are due to obesity and overweight conditions (Muenning *et al.*, 2006).

In recent years the increase of this condition,with all its correlated effects, has been more and more increased especially among young people (Knight *et al.*, 2011). It is therefore essential to apply important strategies to cope with the increase in weight in the population at risk.

1.2 The adipose tissue

1.2.1 Morphogenesis

In human organism there are two types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). However, despite the common origin, the function of the two types of tissue is totally different.

WAT is the main tissue associated with energy storage at the body level; to WAT it is also attributed the function of isolation and mechanical protection of some vital organs. It consists of very large cells, with a diameter of about 50-100 µm, able to accumulate inside large amounts of triglycerides, which gather to form a large oily drop. Due to the considerable size of this drop, the cytoplasm and the nucleus are relegated to the periphery. White adipocytes are not simple fat

reservoirs, but metabolically active cells, able to synthesize and store triglycerides. The WAT mature adipocytes show the expression profile required for the synthesis of triacylglycerols, for glucose uptake and lipogenesis, as well as for lipolysis (Fantuzzi *et al*., 2005). This phenotype makes it possible that, when the body's energy supply is excessive, or when the expenditure is decreased, the excess energy is efficiently deposited at the WAT level as triacylglycerols. On the other hand, in cases of caloric need (when the energy intake is inadequate and/or the energy consumption is increased) WAT mobilizes lipid deposits resulting in the release of fatty acids and glycerol which are carried through the blood into the tissue, where they are oxidized to obtain energy (Large *et al*., 2004). Adipocytes have also the ability to convert excess glucose into reserve triglycerides.

BAT, on the other hand, has significant differences. It consists of smaller cells, whose brownish-red colour (hence the name of ‘‘Brown’’ fat) is due to rich vascularization and to the presence of cytochromes contained in the numerous mitochondria present at the cellular level (Cannon *et al*., 2004; Cinti *et al*., 2005). Unlike white adipocyte, the brown adipocytes do not contain a single large adipose mass but many small drops of triglycerides, known as lipid vacuoles. Therefore the nucleus and the cytoplasm are not located in periphery but well distinguishable within the cell. Besides a morphological there is also a diverse functional nature. Whereas in white adipocytes the triglyceride hydrolysis occurs according to the body's energy requirements, in the brown adipocytes fat degradation occurs in response to a lowering of body temperature. The role of BAT is to metabolize fatty acids to produce heat (Harms *et al*., 2013). BAT is mainly specialized in “nonshivering thermogenesis” (NST). This thermogenesis, also called chemical thermogenesis, is physiologically stimulated by noradrenaline, released by the nerve fibers of the sympathetic system that innervates it, and provides for the production of heat through exothermic biochemical reactions. The transduction of thermogenesis signal occurs mainly through the β_3-adrenergic receptors on the brown adipocytes membrane and it is coupled to the activation of adenylyl cyclase leading to an increase in cytosolic levels of cyclic adenosine monophosphate (cAMP) (Schulz *et al*., 2011).

The cAMP increase also induces hydrolysis of triglyceride reserves through activation of protein kinase A (PKA). The small lipid droplets, typical of brown adipocytes, make this process faster: they are more easily accessible for hydrolysis and rapid fatty acids oxidation. This BAT-specific role is supported by the high presence in mitocondria of uncoupling protein-1 (UCP1), which determines the coupling of the respiratory chain to adenosine triphosphate (ATP) synthetase, thus allowing the use of energy derived from oxidation of fatty acids for heat production (Dulloo *et al.,* 2010). Significant BAT deposits are found in rodents and other animals throughout their life. In humans, however, they are mainly present in newborn, although recent studies have shown that brown adipocytes may persist even in adults (Nedeergaard *et al.,* 2007).

Recently, along with these two types of adipose tissue, the "brite" ("Brown-in-white") or beige adipose tissue has emerged. This tissue is mainly located at the supraclavicular level with intermediate characteristics between the WAT and BAT (**Fig. 1**). This type of adipose tissue is dispersed among the white adipocytes, to which it is similar for the low mitochondrial protein UCP1 concentration; however, like BAT, it responds to cAMP stimulation, thus increasing the activity of UCP1 and mitochondrial respiration. Similarly to brown adipocytes, the activity of beige adipocytes is stimulated by cold, sympathetic stimulation, and natriuretic peptides. The main differences between the two types of adipose tissue (brown and beige), is that the first is rich in UCP1 in basal conditions, while the latter is enriched with this protein only in response to certain stimuli. It is therefore an adipose tissue easily adaptable to energy dissipation (Wu *et al*., 2012; Cui *et al*., 2017; Harms *et al*., 2013).

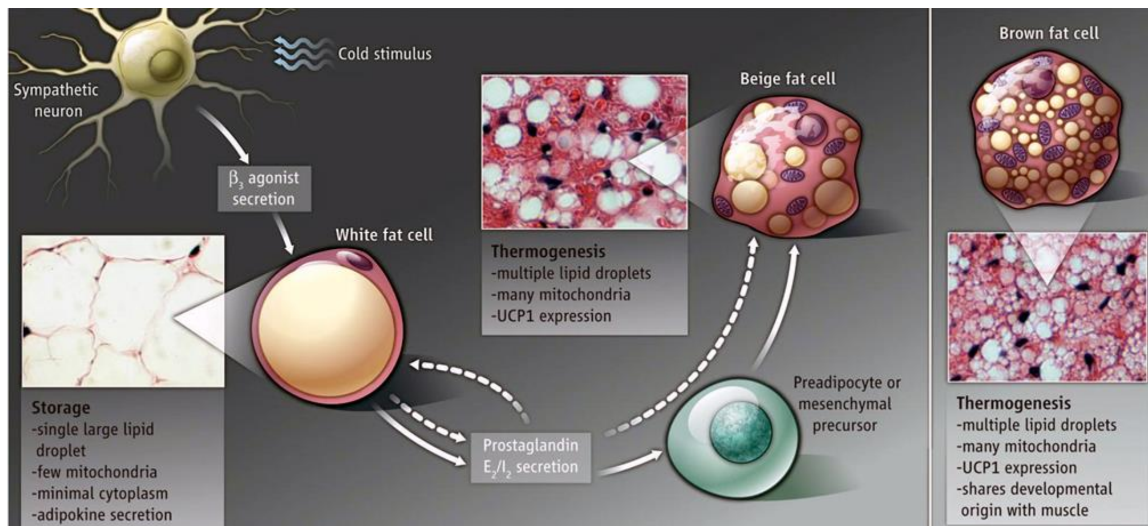


Fig. 1: Different types of adipose tissue and their characteristics (Rui *et al*., 2017).

Most adipose tissue in the adult is composed of WAT subcutaneous and visceral deposits. Subcutaneous adipose tissue affects the entire body surface; in women it is particularly developed in the gluteal-femoral and mammary region, but in men it is mainly present in the abdominal area. A significant portion, about 10% of the total, is deposited around the muscular bundles of the limbs and at the visceral level, while smaller deposits are found in the epicardial region and in the mediastinum (**Fig. 2**).

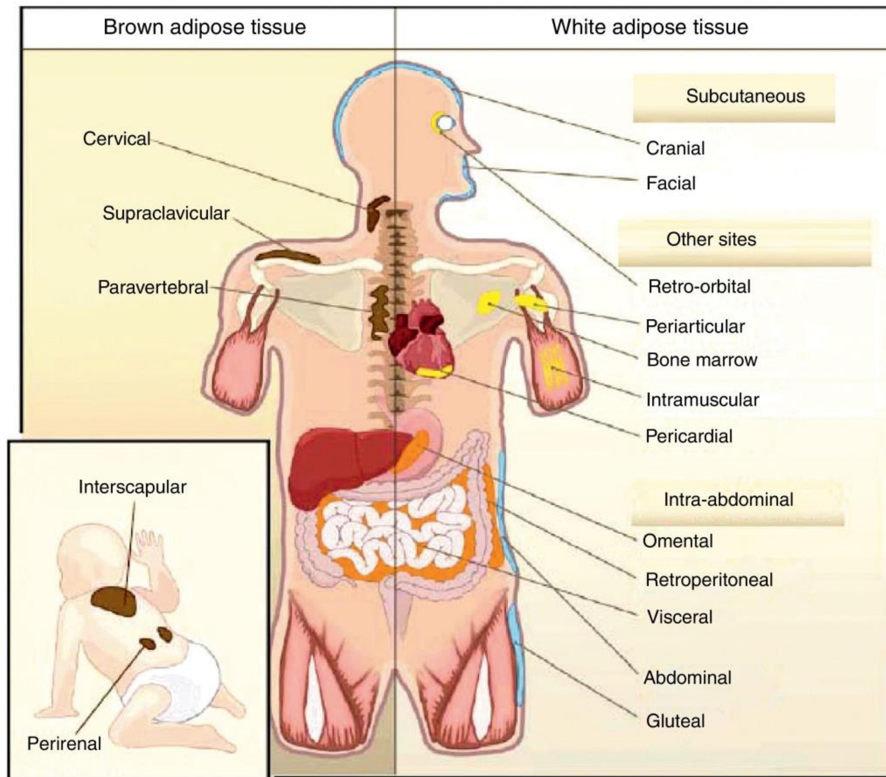


Fig. 2: Body distribution of WAT and BAT in humans (adapted from Gesta *et al*., 2007).

WAT has also a very heterogeneous composition according to body localization (Sanchez *et al*., 2005). If the cell that distinguishes the adipose tissue is the adipocyte, this is not the only type of cell present in the adipose tissue, and not even the most abundant one. Pre-adipocytes, macrophages, neutrophils, lymphocytes, and endothelial cells, are among the different cell types of adipose tissue. The balance between these different cellular forms, as well as their expression profile, is closely related to the maintenance of energy homeostasis. The increase in the form of adipocytes, in the number, and in the type of lymphocytes and infiltrated macrophages, is in fact strictly related to the problems of the metabolic syndrome (Esposito *et al.,* 2006; Lumeng *et al*., 2011).

Therefore, the study of preadipocytes regulation of proliferation and differentiation, as well as the understanding of the interaction between the different cells types present in the adipose tissue, gives us new targets which allow coping with these pathologies.

1.2.2 Adipogenesis

Adipogenesis is a multi-step process whose cellular and molecular events have been extensively studied in recent years, mainly due to some cell lines generation, such as the murine preadipocyte 3T3-L1 line (Green *et al*., 1975). They have allowed the understanding of both the differentiation of preadipocytes in mature adipocytes and the mechanisms underlying the main metabolic functions of the cell, such as lipolysis, incorporation of insulin-mediated glucose, and lipogenesis.

During this process, an undifferentiated mesenchymal cell becomes a pre-adipocyte, which in turn differentiates into a mature adipocyte, a cell used for fat storage. Until few years ago, adipogenesis was considered a function which ended in the first years of life with the presence of a fixed number of adipocytes predestined at birth. On the contrary, today it is widely accepted that the body fat is the place of a continuous cell turn-over through which the mesenchymal stem cells are engaged in the processes of proliferation in preadipocytes, stopping their growth and differentiating into mature adipocytes. The number of adipocytes thus depends on a balance between adipogenesis and apoptosis (Lefterova *et al*., 2009).

Adipogenesis occurs therefore in response both to normal cell renewal and to the need of increasing the fat reserves that happens when the nutritional needs exceed (Hausman *et al*., 2001). Two phases of adipose tissue growth can be distinguished: a first one is characterized by a marked hyperplasia, which increases the number of cells, and a second phase in which the number of adipocytes is apparently stable and instead shows a hypertrophic growth.

Hyperplasia is caused by the differentiation of precursors to mature adipocytes and it is an irreversible process, in contrast to hypertrophy (Avram *et al.,* 2007).

Some authors have developed a theory according to which adipose tissue initially grows thanks to the combination of hyperplasia and hypertrophy; then, as the number of cells rapidly reaches a plateau, cell hypertrophy continues until reaching a "maximum" of cell size (Otto and Lane, 2005).

When the "maximum" is reached, according to the "critical fat cell size hypothesis", adipocytes produce and release a series of paracrine factors that control the proliferation of pre-adipocytes and they are therefore involved in the development of obesity (Hausman *et al*., 2001). This activation is mainly induced by insulin-like growth factor (IGF) released by hypertrophic adipocytes and free

insulin, unable to bind to the specific receptor on adipocyte. There is therefore an initial differentiation of stem cells towards adipoblasts and pre-adipocytes and a subsequent activation of stem cell mitosis to restore the basal number of them in the stroma (Arner *et al*., 2009).

Once the differentiative stimulus has been induced, the cells are subjected to profound changes at transcriptional and morphological levels (Avram *et al*., 2007). One of the first events of adipogenesis is growth arrest, which normally occurs through contact inhibition. Following this, preadipocytes must receive an appropriate combination of mitogenic and adipogenic signals to continue through the next stages of differentiation, which will lead to the progressive acquisition of morphological and biochemical characteristics typical of mature adipocytes (Tang *et al*., 2012; Ali *et al*., 2013) (**Fig. 3**).

In particular, the adipogenic process is initialized by the drastic transition from the elongated fibroblast to the spherical one, typical of adipocytes. The morphological modifications are then accompanied by changes in extracellular matrix (ECM) and cytoskeleton components. The terminal stage of differentiation is instead characterized by the activation of a transcriptional cascade that leads to an increase in the expression of key proteins involved in the synthesis of triglycerides, an increase in the number of glucose transporters and increased sensitivity of insulin receptors. In this last phase, the newly formed adipocyte becomes in fact a highly specialised endocrine cell, able to secrete important hormones related to the regulation of energy homeostasis (Arner *et al*., 2009).

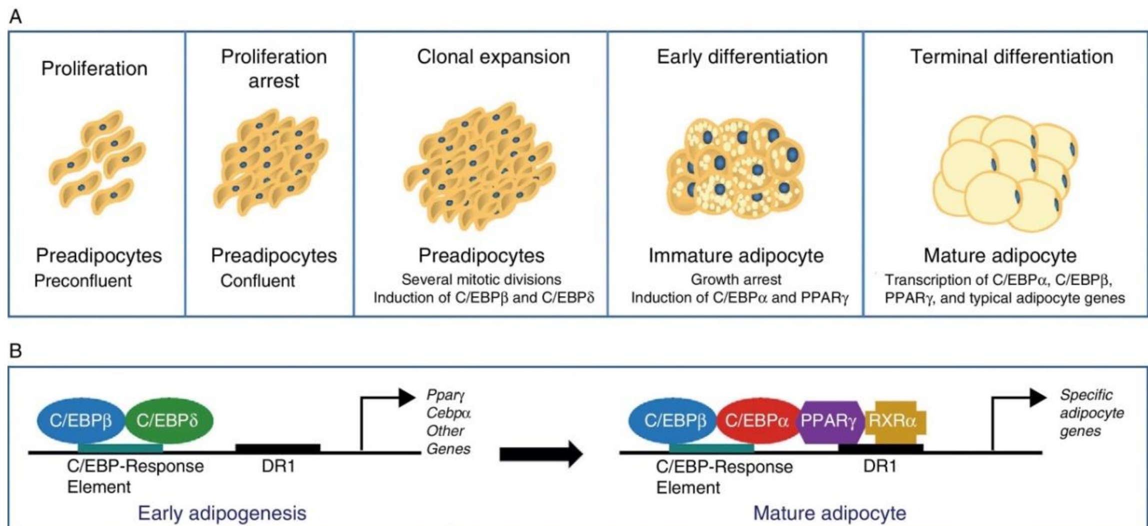


Fig. 3: Differentiation of preadipocytes into adipocytes (Ràfols *et al*., 2014). (A) Scheme of the transition process from preadipocyte to mature adipocyte including the different stages. (B) Sequential model of transcriptional control during adipogenesis.

During adipogenesis the increase in insulin sensitivity is observed through the increased synthesis of its receptor and of the insulin-dependent glucose transporter type 4 (GLUT-4); it is also observed a high transcription of the genes coding for fatty acid-binding protein 4 (FABP4), fatty acid transport protein 1 (FATP1), encoding for a fatty acid transporter, and for lipoprotein lipase (LPL), involved in lipid "storage" and metabolism control (Furuhashi *et al*., 2014; Wu *et al*., 2006; Mead *et al*., 2002). However, the different steps involved in the transition from pre-adipocytes to adipocytes are mainly regulated by the activation of a transcriptional cascade involving the nuclear receptor PPAR-γ (peroxisome proliferator-activated receptor γ) and some members of the C/EBPs family (CAAT/Enhancer binding proteins) (Nerlov 2007; Wang *et al*., 2017).

1.2.2.1 PPAR-γ

Transcription factors PPARs are members of the nuclear receptor superfamily. Up to now three different subtypes of PPARs (α, β/δ, and γ) have been identified; their actions result in a wide and diverse range of biological effects based on tissue localization and on the chemical profile of the ligand involved in the activation (D’amore *et al*., 2013; Wang *et al*., 2010).

In particular, PPAR-α is found in a variety of tissues including liver, heart and skeletal muscles, in which it has the function of fatty acids oxidation (Semple *et al*., 2006; Kiec-Wilk *et al*., 2005; Ferrè *et al*., 2004), and anti-inflammatory activity (Kiec-Wilk *et al*., 2005).

PPAR β/δ is ubiquitous, but mostly expressed in skeletal muscles (Semple *et al*., 2006; Gurnell, 2005). During prenatal development, it promotes the formation of organs through the regulation of cytotrophoblast migration, and influences stem cell differentiation (Kiec-Wilk *et al*., 2005). PPAR-γ instead has been identified as one of the most important regulators of adipogenesis (**Fig. 4**).

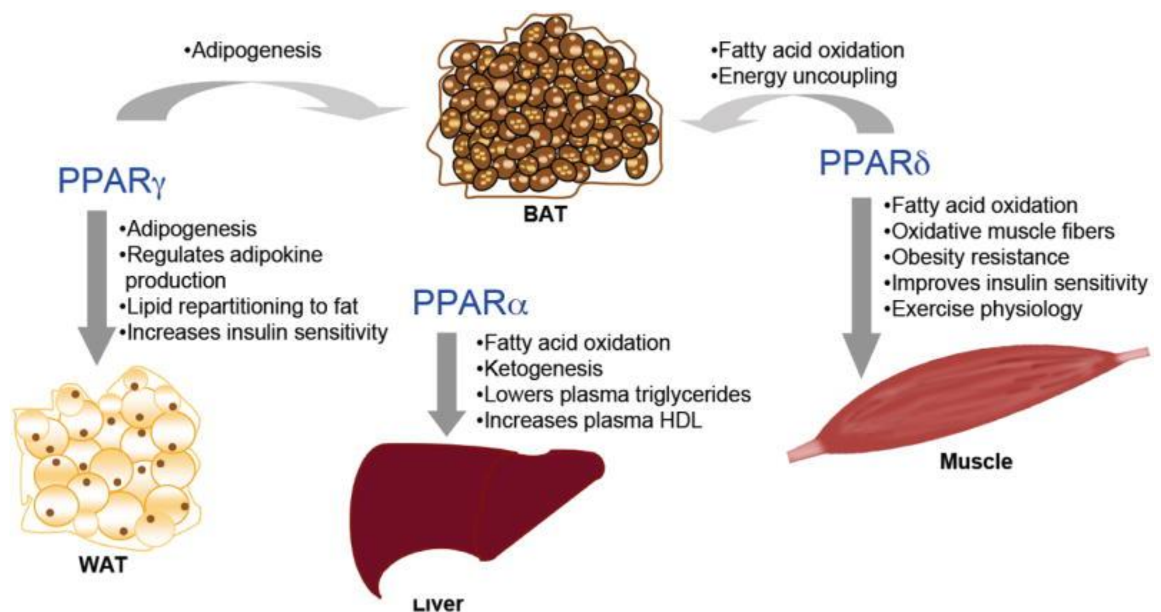


Fig. 4: Physiological and/or pharmacological PPARs roles in energy metabolism (Wang *et al*., 2010).

There are two isoforms of PPAR-γ (PPAR-γ1 e PPAR-γ2), originating from an alternative splicing, and they are both expressed in the adipose tissue, but only the isoform 2 is a specific marker of adipose tissue (Imai *et al*., 2004). The gene coding for the protein PPAR-γ not only plays a key role in the development of fat cells, but also for the maintenance of differentiation. Its silencing in the already differentiated 3T3-L1 adipocytes, in fact, causes de-differentiation with lipid loss and decreased expression of adipocytes typical markers (Feve *et al.,* 2005; Semple *et al.,* 2006; Kiec-Wilk *et al*., 2005). Up to now in fact, no factor capable of

promoting adipogenesis in absence of PPAR-γ has been identified (Agostini *et al.*, 2006).

Some studies have recently shown that the downregulation of PPAR-γ by stressors agents such as tumor necrosis factor-α (TNF-α) leads to triglyceride storage alteration in the fat cells (**Fig. 5**).

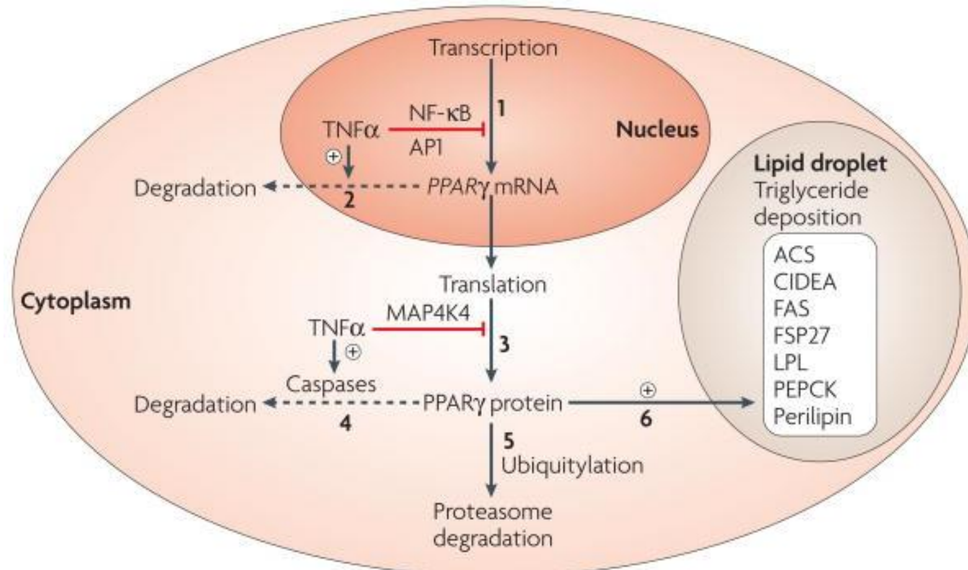


Fig. 5: Adipocyte dysfunction linking obesity to Insulin resistance (Guilherme *et al.*, 2008).

PPAR-γ expression can be regulated at the transcriptional level by TNF-α through the activation of Nuclear Factor-κB (NF-κB) and Activator protein 1 (AP1) protein, that negatively regulates PPAR expression (Ye *et al.*, 2008; Li *et al.*, 2018). In the adipocytes there is a rapid turnover of PPAR gene expression (Tang *et al.*, 2006) and the treatment of cultivated adipocytes with TNF-α could determine gene degradation. Translational control of PPAR is mediated by mitogen activated protein kinase-4 (MAPK4), a kinase upregulated by TNF-α (arrow 3). In addition, caspase activation through TNF-α could trigger the PPAR protein degradation in adipocytes (dashed arrow 4). The regulation of PPAR activity is also negatively regulated by kinase-mediated phosphorylation (Diradourian *et al.,* 2005) and ubiquitylation (Hauser *et al*., 2000), which promotes PPAR degradation through a proteasome-dependent pathway (arrow 5). The multiple-level action of TNF-α could therefore lead to a decrease in PPAR activity. A precise regulation of PPAR expression and function can contribute to the control of triglycerides biosynthesis and lipid droplets hydrolysis and deposition. This can take place thanks to the regulation of triglyceride metabolism enzyme expression as phosphoenolpyruvate carboxykinase (PEPCK), fatty acid synthase (FAS), acyl-CoA synthetase (ACS), LPL, and lipid-droplet proteins, including cell death activator (CIDE-A), fat-specific protein 27 (FSP27), and perilipin (Langhi *et al*., 2015) (arrow 6).

1.2.2.2 C/EBP

The C/EBPs transcription factor family includes 3 members: C/EBP-α, c/EBP-β, c/EBP-δ. These are the first transcription factors involved in adipocyte differentiation (Otto *et al*., 2005) and they are expressed at specific times during adipogenesis. The earliest are β and δ, which, in turn, promote the expression of c/EBP-α and PPAR-γ, essential for adipocytes total differentiation. The expression of C/EBP-β appears to be essential in the early stages of differentiation and this shows how animals deficient in C/EBP-β display a reduction of adipose tissue (Lefterova *et al*., 2014).

Murine models with suppression of c/EBP-β and c/EBP-δ, maintain the ability to generate adipose tissue, although the efficiency with which this phenomenon occurs is reduced. Surprisingly, they express normal levels of PPAR-γ, c/EBP-α, and FABP4, in spite of a reduced total fat mass, indicating, therefore, that there may be compensatory mechanisms involved in the activation of adipocyte genes (Lee *et al.,* 2004). Differently, the expression of C/EBP-α is required for adipogenesis with PPAR-γ, although the latter appears to be the dominant process (Zuo *et al*., 2006) (**Fig. 6**).

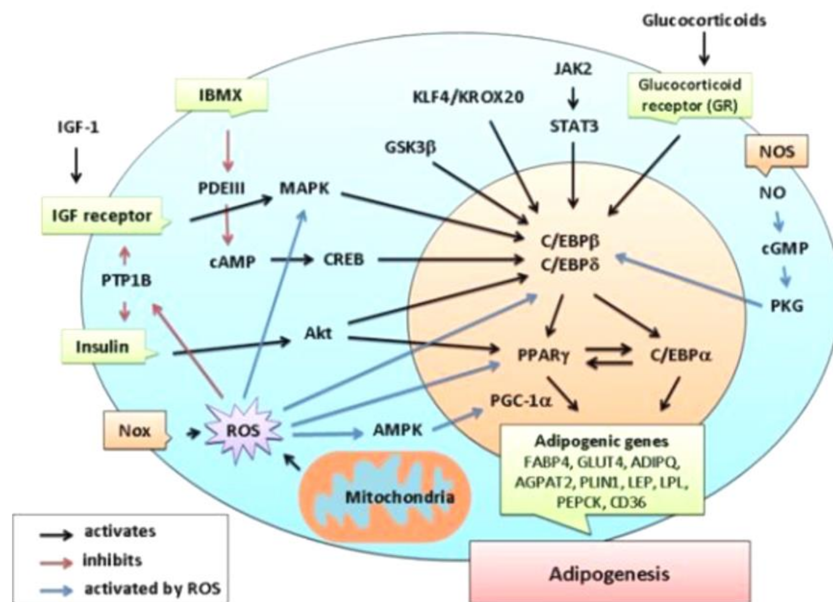


Fig. 6: Diagram of the transcriptional activity of PPAR-γ and C/EBPs during adipogenesis (adapted from Lefterova *et al*., 2014).

1.2.3 Physiology of adipose tissue

For a long time, adipose tissue has been considered an organ with a poorly active role in global energy homeostasis. It was believed that its function, in addition to providing a thermal and mechanical isolation, was only to store excess energy in the form of triglycerides with high caloric density, and to return it, according to needs, as free fatty acids. In recent years the concept of adipose tissue has radically changed, and nowadays it is considered as an endocrine organ that secretes many factors with autocrine, paracrine, and endocrine function (Sikaris *et al.*, 2004; Waki *et al.*, 2007).

The adipose tissue is involved in the regulation of the fat mass and nutrient homeostasis, as well as in the immune response, in blood pressure control, in haemostasis, and in thyroid and reproductive system functions control (Trayhurn *et al.*, 2005; Grant *et al.*, 2015).

Among the various substances secretes by WAT, fatty acids represent the most important molecules. These are esterified and stored in dynamic organelles called lipid droplets (LD) that during periods of excessive food availability act as a deposit for fatty acids and excess cholesterol which would otherwise be harmful to cells (Farese *et al.*, 2009). In addition to the adipogenesis process, two other important metabolic activities occur in the adipose tissue: the synthesis of fatty acids and their storage (lipogenesis), and mobilization or triglyceride hydrolysis (lipolysis). The first one occurs during periods in which there is a situation of excess energy, the second one during periods of caloric deficit and energy demand by other organs or tissues. Both lipogenesis and lipolysis are regulated by the integration of endocrine and neural mechanisms that cooperate in order to maintain the level of balanced and constant body fat under normal conditions (Ameer *et al.*, 2014; Duncan *et al.*, 2007).

Lipogenesis is a process regulated by LPL action. Synthesized and secreted by adipocytes, this enzyme is transported to the endothelium where it acts by hydrolyzing triglycerides from lipoproteins, thus releasing fatty acids and monoacylglycerol. LPL glycosylation is an important adjustment step both for its secretion and its enzymatic activity. LPL activity increases after meals, probably due to the stimulation by insulin itself, allowing the clearance of lipids from the

bloodstream and their storage in adipose tissue. LPL action is also affected by other factors, such as adenosine, which stimulates it, and cortisol, which acts by promoting insulin action; LPL activity is therefore reduced in the absence of insulin. Among the hormones that also inhibit LPL action catecholamines are included.

Glucose metabolism is essential within the adipocyte: it provides energy and allows to maintain a normal degree of free fatty acids esterification; in fact, during glycolysis, α-glycerophosphate is formed, and with it free fatty acids are esterified to form triglycerides (Herman *et al.*, 2006). Adipocytes are also able, through the lipolysis, to release in the circulation fatty acids that are used by most tissues as an energy source when glucose is not present in sufficient concentration, feeding mitochondrial systems of fatty acid oxidation and creating metabolic intermediates that act as substrates or signalling molecules (Zechner *et al.,* 2012).

Lipolysis is largely determined by the action of hormone-sensitive lipase (HSL), an enzyme that hydrolyzes triglycerides by releasing fatty acids and glycerol (Jaworski *et al.*, 2007). The enzyme activity depends on its phosphorylation, and the hormones that affect lipolysis regulate this state. The main regulatory mechanism occurs through the action of PKA, an enzyme whose function is activated by the increase in cAMP resulting from stimulation of adenylate cyclase. There are also other protein kinases that mediate HSL phosphorylation, such as the mitogen-activated protein kinase (MAPK) and the AMP-activated protein kinase (AMPK). The lipolytic stimuli also include catecholamines, glucagon, growth hormone (GH), cortisol, and thyrostatic hormone (TSH).

The most important anti-lipolytic hormone is insulin, whose action negatively regulates HSL phosphorylation. The action of the HSL is also increased by the perilipin protein, which is present on the membrane of the lipid-intracellular droplets and acts as a protective layer to prevent the action of the enzyme (Brasaemle *et al.,* 2007). A local antilipolitic action is also carried out by molecules directly produced by adipocytes, including adenosine and prostaglandins. The correct functioning of the lipogenesis/lipolysis balance is necessary to monitor all other metabolic processes in the organism. In presence of nutrients and energy excess or obesity, the adipose tissue ability can be overwhelmed, causing stress, injury and malfunctioning. For example, insulin

resistance under these conditions leads to higher levels of basal lipolysis and a decreased ability to synthesize and esterify fatty acids for storage or neutralization by downregulation of synthetics mechanisms (Morigny *et al*., 2016; Ranganathan *et al*., 2006).

This dysfunctional state contributes to systemic lipotoxicity, because the excess of fatty acids released or absorbed from diet, are moved into circulation and deposited in organs that are not predisposed to store lipids. The esterification purpose is to prevent the harmful effects of fatty acids (Listenberger *et al*., 2003) by increasing the storage capacity of LD in adipocytes and by determining protection against the development of diabetes and insulin resistance (Schaffer *et al.,* 2016; Sezer *et al*., 2017).

The increase of lipolysis is also linked to the secretion of FABP4 (Cao *et al*., 2013; Ertunc *et al*., 2015), which is an important mediator of immunometabolic local responses in adipose tissue and links the lipolytic state to glucose metabolism into the liver and other sites (Cao *et al*., 2006). Although adipocytes represent a specialized site for neutralizing fatty acids, this ability is not infinite and these cells are not entirely insensitive to the accumulation of excess lipids that can lead to various inflammatory responses (Sezer *et al*., 2017). Among the other lipid molecules secretes by WAT are prostanoids, cholesterol, and retinol, which are stored to be subsequently released (Trayhurn *et al*., 2005), and steroid hormones (sexual steroids and glucocorticoids), which at the level of the WAT can move from inactive to active forms and vice versa, with significant autocrine and paracrine roles (Li *et al*., 2017). In addition to these lipid substances, adipocytes and associated cells also produce and release, in systemic circulation, a series of hormones, factors and signals protein, called adipokines or adipocytokines, important for the maintenance of energy homeostasis, and their alterations contribute to the onset of complications associated with obesity (Fernández-Sánchez *et al*., 2011).

1.2.4 Adipose tissue as an endocrine organ

All molecules produced and secreted by adipose tissue with autocrine, paracrine, or endocrine functions are called adipokines. The term "adipokine" should be used to designate proteins synthesized and secreted directly from adipocytes (Trayhurn *et al.*, 2004). However, this term is used generically for all proteins secreted by WAT, although they are mainly synthesized by other cell types, such as infiltrated macrophages, present in the adipose tissue (Weisberg *et al.*, 2003). Adipokines have highly diversified chemical structures and important physiological roles. They include proteins involved in the regulation of energy intake and energy balance (leptin), in the regulation of arterial blood pressure (angiotensinogen), in vascular hemostasis (PAI-1), in lipid metabolism (RBP-4, CETP), in carbohydrate homeostasis (adiponectin, resistin, visfatine), and in angiogenesis (VEGF), as well as growth factors (TGF) and acute phase and oxidative stress proteins (hepatoglobulin, and A1-acid glycoprotein) (Antuna-Puente *et al.*, 2008). It is also interesting to note how, many of them, are linked to the immune system, such as the classical cytokines TNF-α, IL-1, IL-6, IL-8, IL-10, IL-4, IL-13, and MCP-1. This can therefore underline the close relationship between inflammation and obesity (Ouchi *et al.*, 2011; Gregor *et al.*, 2011).

This wide range of factors and protein signals suggests that adipose tissue is a complex organ, highly integrated in the physiology and metabolism of mammals, capable of establishing communication links with other tissues and organs, including the central nervous system (CNS), the liver, the skeletal muscles, and the surrenal cortex, through effects on the neuroendocrine pathways (Mathieu *et al.*, 2009). It is important to emphasize, however, that endocrine function is not only typical of the WAT, because many of these factors are also synthesized by BAT (Cannon *et al.*, 2004; Villarroya *et al.*, 2013). According to their effect in the adipose tissue, these adipokines can be classified as pro- or anti-inflammatory agents (**Fig. 7**).

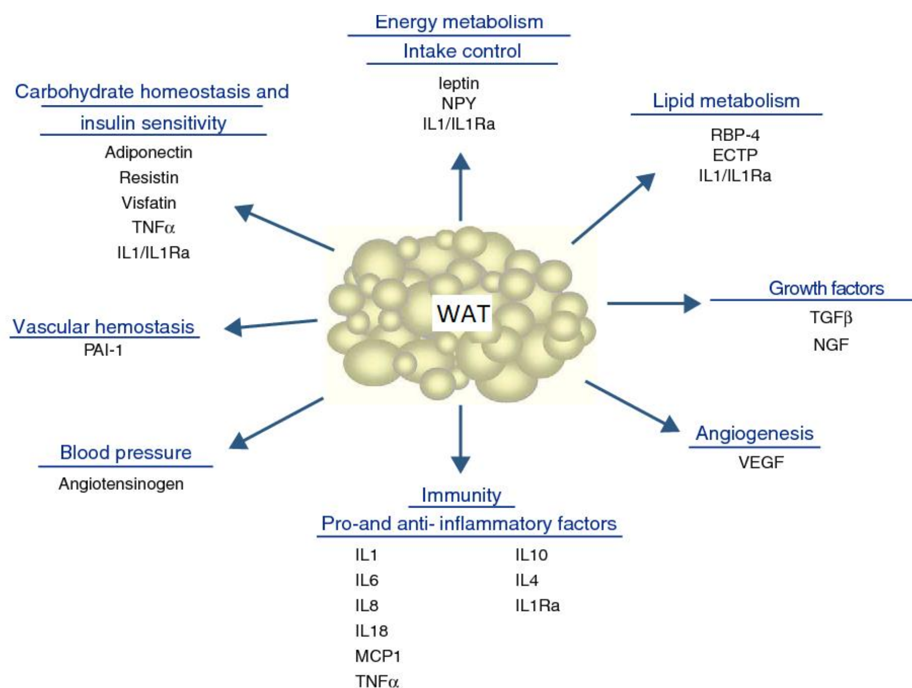


Fig.7: Physiological and metabolic processes regulated by WAT through adipokine secretion (Ràfols *et al*., 2014). CETP: cholesterol ester transfer protein; IL1: interleukin 1; IL1Ra: interleukin receptor antagonist-1; IL4: interleukin 4; IL6: interleukin 6: IL8: interleukin 8; IL10: interleucin-10; IL18: interleukin-18; MCP-1: monocyte chemoattractant protein-1; NGF: nerve growth factor; NPY: neuropeptide Y; RBP-4: retinolbinding protein-4; TGFβ: transforming growth factor β; TNFα: tumor necrosis factor alpha: VEGF: vascular endothelial growth factor.

1.2.5 Pro-inflammatory Adipokines

1.2.5.1 Leptin

The identification in 1994 of the Leptin gene (OB) and its receptor has started the endocrine era of adipocyte. It is a 16 kDa hormone formed by 167 amino acids, located on chromosome 6 in mice and chromosome 7 in humans. It is mainly secreted by adipocytes proportionally to the mass of adipose tissue and to nutritional conditions. Leptin is mainly produced in WAT and it is strongly involved in the regulation of lipid metabolism and energy consumption (Lastra *et al*., 2006; Kelesidis *et al*., 2011; Moon *et al*., 2013).

In obesity conditions it has shown an influence on dietary behaviour through hypothalamic regulation in CNS. Once secreted by adipose tissue, it circulates in the blood linked to the plasma proteins, and diffuses in the CNS through the binding to the capillaries in the median eminence and, through saturable transport,

through the choroid plexus receptor. In the ventromedial nucleus of the hypothalamus, leptin stimulates the cytokine receptor kinase 2 (CK2), the synthesis of the melanocyte stimulating hormone and cocaine- and amphetamine-regulated transcript (CART) which, via paracrine mechanisms, stimulates receptors 3 and 4 of the melanocortin lateral nucleus, causing satiety (Sikaris *et al.,* 2004; Fonseca-Alaniz *et al*., 2007).

The functions of leptin are not limited in the hypothalamus and are not exclusively concerned with the maintenance of energy homeostasis (Baratta *et al*., 2002), but involve the entire CNS and peripheral nervous system (PNS) (Bjørbæck *et al*., 2004). Leptin, in fact, is involved in several physiological processes such as: regulation of metabolism, growth, development, and regulation of certain endocrinological and immunologic processes, reproduction, cardiovascular physiopathology and maintenance of respiratory function (La Cava *et al.,* 2004). The discovery of leptin has thus confirmed the existence of a channel of communication between adipose tissue and brain, which aims to regulate the accumulation of fat in the adipocytes (**Fig. 8**).

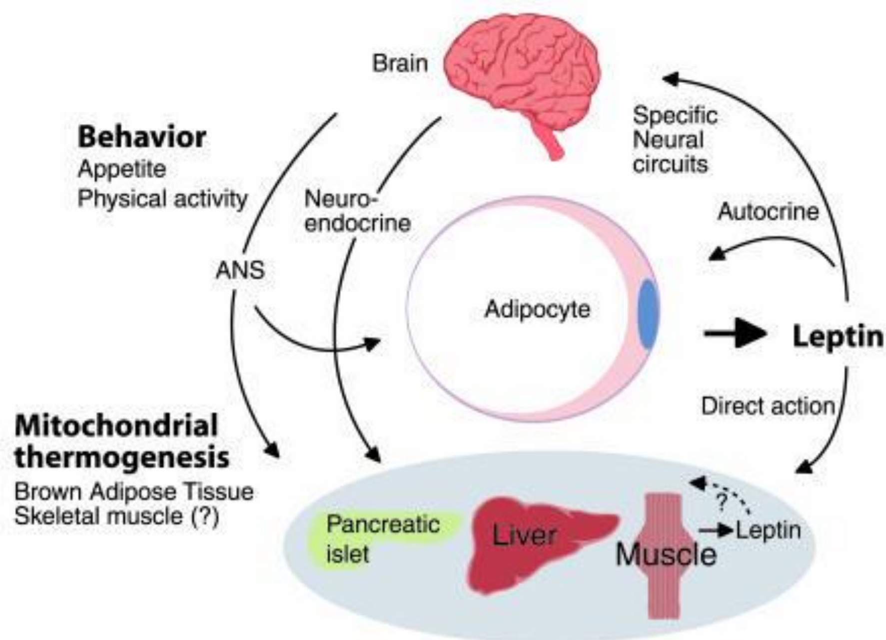


Fig. 8: Effects of leptin (Kahn *et al*., 2000).
Leptin exerts multiple actions to regulate glucose homeostasis through autocrine, paracrine, endocrine, and neural pathways.

Leptin inhibits lipogenesis and stimulates lipolysis, reducing intracellular lipid levels in skeletal muscles, liver and pancreatic β cells, thus improving insulin sensitivity. The limbic system stimulates dopamine reuptake, decrease appetite, and, through the nucleus *locus coeruleus*, activates the sympathetic nervous system (SNS) that increases resting energy expenditure (Fonseca-Alaniz *et al.*, 2007; Minokoshi *et al.,* 2012). Catecholamines influence the leptin secretion while other regulators of its synthesis are glucocorticoids (Sikaris *et al*., 2004); however, it has been shown that the main determinant in leptin secretion is glucose metabolism, because leptin concentration in bloodstream decreases in fasting conditions or caloric restriction and increases in response to food intake (Dulloo *et al*., 2010). Obesity is associated with increased leptin levels and it has been shown that the apparent decrease in anorectic effects and weight loss are the result of a mechanism of resistance to it (Fonseca-Alaniz *et al.,* 2007). The obese subjects therefore present leptin resistance (Zhou *et al*., 2013). This condition also causes altered leptin transport through the brain barrier, hyperleptinemia (Kievit *et al*., 2006), autophagy (Quan *et al.*, 2012), and ER stress (Ozcan *et al.,* 2009). Besides intervening in the body weight regulation, leptin regulates puberty and reproduction, placental and fetal functions, immune response, muscular and hepatic insulin sensitivity (Kelesidis *et al.,* 2010). In patients with lipoatrophy the lack of adipose tissue causes severe hypolepinemia, which is associated with severe insulin resistance, hepatic steatosis and dyslipidemia. In these patients, in addition, leptin treatment results in a marked improvement of glucose metabolism, dyslipidemia and hepatosteosis. Furthermore, hyperleptinemia, which belongs to the majority of the obese subjects, seems to have a proatherogenic role contributing to insulin resistance, altering endothelial function, and promoting platelet aggregation and arterial thrombosis (Martens *et al*., 2006). In inflammation, leptin acts directly on macrophages by increasing the phagocytic activity and the production of proinflammatory cytokines; when leptin is administered, higher levels of C-reactive protein are produced, thus proving its inflammatory effect (Steffes *et al*., 2006). In fact, leptin activates monocytes and macrophages, leading to the production of MCP-1 and VEGF in the hepatic stellate cells (Aleffi *et al*., 2005). Other inflammatory signals such as TNF-α and lipopolysaccharide (LPS) also stimulate the expression of leptin and its receptor

(Gan *et al*., 2012). Leptin also increases the production of pro-inflammatory Th1 cytokines and suppresses the production of anti-inflammatory Th2 cytokines, such as IL-4 (Iikuni *et al*., 2008). When there is weight loss the circulating hormone levels decrease and, in turn, also those of the inflammatory markers associated with obesity (Hukshorn *et al*., 2004). Leptin, besides promoting oxidative stress and vascular inflammation, stimulates the proliferation and migration of endothelial cells and smooth muscle ones, favouring the development of atherosclerosis (Cachofeiro *et al*., 2006). It is also important to emphasize that leptin can also be produced in the placenta, in the spinal cord, in the muscle, and perhaps in the brain (Sikaris *et al*., 2004).

1.2.5.2 Interleukin-6

Interleukin-6 (IL-6) is a cytokine with pro- and anti-inflammatory action (Fonseca-Alaniz *et al*., 2007). It is produced by macrophages, adipocytes (Cachofeiro *et al*., 2006), immune system cells, fibroblasts, endothelial cells, and skeletal muscles (Sanchez *et al*., 2005). IL-6 is highly expressed in adipose tissue and it is positively related to obesity in humans. Circulating cytokine levels are related to body mass index (BMI), insulin resistance, and carbohydrate intolerance (Lastra *et al*., 2006). Elevated plasma levels of IL-6 are considered as a predisposing factor for type 2 diabetes and myocardial infarction (Qu *et al.,* 2014). IL-6 also influences glucose tolerance through the negative regulation of visfatin; it also inhibits the secretion of adiponectin (Fonseca-Alaniz *et al.,* 2007) and in animal models increases triglycerides levels by increasing gluconeogenesis and glycogenolysis and inhibiting glycogenesis. IL-6 also induces hepatic C-reactive protein (CRP) production, which is known to be a risk factor for cardiovascular complications (Sacheck *et al*., 2008). High levels of IL-6 have been detected in atherosclerotic plaques in humans. In addition, IL-6 alters endothelium-dependent dilatation in veins, for this reason it appears to be an important aggravating factor of coronary heart disease (Neal *et al*., 2008). IL-6 activity is however controversial. The peripheral administration of IL-6 leads to an interruption of insulin signal due to the increase of suppressor of cytokine signaling-3 (SOCS3) expression in hepatocytes, suggesting that the expression of IL-6 in obesity determines insulin resistance (Kim *et al*., 2009). In contrast, *in*

vivo studies, have found that IL-6 deficient mice show obesity and hepatic inflammation, and IL-6 intake reverses insulin resistance (Matthews *et al*., 2010). IL-6 intake therefore would improve energy expenditure by reducing obesity. Thus the role of IL-6 in obesity and insulin resistance depends on specific expression sites integrated with other adipokines and cytokines.

1.2.5.3 Tumor necrosis factor-α

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine produced mainly by monocytes, lymphocytes, adipose tissue, and muscles (Ouchi *et al*., 2011). It appears involved in several systemic inflammatory responses and its irregular production contributes to the pathogenesis of the metabolic syndrome resulting in a higher incidence of obesity, diabetes, and cardiovascular diseases (Sanchez *et al*., 2005). The high levels of TNF-α are related to an increase in adipose tissue metabolism. In fact, it is also secreted by preadipocytes and adipocytes and its production is increased in obese subjects. TNF-α is also capable of suppressing the differentiation of preadipocytes and inducing delipidation and dedifferentiation of adipocytes (Kurebayashi *et al.,* 2001). Therefore it appears to be closely related to insulin resistance and obesity (Tzanavari *et al.,* 2010).

In several *in vitro* and *in vivo* studies, TNF-α has been shown to induce insulin resistance (Borst *et al.,* 2004; Swaroop *et al.,* 2012). In this condition, in particular, there is an increase in the release of free fatty acids in adipocytes, blocking of adiponectin synthesis, and reduction of tyrosine phosphorylation in insulin receptor, which is essential for progression of intracellular hormone signalling (Lastra *et al*., 2006). Thus, TNF-α appears positively related to insulin resistance and its neutralization should improve insulin sensitivity. However, all these aspects are still controversial. Recent studies have shown that a short-term intake of TNF-α significantly suppresses inflammation in obese subjects with type 2 diabetes, but does not show improvement in insulin sensitivity (Swaroop *et al.,* 2012).

In contrast, long-term treatment with TNF-α causes remission of inflammatory diseases such as rheumatoid arthritis, greatly improving insulin sensitivity (Gonzales-Gay *et al*., 2006; Stanley *et al.,* 2011). TNF-α also plays an important role in inflammation by determining activation of NF-κB, resulting in an increase

in adhesion molecules expression on the surface of muscle cells, inflammatory state of adipose tissue, endothelial dysfunction, and atherogenesis (Lastra *et al.*, 2006).

1.2.5.4 Monocyte chemoattractant protein-1 and CC-Chemokine receptor type 5

Chemokines and their receptors play an essential role in mediating immune cell infiltration in adipose tissue. Monocyte chemoattractant protein-1 (MCP-1) (also called CCL2) and CC-Chemokine receptor type 5 (CCR5) are respectively a typical chemokine and a chemokine receptor that mediate inflammatory responses and are significantly increased in obese subjects. *In vivo* studies have shown how deficient mice in MCP-1 receptor exhibit reduced macrophagic infiltration, inflammation and insulin resistance (Weisberg *et al.*, 2006). In addition, the deletion of the CCR5 receptor in obese rats show improved inflammation, insulin sensitivity and hepatic steatosis resulting in reduced macrophagic infiltration and differentiation of macrophages into the anti-inflammatory M2 isoform (Kitade *et al.*, 2012; Kanda *et al.*, 2006). However, MCP-1 role in inflammation and insulin resistance is still unclear. In another study, obese mice deficient in MCP-1 do not show differences in the accumulation of macrophages and inflammation in the adipose tissue (Kirk *et al.*, 2008). However, the reason for this difference is still unclear: it is possible that a deficiency of MCP-1 could be compensated by other chemokines to it related.

1.2.5.5 Other Pro-inflammatory Adipokines

In addition to Leptin, IL-6, TNF-α and MCP-1, WAT expresses and releases a variety of other pro-inflammatory cytokines and chemokines including Retinol-binding protein 4, Resistin, Angiotensinogen and Plasminogen Activator Inhibitor–1.

The Retinol-binding protein 4 (RBP4) is a specific protein for circulating transport of retinol (vitamin A). It is expressed in the liver, in adipocytes, and in macrophages, and shows elevated plasma levels in different animal models of obesity and insulin resistance. Recently, it has been shown that retinol-binding protein constitutes an adipocitary signal that can contribute to the pathogenesis of

T2DM in obesity. The increase of its plasma concentration seems to induce the hepatic expression of neoglucogenetic enzymes and contributes to the insulin resistance in skeletal muscles (Yang *et al*., 2005). Recent *in vivo* studies have shown that the intake of recombinant RBP4 in healthy rats determines insulin resistance (Yang *et al.,* 2005). The expression of RBP4 seems, in fact, to be inversely related to GLUT-4 in adipocytes, thus determining induction of insulin resistance condition by inhibiting insulin-induced insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation. Clinical studies have also shown that increased levels of RBP4 are closely associated with high blood pressure, high levels of triacylglycerol, high BMI (Graham *et al*., 2006), subclinical inflammation, and renal diseases (Akbay *et al*., 2010). RBP4 stimulates primary human endothelial cells to produce pro-inflammatory molecules such as vascular cell adhesion molecule-1 (VCAM-1), MCP-1, and IL-6, determining endothelial inflammation progression in cardiovascular disorders and microvascular complications in diabetes (Farjo *et al*., 2012).

The Resistin (ADSF/FIZZ3/XCP1), a 10 kDa polypeptide with 114 amino acids, has been identified as a pulmonary inflammation and insulin resistance inducer (Mojiminiyi *et al.,* 2007). It belongs to the rich cysteine family and circulates as hexamer or trimer. The hexameric form is the most abundant but the less active, while the trimeric instead determines insulin resistance. Resistin is involved in the activation of the SOCS3 protein which triggers suppression of the insulin mediated signal in adipocytes (Steppan *et al.,* 2005). Studies in mice have shown that, as a result of the deficiency of this protein, there is also a better glucose tolerance and insulin sensitivity (Qi *et al*., 2006). Instead the resistin function in humans is still controversial. Monocytes and macrophages are the main source of resistin in humans; its expression in adipocytes is however restricted only to rodents. Inflammatory cytokines such as IL-1, IL-6, TNF-α, and LPS induce resistin expression in human macrophages. Resistin also stimulates human peripheral mononuclear cells to produce IL-6 and TNF-α through the NF-κB pathway. Rosiglitazone and PPAR-γ agonists instead suppress its expression in adipose tissue, determining attenuation of inflammatory responses (Bokarewa *et al.,* 2005). Resistin also activates JNK and p38 MAPK determining induction of insulin resistance through the bond in hypothalamus with the Toll-like receptor-4

(TLR4) (Benomar *et al*., 2013). Induction of resistin synthesis can be attenuated by PPAR-γ agonists (Kusminski *et al*., 2005; Lehrke *et al*., 2004). It has been shown that the treatment of patients with T2DM with pioglitazone, a PPAR-γ agonist, results in a decrease in serum resistin levels (Bajaj *et al*., 2004). In addition, several factors, such as pituitary, steroid and thyroid hormones, adrenalin, β_3-adrenergic receptor activation, endothelin-1 and insulin, modulate resistin expression (Kusminski *et al*., 2005; Lehrke *et al.,* 2004).

Adipose tissue is, in addition, an important site for the production of angiotensinogen and angiotensin II. Higher levels of Angiotensinogen can be found in adipose tissue of obese individuals compared to normal weight subjects, and a positive relation between plasma levels of angiotensinogen and adiposity can be detected (Goossens *et al*., 2003). It is clear, therefore, that the increased synthesis of angiotensinogen and angiotensin II can contribute to hypertension that is frequently associated with obesity. In addition, angiotensin II appears to exert disparate proinflammatory effects in adipocyte and stimulates oxidative stress, effects that can be inhibited by blocking angiotensin receptor 1. Angiotensinogen can play an important role in regulating blood and fatty acids flow into adipose tissue (Dulloo *et al.,* 2010). It is expressed in multiple cell types within the adipose tissue; its expression and secretion are higher in visceral tissue compared to the subcutaneous one and its high levels are related to the metabolic syndrome (Fonseca-Alaniz *et al*., 2007). The plasminogen activator inhibitor-1 (PAI-1) is the first physiological inhibitor of plasminogen activators in blood and contributes to thrombosis and to the development of chronic cardiovascular diseases. PAI-1 can play an important role in regulating blood and fatty acids flow of adipose tissue. Plasma levels of PAI-1 are regulated by the accumulation of visceral fat and its high concentration is associated with insulin resistance and proinflammatory cytokines (Sikaris *et al.,* 2004).

1.2.6 Anti-inflammatory Adipokines

1.2.6.1 Adiponectin

Adiponectin, also called Acrp30, ApM1, AdipoQ and GBP28 (Ruan *et al.,* 2016), is a protein of 247 amino acids isolated for the first time in 1995 into adipose tissue (Scherer *et al*., 1995). It is highly expressed in adipocytes with potent anti-inflammatory activities. It presents a collagen-like N-terminal and a globular C-terminal domain, and circulates as a trimer, hexamer and other high molecular weight structures. Under physiological conditions, it is present in blood at elevated concentrations (5-10 µg/ml); in obese subjects, in those with T2DM and in those suffering from cardiovascular disease, the circulating levels of adiponectin are reduced. Proinflammatory factors such as TNF-α, IL-6, reactive oxygen species (ROS) and hypoxia suppress the expression in adipocytes (Li *et al*., 2009). Recently, it has been shown that not only inflammatory signals, but also iron overload in adipocytes, suppress the expression of adiponectin in obese subjects through Forkhead Box O1 gene (FOXO1) (Gabrielsen *et al*., 2012). Conversely, PPAR-γ antagonists stimulate adiponectin expression in adipocytes (Maeda *et al*., 2001). Adiponectin activates AMPK through its receptors AdipoR1 and AdipoR2, resulting in increased fatty acid oxidation and glucose uptake in muscles and suppressing hepatic gluconeogenesis (Yamauchi *et al*., 2002). Exogenous adiponectin intake also leads to improvement in insulin sensitivity (Maeda *et al*., 2002; Kim *et al*., 2007). Moreover, Adiponectin has a direct effect on glucose uptake in skeletal muscles and adipose tissue through an increased translocation of GLUT-4 on the plasma membrane (Ceddia *et al*., 2005; Fu *et al*., 2005).

Adiponectin inhibits, in macrophages, the production LPS-induced of TNF-α by inhibiting the activation of NF-κB pathway and stimulating the production of IL-10 which presents an anti-inflammatory activity (Kumada *et al.,* 2004). It also promotes the differentiation of anti-inflammatory macrophages M2 and modulates T-cells activation and inflammatory NK-cells function. Adiponectin receptors are upregulated at the surface of T-cells after antigenic stimulation and mediate apoptosis of specific T-cells by suppressing the antigen-specific expansion (Wilk *et al*., 2011). Moreover, adiponectin suppresses the production of interferon

gamma (IFN-γ) mediated by Toll-Like Receptor (TLR) in NK-cells without determining cytotoxicity (Wilk *et al.,* 2013). Thus adiponectin shows to possess important antiatherogenic, anti-diabetic and anti-inflammatory properties. Subjects with elevated plasma levels of adiponectin therefore have a significantly reduced risk of major cardiovascular events with consequent reduction of atherosclerosis and hepatic diseases (Hui *et al*., 2012).

1.2.6.2 SFRPs (Secreted frizzled-related proteins)

The secreted frizzled-related proteins (SFRPs) are a family of soluble proteins that consists of five secreted glycoproteins (SFRP1, SFRP2, SFRP3, SFRP4, SFRP5) that act as extracellular signalling ligands. Each SFRP is composed of around 300 amino acids and contains a cysteine-rich domain (CRD) that presents a 30-50% sequence homology with the CRD of Frizzled (Fz) receptors. SFRPs are the main proteins responsible for binding to 'Wingless/Integrated' (WNT) proteins on the plasma membrane (You *et al*., 2013; Garcia Tobilla *et al*., 2016).

SFRPs are responsible for the control of several biological phenomena and human abnormalities development. Aberrant gene expression and abnormal secreted protein levels are releated to a wide range of pathologies in humans (Liu *et al.,* 2018). In particular, the isoform SFRP5 has been identified as an anti-inflammatory adipokine which may be implicated in obesity adipocyte disfunction (Anunciado-Koza *et al*., 2016). Lower SFRP5 levels have been observed in obese rather than in lean subjects. This confirms therefore the relation between this protein and BMI (Hu *et al*., 2013). The expression of SFRP5 is low during the white and brown adipocytes differentiation while it is elevated in mature adipocytes (Wang *et al*., 2014). SFRP5 acts by WNT signalling pathway inhibition in white adipose tissues and recent studies have shown that this protein can play a key role in lipid metabolism, inflammation, and T2DM (Chen *et al.,* 2017).

SFRP5-deficient mouse models exhibit marked glucose intolerance, insulin resistance, as well as adipose tissue inflammation (Carstensen-Kirberg *et al.,* 2016; Ehrlund *et al*., 2013). Recent studies have shown that SFRP5 modulates adipogenesis by increasing adypocite differentiation and lipid accumulation and storage (Van Camp *et al*., 2014). This protein represses the WNT protein action

that reduces the expression levels of pre-adipogenic transcription factors, such as the members of the C/EBP family and PPAR-γ (Ehrlund *et al*., 2013). Therefore, the anti-inflammatory and insulin-sensitizing properties of SFRP5 have been determined by studies that have shown increased insulin sensitivity and decreased macrophage infiltration and pro-inflammatory protein production (Carstensen-Kirberg *et al.,* 2017). SFRP5 acts by inhibition of c-Jun N-terminal kinase (JNK) activation and antagonism of serine phosphorylation on IRS-1.

All this confirms that SFRP5 could represent a promising therapeutic target in the treatment of obesity, T2DM, and other metabolic diseases (Ouchi *et al*., 2010; Lu *et al*., 2013).

1.2.6.3 Other Anti-inflammatory Adipokines

In addition to Adiponectin and Secreted frizzled-related proteins, adipose tissue expresses and releases a variety of other anti-inflammatory cytokines including Omentin–1 and Apelin.

The omental adipose tissue secretes omentin-1, which is mainly expressed by the cells of the omental stromal vascular fraction, but not by adipocytes (Schaffler *et al*., 2005). Omentin-1 increases insulin-induced glucose uptake at the level of visceral and subcutaneous human adipocytes through the increase of AKT/PKB phosphorylation (Yang *et al*., 2006). Interestingly, Omentin-1 attenuates CRP- and TNF-α-induced NF-κB activation in human endothelial cells. Some studies have also demonstrated how Omentin-1 promotes macrophage differentiation in the anti-inflammatory M2 phenotype, thus suppressing inflammatory response and formation of LDL-induced foamy cells. This therefore suggests that Omentin-1 could be an anti-inflammatory adipokin in humans (Tan *et al*., 2010).

Apelin is a protein expressed in many tissues such as lung, mammary gland, and testicles, that has been identified as an endogenous ligand of the G protein coupled to the apelin receptor (APJ) (Lv *et al.,* 2017). It has several physiological functions to regulate multiple metabolic functions (Carpene *et al.,* 2007). Adipocytes produce apelin, and its plasma levels are increased in obese subjects. Apelin improves glucose uptake in an AMPK dependent way and suppresses lipolysis. Apelin deficient mice show insulin resistance suggesting that this

protein can improve glucose homeostasis and insulin sensitivity (Yue *et al.,* 2010-2011). Apelin is also involved in the inflammatory responses of obese individuals. Its expression is positively associated with TNF-α, some studies have shown, in fact, that the treatment with TNF-α induces its expression in adipose tissue. Furthermore, apelin activates JNK and NF-κB thus inducing the adhesion of molecules such as Intercellular Adhesion Molecule-1 (ICAM-1) in HUVEC cells (Lu *et al.,* 2012). However, apelin intake reduces renal inflammation to improve diabetic nephropathy by suppressing the expression of MCP-1, monocytes infiltration, and NF-κB activation (Day *et al.,* 2013). Thus the precise role of apelin in the regulation of inflammatory responses remains undefined.

1.3 Lipotoxicity and inflammation in adipose tissue

1.3.1 Lipotoxicity

The condition of lipotoxicity, typical of obese subjects, causes alteration of the body homeostasis following prolonged exposure to high concentrations of free fatty acids (FFA), sterols and phospholipids. These molecules are important cellular constituents and provide energy for the body metabolic activities, regulating different homeostatic processes inside and outside the cell, including organelles homeostasis, organs communication, immune function, energy metabolism, and cell survival. Although they perform important physiological roles, when in excess or altered they can be highly harmful, causing cell death, chronic inflammation and alterations of the energetic metabolism with consequent diffuse homeostatic alterations. The condition of lipotoxicity plays an essential role in different metabolic diseases such as inflammation and insulin resistance (Hotamisligil *et al.,* 2006).

Chronic low-grade metabolic inflammation, termed "metainflammation", is considered one of the main features of metabolic diseases such as obesity and diabetes, and it is diffused in several metabolic tissues including adipose tissue, liver, muscles, brain, and intestine (Hotamisligil *et al*., 2006). Just as lipotoxicity gives rise to metainflammation, alterations in lipid metabolism and typical cellular signalling pathways can lead to an increase in the onset of many diseases (Fu *et*

al., 2012). Plasma free fatty acids can derive from two different sources: lipolysis of triglyceride-rich lipoproteins within the bloodstream and, secondarily, intracellular lipolysis in adipose tissue. A recent article by Karpe and coll. has described the relationship between plasma FFA concentrations and insulin resistance in obesity (Karpe *et al*., 2011). It is widely known that the increase in plasma FFA in obese people is a consequence of increased release of fatty acids from adipose tissue and a reduction in plasma FFA clearance (Mook *et al.,* 2004).

Increase in FFA and inflammation induced by obesity play a key role in insulin resistance development (Capurso and Capurso 2012). Lipotoxicity can also affect the inflammatory condition through direct effects on different intracellular signalling pathways. For example, exposure to palmitic acid (PA), one of the most common fatty acids present in nature, is implicated in the synthesis of diacylglycerols (DAGs), which can activate new isoforms of protein kinase C (PKC), such as PKC-Θ and PKC-ε, which are linked to the activation of T-cells and to LPS responses (Aksoy *et al*., 2002), as well as to insulin action and metabolic responses (Perry *et al*., 2014).

While the exact mechanisms underlying these signalling pathways and the lipid species modulating PKC isoforms remain the subject of discussion (Zechner *et al.,* 2012), there is strong evidence supporting PKC involvement in important metabolic and inflammatory responses for obesity and type 2 diabetes. The accumulation of PA also leads to the synthesis of ceramide which can activate inflammatory pathways and can inhibit insulin action. Ceramides inhibit the Akt-mediated insulin signalling pathway and the oxidation of mitochondrial fatty acids by interrupting electron transport (Stratford *et al*., 2004; Summers, 2006). However, the inhibition of ceramide synthesis through treatment with miriocin, a serine palmitoyltransferase inhibitor, increases glucose levels and energy metabolism by restoring the insulin signalling pathway in the liver and in muscles (Yang *et al.,* 2009). It is also interesting to point out that the signalling pathway of TLR4 can also lead to increased expression of ceramide biosynthesis enzymes (Holland *et al.,* 2011), suggesting the importance of this pathway in metainflammation and insulin resistance.

Lipids can also affect cells fate and function by involving receptors on the cellular surface or kinases in the cytoplasm. Fatty acids such as PA, can directly activate

inflammatory pathway by increasing TLR4 signalling pathway (Huang *et al*., 2012) and stimulating molecules such as Protein kinase RNA-activated (PKR) (Nakamura *et al*., 2010). In response to potentially harmful lipids, such as PA and oxidized cholesterol, PKR can activate JNK leading to recruitment of the transcription factor AP-1, and therefore to the expression of genes that promote inflammatory process and the activity of inflammasome (Takada *et al*., 2007; Peng *et al*., 2015).

1.3.2 "Chronic low-grade inflammation" in adipose tissue

When a positive energy balance condition is created in the organism morphological and metabolic changes occur in the adipose tissue; they are accompanied by a greater expression of pro-inflammatory cytokines, such as IL-6 and TNF-α, and less production of anti-inflammatory molecules, such as adiponectin (Gregor *et al*., 2011). These modifications, in obesity, do not only have local effects, but they are accompanied by an increase in serum concentrations of acute phase proteins, contributing to "chronic low-grade inflammation" (Wellen *et al*., 2003; Ouchi *et al.,* 2011). This process seems to have an important physiopathological role in the development and progression of complications releated to obesity (Hotamisligil, 2006).

1.3.3 Inflammatory cells infiltration in adipose tissue

Another important mechanism contributing to chronic inflammation state in obesity, seems to be linked to the infiltration of inflammatory cells (mainly macrophages and lymphocytes) into adipose tissue. Many studies have shown that the adipose tissue of obese subjects is markedly infiltrated by macrophages, which actively participate in the adipose tissue inflammatory process (Weisberg *et al.,* 2003; Clement *et al.,* 2004). These cells would be recalled by the peripheral circulation, through the production and release in the circulation of chemokines by hypertrophic adipocytes, such as TNF-α, MCP-1, and VCAM. In this process preadipocytes and endothelial cells also contribute, that in turn, by secreting MCP-1, actively attract macrophages into adipose tissue (**Fig. 9**).

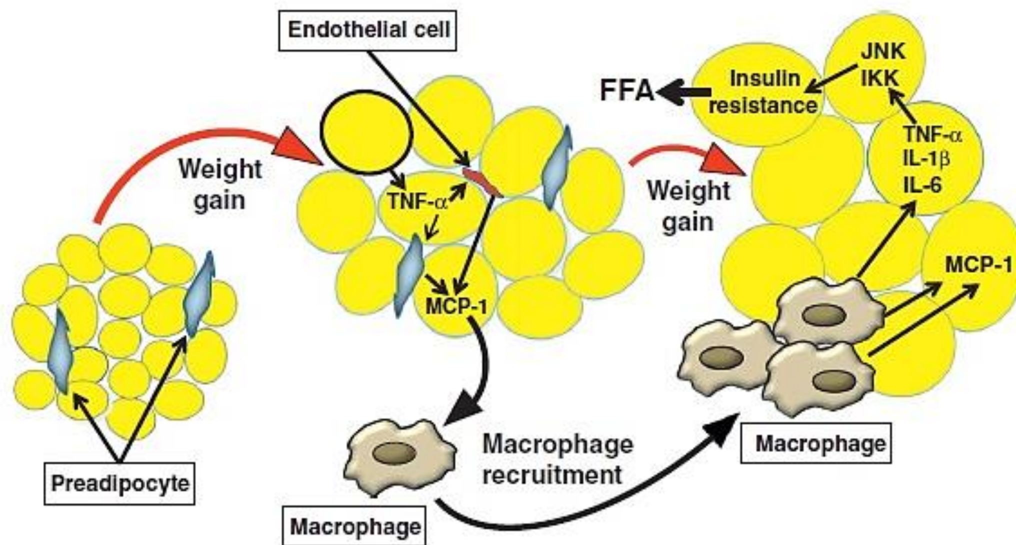


Fig. 9: Inflammatory cells infiltration in adipose tissue (Adapted from Capurso and Capurso 2012).
The adipose tissue of obese subjects is characterized by a latent inflammatory state (low-grade inflammation) and by infiltration of macrophages (a phenomena that progressively increase with the development of obesity). The adipose tissue of obese subjects is infiltrated by macrophages that actively participate in the establishment of an inflammatory condition in adipose tissue.

Once penetrated into the adipose tissue and locally activated, the macrophages, in synergy with adipocytes and other trigger cells, perpetuate a vicious circle of other macrophages recruitment and inflammatory cytokines production compromising adipocytes function. The inflammatory cytokines IL-6, IL-1β and TNF-α activate the serin-kinases JNK and IKK, which promote serine phosphorylation of IRS-1 (serine 307-312), resulting in insulin receptor and signal functional arrest. This mechanism, which begins in the peri-visceral adipose tissue, promotes insulin resistance in adipocytes. With the increase of adipose mass, many other factors contribute to the stimulation of the new macrophages recruitment, adhesion and infiltration into adipose tissue. Among these increased leptin and reduced adiponectin secretion, endothelial damage (caused by increase in "shear stress") or oxidative damage (induced by increase in FFA) and increased expression by adipose tissue of C-C chemokine receptor type 2 (CCR-2), are

involved in the recruitment of monocyte-macrophages by adipose tissue (Suganami *et al*., 2010).

Whatever the initial causal factor, recent studies have shown an increase in this inflammatory infiltrate along with obesity development. Furthermore, the non-adipocyte component of adipose tissue has high secretory capacities of proinflammatory cytokines, greatly amplifying tissue inflammatory response (Weisberg *et al*., 2003; Duffault *et al*., 2009).

In vivo studies have also observed that the genes that preside the synthesis and secretion of those factors that attract macrophages in adipose tissue and that give rise to inflammation are expressed in the adipose tissue of the obese subject before insulin resistance onset. This confirms that macrophages are an important source of inflammation of adipose tissue, related to insulin resistance (Weisberg *et al*., 2003; Clement *et al.,* 2004). Following the observation that pro-inflammatory macrophages are recruited into adipose tissue, growing interest has been devoted to the relationship between adipocytes and inflammatory cells (Wellen *et al.,* 2003). The achievement of adipocyte growth "critical size" and the subsequent state of cell hypoxia have been proposed as the first phases of an event sequence that triggers activation of a phlogistic response characterized by leukocyte recruitment and inflammatory mediators increased secretion (Frain *et al.,* 2003; Rausch *et al.*, 2008).

Several *in vivo* studies, in different murine and human cellular models, have shown that obesity development is associated with an increased release of chemotactic factors by adipocytes, such as MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), CCR2, and CCR5 chemokines receptors, and the colony stimulating factor-1 (CSF-1), necessary for macrophage proliferation and survival (Huber *et al.,* 2008; Curat *et al.,* 2004). Studies carried out on MCP-1 knockout mice and mice with an over-expression of the gene, also support the important role of this chemokine in attracting macrophages into adipose tissues and in reducing insulin sensitivity in a high fat diet-induced obesity condition (Kanda *et al.,* 2006). In this state, deficient MCP-1 mice, in fact, in comparison with the controls, show a reduced macrophagic content and an insulin sensitivity improvement. Following overweight and obesity conditions, dynamic phenotypic modifications in the macrophagic population occur (Anderson *et al.,* 2010). The

macrophages are divided into two classes. The "classically activated" M1 macrophages are stimulated by inflammatory cytokines such as IFN-γ and LPS antigen and, by secreting proinflammatory factors such as TNF-α and IL-6 in response to bacterial stimuli, cause inflammatory conditions (Zeida *et al.,* 2007). M2 macrophages are instead induced by anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 and, therefore, are "alternately activated" (Anderson *et al.*, 2010; Sell *et al.*, 2010).

The initial inflammatory response activates M1 macrophages, which become able to eliminate invading microorganisms and promote the inflammatory response. In particular, Cinti and coll. have highlighted a characteristic organization of the M1 macrophages, which seem to form a "crown-like structure" in relation to sites of adipocyte suffering and death (Murano *et al.*, 2008). These structures also seem to have the role of phagocyte cellular debris, especially LD. It is possible to observe in them the formation of multinucleated giant cells, a sign of a chronic inflammation development (Cinti *et al.,* 2005). On the other hand, during the inflammation resolution phase the macrophages are re-polarized in M2 direction, losing reactivity to the inflammatory stimuli and assuming the ability to eliminate damaged cells and tissues, and to promote lipid metabolism, angiogenesis and tissue remodeling (Anderson *et al.*, 2010; Sell *et al.*, 2010). M2 macrophages are dominant in adipose tissue of lean subjects. The anti-inflammatory cytokines secreted by these cells are able to suppress inflammation and maintain insulin sensitivity in adipose tissue. The factors that determine the differentiation of macrophages on adipose tissue are still unknown. The scavenger and mannose receptors pronounced expression, together with high endocytic activity, implies a role of adipose tissue macrophages in the intake of lipids and lipoproteins (Zeyda *et al.*, 2007). This condition supports the idea that the macrophages recruitment in adipose tissue, induced by a condition of obesity, is due to an increase in the necrotic adipocytes number that must be removed (Cinti *et al.*, 2005). However, the factors that lead macrophages into adipose tissue to change their pro- and anti-inflammatory phenotype, is one of the most tipical problems of obesity (Bai *et al.*, 2015). In any case, the analysis of macrophages gene expression in adipose tissue shows how they are the primary source of TNF-α and other proinflammatory molecules such as IL-6 in adipose tissue. This demonstrates that macrophages

contribute, in an important way, to the amplification of inflammation and to the development of dysfunctions and metabolic complications induced by obesity (Fain *et al*., 2004). Other cell types such as neutrophils, eosinophils (Wu *et al*., 2011) and dendritic cells (Bertola *et al*., 2012), through the production of pro- and anti-inflammatory cytokines in adipose tissue, also play an important role in the onset of inflammation and insulin resistance following the condition of obesity. Neutrophils, in particular, are the first immune cells involved in inflammatory responses. By rapidly infiltrating in adipose tissue, they accelerate the inflammatory responses going to call other cells in inflammatory sites. Thus, blocking the neutrophils and macrophages action results in inflammatory responses and insulin sensitivity improvement in adipose tissue (Talukdar *et al.,* 2012).

PA, ceramide and DAGs are widely studied lipid mediators that lead to the immune cells activation, suggesting also a lipotoxic inflammatory connection in adipose tissue. In addition to the direct sensors and stress kinases activation, lipids can also act as antigens that are presented to the immune cells by Cluster of differentiation-1 protein (CD-1). Recently, the role of NK-cells, in the inflammation and insulin resistance conditions induced by obesity, has also been considered. In fact, the NK-cells generates TH1 and Th2 cytokines such as IFN-γ and IL-4 to rapidly regulate the immune responses (Bendelac *et al*., 2007). Therefore a reduction in these cell types determines in lean and obese subjects, anti-inflammatory cytokine production (Schipper *et al*., 2012). However, the NK-cells role remains controversial. Some studies suggest a beneficial effect of the NK-cells (Lynch *et al*., 2012), other, in contrast, attribute a key role in inflammatory and dysfunctional state (Mantell *et al*., 2011; Wu *et al*., 2012). Thus the immune cells are all involved in the development of inflammation and insulin resistance in the adipose tissue and their inhibition could cause their remission.

1.3.4 Molecular mechanisms underlying the inflammatory process in adipose tissue

1.3.4.1 Reticulum Stress as the origin of inflammation

The main cause of obesity-induced inflammation has not yet been fully understood, but certainly stress conditions in endoplasmic reticulum (ER) contribute to the onset of inflammatory condition. Obesity causes ER stress in the liver and adipose tissue due to the excess lipids accumulation and disturbed energy metabolism (Ozcan *et al.,* 2004). The ER stress determines the initiation of a signal cascade that involves the production of the unfolded protein response (UPR) with protective but also apoptotic and inflammatory effects. During ER stress, trans-membrane proteins are activated, including the protein kinase/endoribonuclease inositol-requiring enzyme 1 (IRE1), an ER-transmembrane protein which initiates the non-spliceosomal mRNA splicing for the transcription factor X-box binding protein 1 (XBP-1) with consequent protective responses to ER stress.

IRE1 modulates ROS production and also determines the initiation of an inflammatory cascade by activating several inflammatory kinases such as JNK (JNK-MAPK4-AP1), PKR, IKK, and the main inflammatory transcription factor NF-κB (Nakamura *et al*., 2010). Obesity also induces ER stress causing IRS phosphorylation in the serinic site with consequent insulin signal alteration. The link between ER stress and inflammatory condition with consequent insulin resistance is also supported by experiments carried out in heterozygous mice XBP-1 +/- in which the protective responses to the ER stress were partially blunt with consequent increased insulin resistance development (Ozcan *et al*., 2004). On the contrary, the chemical chaperones administration that reduces ER stress conditions restores insulin sensitivity in obese mice (Ozcan *et al*., 2006). Moreover, ER stress determines downregulation of GLUT-4 expression in adipocytes (Miller *et al*., 2007). Therefore, ER stress directly influences cellular insulin targets through the inflammatory signals activation, thus contributing to insulin resistance. Adipose tissue is the subject of continuous research to understand stress-sensitive pathways and to discover new potential therapeutic targets able to diminish obesity-induced metabolic diseases (**Fig. 10**).

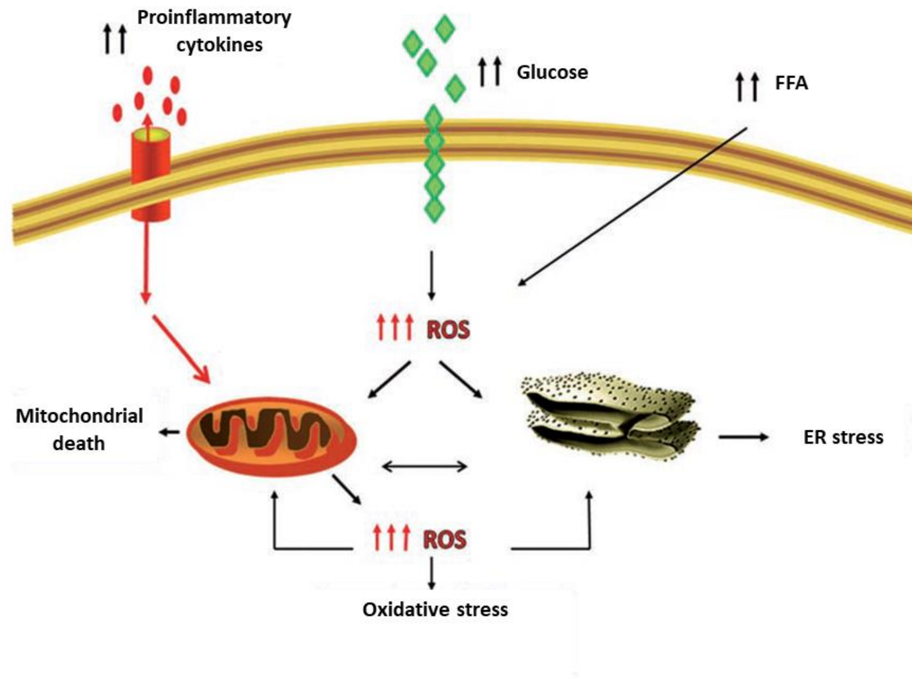


Fig. 10: Reticulum Stress as the origin of inflammation (adapted from Ozcan *et al*., 2004). Elevated levels of glucose and free fatty acids induce oxidative and endoplasmic reticulum stress, resulting in cellular damage.

1.3.4.2 Cell signaling pathway modulated by the transcription factor NF-κB

The adipose tissue low-grade chronic inflammation is correlated with chronic activation of proinflammatory cell signalling pathways. Fatty acids, especially the saturated ones (FFA), are the major responsible for this condition. In obesity, adipocytes exposure to high concentrations of fatty acids activates TLRs, such as TLR-2 and TLR-4, which lead to the inflammatory responses initiation by modulating a series of kinases leading to NF-κB translocation in the nucleus, increasing also the inflammatory cytokines production.

NF-κB is a redox-sensitive transcription factor with a key role in the induction of genes involved in various processes, such as inflammation, immune and stress response, apoptosis, cell development and growth. The main NF-κB targets consist in genes coding for chemokines, cytokines, immunoreceptors, and cellular adhesion molecules (Speciale *et al.,* 2011). In mammals, the NF-κB family is composed of several proteins, including NF-κB1 (P50/p105), NF-κB2 (P52/P100), p65 (RelA), RelB and c-Rel that share the Rel homology domain. The most widespread form in human is a heterodimer composed of a subunit p50

or p52 and p65, which contains the domains required for gene induction (**Fig. 11**) (Liu *et al.*, 2017). The biologically active NF-κB form can be found as a homodimer or heterodimer, who is able to move into the nucleus and to regulate several genes transcription that present a common recognition sequence in their promoter. NF-κB is maintained in the cytoplasm of the cells in a latent form, complexed with the inhibiting protein IκB. The identified members of the IκB family proteins are seven [IκB-α (MAD-3, PP40), IκB-β, IκB-γ/p105, BCL-3, IκB-δ/100, IκB-ζ and IκB-ε], that are characterized by multiple repeated sequences of 33 amino acids, defined as repeaters and considered responsible of interaction with the Rel NF-κB domain (Lawrence *et al.,* 2009). NF-κB activation through the canonical or non-canonical signalling pathway is an important mechanism regulating the immune and inflammatory responses of the organism (Baker *et al*., 2011). Several pathogenic agents, oxidants, cytokines, chemokines and growth factors induce, through specific receptors or oxidative stress, molecular signals that eventually lead to NF-κB activation. Cell stimulation by several inductor agents triggers a series of cascade reactions, resulting in activation of NF-κB that enters into the nucleus where it binds to the DNA κB binding sequence transcribes various genes for cytokines, chemokines, and other inflammatory markers (Liu *et al*., 2017). IκB-α is phosphorylated by IκB kinase (IKK) which induces proteasome-dependent degradation, this allows a rapid NF-κB translocation in the nucleus where it binds, with high DNA affinity, in correspondence with the consensus sequences 5'- GGGPuNNPyPyCC-3' (p65/p50) or 5'-GGGPuNPyPyCC -3' (p65/c-Rel) determining transcription activation. F-κB nuclear translocation causes the transcription of several proinflammatory genes, such as cytokines (TNF-α, IL-1β, IL-6) and inducible enzymes (iNOS and COX-2) (Janssen-Heininger *et al*., 2000; Liu *et al*., 2017)(**Fig.11**).

Recent studies have shown that TLR4 represents the main responsible for most inflammatory processes into adipose tissue. TLR4 deficient mice are substantially protected from the insulin resistance condition induced by elevated free fatty acids in obesity (Shi *et al*., 2006). These studies therefore suggest that TLR4 is a sensor of high concentrations of FFA, leading to the onset of inflammatory and insulin resistance processes.

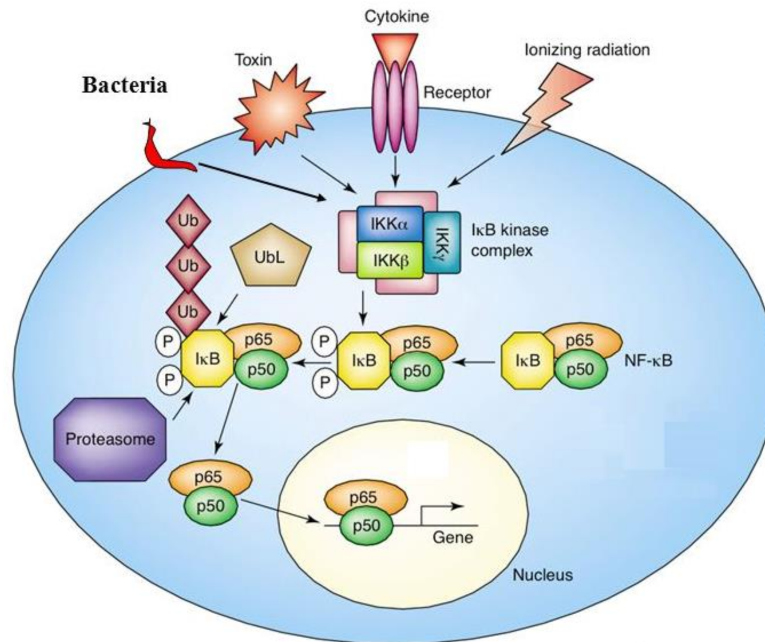


Fig. 11: NF-κB pathway (adapted from Perkins *et al.,* 2007).
NF-κB, in the inactive state, is located in the cytosol complexed with the inhibitory protein IκB. Following the interaction with integral membrane receptors, a variety of stressful extracellular agents (bacteria, toxins, cytokines, ionizing radiations) can activate the enzyme IκB kinase (IKK). IKK, in turn, phosphorylates the IκBα protein, which following the dissociation from NF-κB, is degraded by the proteasome. Activated NF-κB is then translocated into the nucleus where it binds to specific DNA sequences called response elements (RE). The DNA/NF-κB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA. In turn, mRNA is translated into protein, resulting in a change of cell function.

1.3.5 Obesity, inflammation and reduced insulin sensitivity

In recent years there are increasingly evidences that obesity is associated with inflammation that is in turn involved in insulin resistance development. In obesity, hyperlipidemia and hyperglycemia (due to nutrients excess), lipolysis and gluconeogenesis determine mitochondrial dysfunction, ER and oxidative stress, stimulating the activation of inflammatory kinases JNK and IKK-β. They increase inflammatory genes expression, amplifying systemic inflammation and determining insulin resistance (Samuel and Shulman, 2012).

In obese patients, a chronic low-grade inflammation results in increased plasma levels of CRP, inflammatory cytokines (such as TNF-α, IL-6, MCP-1 and IL-8), leptin (Likuni *et al.,* 2008) and osteopontin (Kiefer *et al*., 2008). Aging is also a factor associated with chronic low-grade increases in inflammatory markers

circulating levels. Particularly, TNF-α and IL-6 increased levels have been described as indicators of morbidity and mortality cause in elderly people. In agreement with the close relationship between inflammation and insulin resistance, the inflammatory condition is particularly prominent in elderly diabetic patients (Bruunsgaard *et al*., 2003). These conditions are particularly evident in some murine obesity models widely used to study their pathogenesis. TNF-α neutralization in rats provided the first convincing evidence that inflammatory mediators can cause insulin resistance (Dominguez *et al*., 2005). In fact, deficient TNF-α (TNF-/-) or TNF-receptor 1 gene (TNFR1) knockout mice are protected from insulin resistance caused by diet and genetic obesity (ob/ob mouse) (Uysal *et al*., 1997). *In vitro* experiments with murine adipocytes have also demonstrated that TNF-α activates several pathways related to insulin resistance, modifying insulin receptor IRS-1, GLUT-4, adiponectin, and PPAR gene expression, and determining IKK JNK kinases and SOCS3 proteins activation (Shi *et al*., 2004). In the same way, other inflammatory cytokines also interfere with the insulin signalling pathway. IL-1, mainly produced in monocytes and macrophages, through a precursor present in the cytosol causes the serine phosphorylation of IRS-1 by ERK activation (Jager *et al.,* 2007; Spranger *et al.,* 2003); IL-6, instead, blocks insulinic signal mediated by IRS in liver and muscle through SOCS proteins activation (Weigert *et al.,* 2006). In addition, high circulating IL-6 levels result in a compromised glucose absorption in skeletal muscles (Franckhauser *et al.,* 2008). However, IL-6 role in obesity, following the insulin resistance condition, remains controversial. Some studies have shown that the over-expression of IL-6 also reduces body weight, and in presence of high concentrations, it can also improve insulin sensitivity (Pedersen *et al*., 2007). In contrast to this, anti-inflammatory mediators such as IL-10 and adiponectin exert insulin-stimulating effects. Particularly the probable mechanism by which chemokines such as MCP-1 and IL-8 affect insulin sensitivity is through macrophages recruitment to adipose tissue. Different cytokines and proteins, elevated in obesity, such as TNF-α and leptin, determine activation of macrophages that, after tissues infiltration, become the main source of inflammatory mediators, determining activation of numerous inflammatory pathways (**Fig.12**).

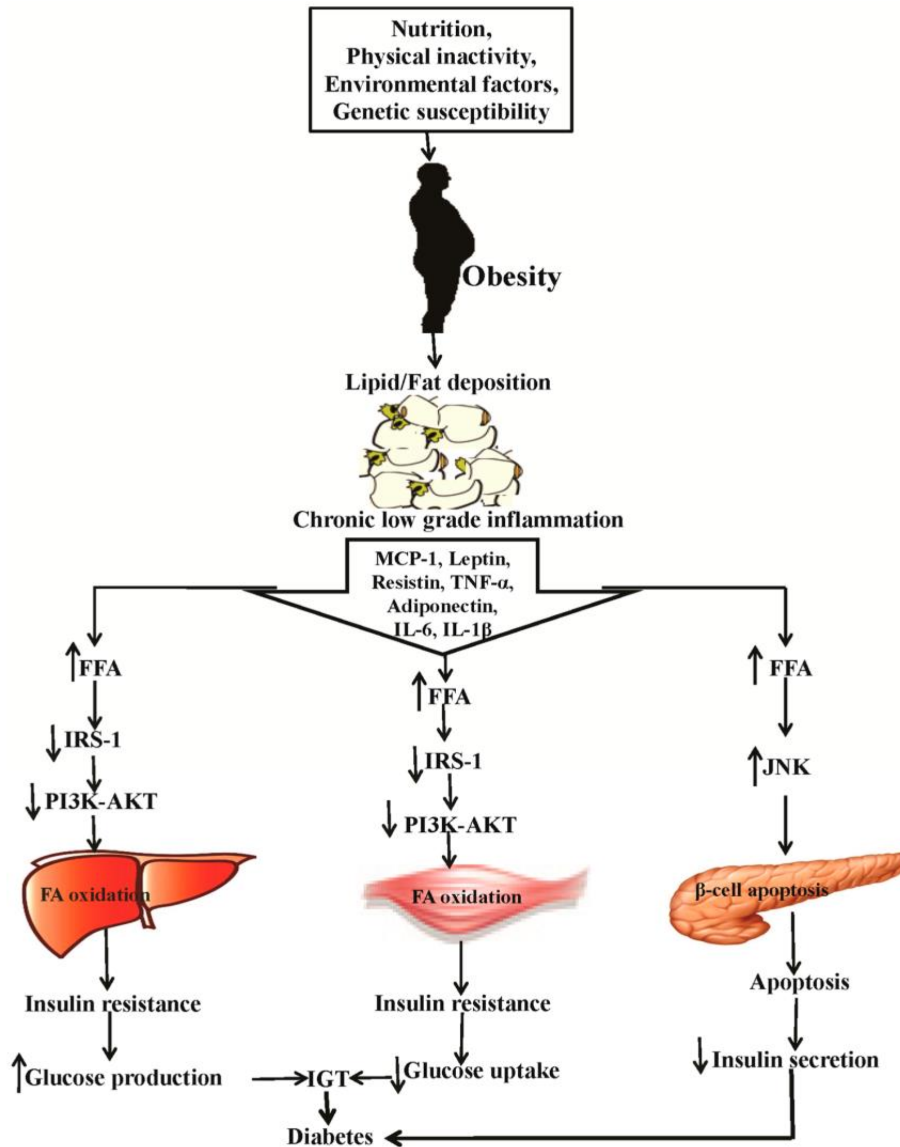


Fig. 12: Schematic diagram of the link between obesity and diabetes as well as their effects in skeletal muscles, liver, and pancreas to stimulate different inflammatory cytokines, metabolic enzymes and signalling pathways (adapted from Kawser *et al*., 2006).

Nutrition, physical inactivity, environmental factors and genetic susceptibility cause obesity, and this condition leads to chronic low-grade inflammation resulting in increased secretion of MCP-1, leptin, resistin, TNF-α, Adiponectin, IL-6, and IL-1 β. Chronic inflammation results in increased secretion of FFA from the liver, skeletal muscles, and pancreas. Increase in FFA levels also leads to a reduction in IRS-1 and PI3K-Akt expression in the liver and skeletal muscles, and increased JNK expression in the pancreas, leading to hepatic and muscular insulin resistance and pancreatic apoptosis. Insulin resistance also causes an increase of glucose production and a decrease of its absorption resulting in reduced insulin secretion due to increased apoptosis in pancreatic β cells. MCP-1: monocytes-chemo-attractive protein-1; TNF-α: tumour necrosis factor α; IL-6: Interleukin-6; IL-1 β: interleukin 1 β; FFA: free fatty acid; IRS-1: insulin receptor substrate 1; PI3K: Phosphatidyinositol 3-kinase; Akt: serine/threonine kinase; JNK: C-Jun N-terminal kinase; FA: fatty acid; IGT: Reduced glucose tolerance. (↓) decrease, (↑) increase.

Since macrophages are not only the main source but also inflammatory mediators target, they are supposed to be central players in a vicious circle that causes inflammation and insulin resistance.

1.4 Insulin resistance in adipose tissue

Insulin resistance is a condition which determines a reduction of cells sensitivity to insulin activity, resulting in lower biological effect of this hormone and drastic alterations in homeostasis. Insulin resistance is manifested by an initial impaired glucose tolerance that may eventually turn into type 2 diabetes, due to insulin inability to compensate hyperglycemia values (Reaven *et al.*, 2000). Insulin resistance genesis is multifactorial: it involves both genetic and environmental factors. The genetic component has been demonstrated by the fact that insulin resistant subjects have positive familiarity for T2DM (Wilcox, 2005). A single specific responsible gene has not been identified yet, but mutations on different genes, in particular those of glucose carriers, receptor and insulin gene, mitochondrial DNA and TNF-α have been studied. For this reason, insulin resistance is considered a polygenic pathology (Olefsky *et al.,* 2010). The association of metabolic syndrome with obesity, insulin resistance and chronic low-grade inflammation has been evident for several years. However, although many of the molecular and cellular mechanisms that diminish insulin sensitivity in obese patients and related to the inflammatory condition, have recently been clarified, the pathogenesis of obesity-induced insulin resistance is still obscure in many respects.

1.4.1 Metabolic effects of insulin

Insulin is a peptide hormone synthesized by the β cells of the pancreas, inside which it is stored and released following glucose increase in the bloodstream. It has an important effect in regulating carbohydrates, proteins, and fats metabolism. Insulin reduces glucose, fatty acids and amino acids plasma levels and promotes their storage. When these nutrients come in the bloodstream, insulin promotes their cellular uptake and conversion, respectively, into glycogen, triglycerides, and proteins. It exerts its main effects both by acting on the transport of specific

plasmatic nutrients in the cells, as well as by acting on the enzymatic activities involved in specific metabolic pathways (Sherwood, 2012). Insulin plays a key role in maintaining energetic metabolism homeostasis and coordinates storage and use of molecules in adipose tissue, liver, and skeletal muscles (**Fig. 13**).

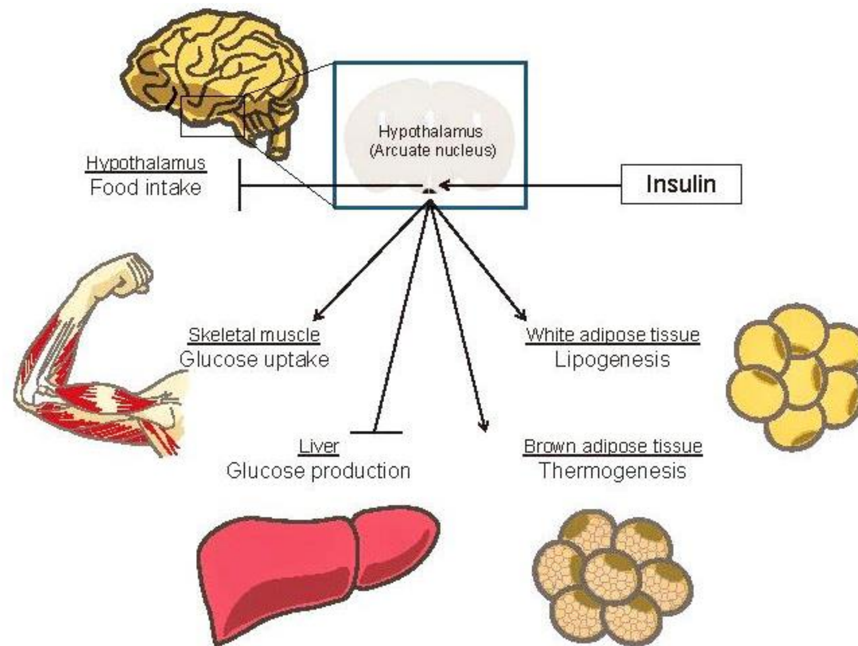


Fig. 13: Metabolic effects of insulin (from Dimitriadis *et al*., 2011).

The maintenance of glycemic homeostasis is one of insulin main functions which, thanks to the recruitment of specific transporters, decreases blood glucose concentration by promoting cells uptake. The glucose transport between blood and cells is carried out by transporters, known as "GLUT", placed in the plasma membrane, which allows the passive facilitated diffusion of glucose through it. There are several isoforms of "GLUT" but certainly the most important are the "glucose-constitutive transporter-GLUT1" and the "insulin sensitive transporter-GLUT4" (Thorens *et al*., 2009).

Insulin exerts multiple effects in order to lower fatty acids blood levels and to promote their storage as triglycerides. It promotes the entry of fatty acids from blood to adipocytes, and increases glucose transport in the adipose tissue by recruiting GLUT-4. Glucose is a precursor of fatty acids and glycerol formation, fundamental elements for triglycerides synthesis. Insulin also inhibits lipolysis by

reducing fatty acids release from adipose tissue into the bloodstream. These functions favour fatty acids removal from the blood and promote their storage in the triglycerides form (Levy *et al.*, 2007). Insulin, therefore, regulates systemic energy homeostasis by coordinating FFA and glucose storage, mobilization, and use in adipose tissue, liver, and skeletal muscles. Insulin resistance development determines important metabolic consequences in these insulin target tissues and it is widely recognized as a fundamental defect that precedes the insulin resistance syndrome development (Franckhauser *et al.*, 2008; Lechleitner *et al.*, 2008). Thus, understanding of insulin mechanism of action in the main target tissues and if the molecular mechanisms that determine insulin resistance development, are particularly important to try to improve insulin sensitivity in the treatment of type 2 diabetes and to its associated complications.

Type 2 diabetes is characterised by high levels of FFA and glucose, and results in a series of abnormalities, such as central obesity, dyslipidemia, increased inflammatory markers, adiponectin reduced plasma levels, damaged fibrinolysis, vascular abnormality and hypertension, associated with an increased risk of cardio and cerebrovascular diseases (Ahmad *et al.*, 2010). The investigation of insulin signal transduction defects in the main insulin-reactive tissues and the consequent systemic insulin resistance development has been facilitated by genetic studies carried out on a model of knock-out mice for the insulin receptor and other insulin signalling pathway components. Recent studies have also identified a variety of factors involved in insulin resistance and type 2 diabetes related to obesity condition (Taylor *et al.*, 2012; Jianping *et al.,* 2013). Many of these factors such as FFA, TNF-α, leptin, IL-6, adiponectin and resistin are secreted by adipose tissue. Recent evidences, concerning the biological and physiological pathways that control insulin resistance, have also shown that adipose tissue is the cornerstone of endocrine, autocrine, paracrine, metabolic and inflammatory signals. Adipose tissue modulates several processes that regulate body homeostasis, inflammatory and immune response, blood coagulation and reproduction (Ruan *et al.,* 2003).

1.4.2 Insulin resistance and signalling pathways

It is necessary to consider the insulin receptor structure and its metabolic activity in order to understand the FFA and other factors role in insulin resistance development (**Fig. 14**).

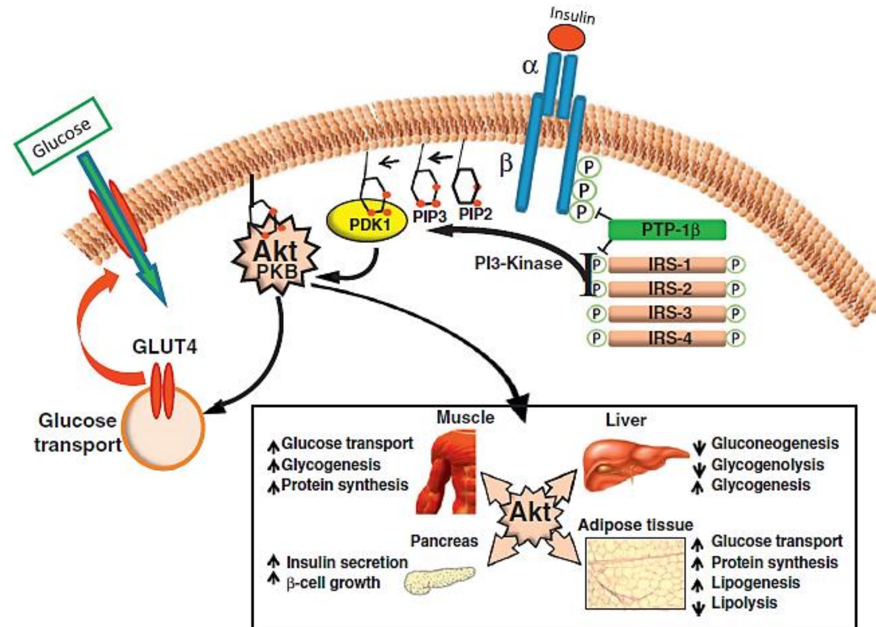


Fig. 14: Insulin signalling pathways (adapted from Capurso and Capurso 2012). When insulin binds to insulin receptor, a cascade self-phosphorylation process is initiated. The IRS-1 and -2 phosphorylation determines phosphatidyl-inositol 3-kinase (PI3K) anchorage and activation which in turn phosphorilate phosphatidyl-inositol 3.4 biphosphate ([PI (3, 4) P2] converting it to phosphatidyl-inositol 3,4,5 triphosphate [PI (3,4,5) P3].These nucleotides act as anchorage sites for some protein kinases, anchoring them to the cellular membrane. The [PI (3,4,5) P3] anchored to the plasmatic membrane binds the PDK-1 kinase (phosphoinositol-dependent kinase-1), which in turn determines the protein-kinase B phosphorylation, also called AKT, and its consequent activation. Activated AKT is responsible for insulin numerous metabolic effects in adipose tissue, muscle tissue, liver, and pancreas.

The insulin receptor (IR) is part of the large "tyrosine kinase" receptor family, and it is an insulin-activated trans-membrane receptor (Ward *et al.*, 2009). IR is a tetrameric protein formed by a pair of two sub-units in an extracellular position, the "α-subunits", and a pair of two sub-units in transmembrane position, the "β-subunits" (Greene *et al.*, 2003) connected by sulfhydryl bridges.

When insulin binds to the insulin receptor, the first substrate to be phosphorylated is the transmembrane "β-subunits", with an autophosphorylation mechanism

(Greene *et al.,* 2003). This preliminary autophosphorylation activates certain anchor sites on the "β-subunits" that bind other substrates, among which the main one is the IRS 1-4 complex. Therefore the insulin extracellular binding to the insulin receptor induces the intracellular binding of the IRS 1-4 complex to the insulin receptor β-subunits in the "src homology 2" (SH2) domains.

By anchoring the IRS complex to the insulin receptor, numerous tyrosine residues of the IRS complex are in turn phosphorylated through a cascade of phosphorylation reaction. Once phosphorylated, IRS-1 and -2 protein complexes is an anchor for other intracellular protein substrates containing the SH2 domain. In this way, the IRS-1 complex becomes an activator of many protein-kinases that have "system signal" functions. Among these protein kinases, the most important is phosphatidylinositol-3-phosphate (PI3K) (**Fig. 14**), which converts phosphatidyl-inositol 3.4 biphosphate [PI (3,4) P2] to phosphatidyl-inositol 3,4,5 triphosphate [PI (3,4,5) P3]. These nucleotides are used to anchor other protein-kinases located downstream of the metabolic cascade on the surface of the plasma membrane. Among them the most important is undoubtedly the AKT kinase; also known as "protein kinase B", that plays a key role in the insulin metabolic effects, such as the translocation of GLUT-1 and GLUT-4 glucose transporters from the cytoplasm to the cellular membrane, glycogen and protein synthesis, and anti-lipolytic effect exerted by insulin on adipose tissue (Shepherd *et al*., 2005) (**Fig. 14**). GLUT-4 translocation on the cellular membrane in insulin-sensitive tissues, and in particular in the striated muscles and adipose tissue, causes a marked increase in glucose uptake by these tissues (Saltiel *et al*., 2001). The insulin receptor, after being activated by insulin and initiating cascade phosphorylation processes, must be rapidly deactivated. The insulin signal activation takes place basically through a tyrosine phosphorylation mechanism of downstream substrates, while the signal deactivation happens through a mechanism that involves the serine site phosphorylation. It is still insulin responsible for deactivating the IRS-1 complex through serine phosphorylation (Greene *et al*., 2003). After activating the IR and the tyrosine-phosphorylative events cascade, insulin determines the translocation on the cellular membrane, and the consequent activation, of a serin-kinase, the theta isoform of the Protein-kinase-C (PKC- Θ), which is known to be negatively associated with cellular insulin sensitivity

(Standaert *et al*., 1999; Leitges *et al.,* 2002). The so activated PKC-Θ (phosphorylated) with an autophosphorylation mechanism, binds to the IRS-1 complex which in turn is phosphorylated in the serine site (serine 307 in rodents and serine 312 in humans) (Sun *et al*., 1999). The serin-phosphorylated IRS complex is thus distinguished from the insulin receptor and it is degraded by the proteasome system (Greene *et al.,* 2003). In this way, the IR is "switched off" and subsequently degraded. Therefore, when the insulin signal starts, the IRS-1 complex is tyrosine-phosphorylated by the insulin receptor. IRS-1 activates the PI3-kinase (PI3K) metabolic cascade; PI3K also provides for the IRS-1 serine 307 phosphorylation, thereby blocking the IRS-1 tyrosine-phosphorylation process operated by IR and the insulin signal propagation (Rui *et al.,* 2001). Despite the insulin signal complexity, there is agreement that the IRS-1 phosphorylation on serine/threonine residues blocks the IRS-1 tyrosine-phosphorylation, thus blocking the cascade signal propagation activated by insulin binding to IR and "extinguishing", in this way, the receptor activity (Greene *et al*., 2003). With the obesity onset, the mechanisms that regulate IR activity are seriously altered by factors that initiate the "shutdown" reactions of receptor activity resulting in IRS-1 serine phosphorylation. Among the various factors involved in this process, circulating FFA excess, TNF-α hypersecretion and alterations in circulating adipokines, appear to play a dominant role (**Fig. 15**).

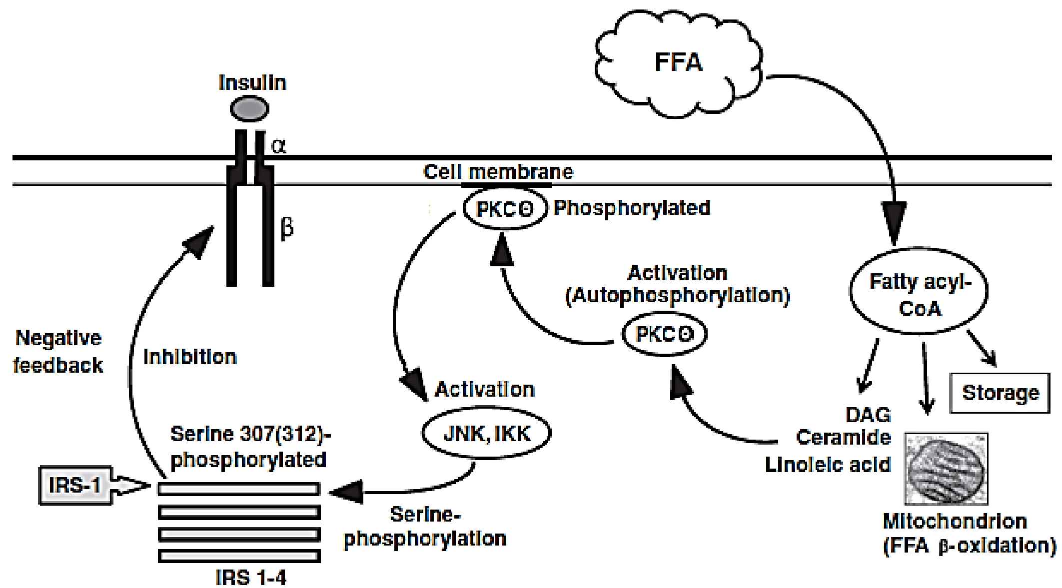


Fig. 15: FFA effect in insulin signal (adapted from Capurso and Capurso 2012). FFA, in their activated form (fatty acyl-CoA) are metabolized through two pathways, oxidation or deposition in adipose tissue. When the FFA flow exceeds the metabolic capacities of these two pathways, as generally happens in obesity, FFA and their intermediate metabolites [linoleic acid, diacylglycerol (DAG), phosphatidic acid (PA), lysophosphatidic acid (LPA), ceramide] accumulate in the cell and activate phosphokinase-C-Theta (PKC-Θ). The phosphorylated PKC-Θ in turn activates two serin-kinase, the c-JUN NH2-terminal kinase (JNK) and the inhibitor kappaB kinase (IKK). These kinases bind to IRS-1 substrate and promote serine phosphorylation that represents a stop signal. This condition causes interruption of insulin signal and thus insulin resistance onset.

1.4.3 FFA and insulin receptor inactivation

It has long been known that FFA excess promotes insulin resistance (Sears *et al.*, 2015). According to numerous studies, FFA would determine insulin resistance with a mechanism of insulin signal inhibition that would be provoked by phosphorylation cascade activation induced by some serin kinases (Fratantonio *et al.*, 2017).

In obesity, there is a FFA hyperinflux in the bloodstream, with an increase in the FFA uptake by muscle tissue, liver and adipose tissue. Activated FFA are normally metabolised through two pathways: oxidation or deposition (**Fig. 15**). However, when the FFA hyperflux exceeds the metabolic capacities of these two pathways, FFA and their metabolites (linoleic acid, diacyl glycerol, phosphatidic acid, lysophosphatidic acid, ceramide) accumulate in the tissues where they determine the activation of some serine-kinases, which in turn, as it has already

been said, blocks the insulin signal. Recent studies have also clarified the FFA role in insulin receptor inactivation and degradation process (Gao *et al.,* 2004).
The FFA, or some of their intermediate metabolites, activate the PKC-Θ serine kinase, which is phosphorylated on threonine 538 residue. Activated PKC-Θ serine kinase triggers a cascade reaction that activates two other serine-kinase, c-Jun NH2-terminal kinase (JNK), main kinase that plays an essential role in regulating glucose metabolism, and the IκB-kinase inhibitor (IKK) (Pal *et al.*, 2016). These serine kinases, being typical mediators of inflammatory signalling pathways, provide an inhibitory crosstalk between inflammation and insulin signalling at the molecular level. They bind to the IRS-1 complex, determining its serine phosphorylation (**Fig. 15**) with consequent functional blockade and insulin resistance onset by blocking the interaction between insulin receptor and IRS-1 complex (Aguirre *et al.*, 2002) and its subsequent degradation (Greene *et al.,* 2003). The inactive insulin receptor is then internalized in the cell and catabolized in the lysosomal compartment. This metabolic pathway occurs in many tissues, particularly in myocytes, hepatocytes, and adipocytes (Gao *et al.,* 2004).
Other important molecular mediators that cause insulin signalling pathway inhibition are the SOCS 1-3 proteins, negative regulators of cytokine signals, and nitric oxide (NO). SOCS proteins are activated during inflammation by different inflammatory cytokines (e.g. IL-6 and TNF-α) and are involved in the IRS-1 and -2 tyrosine phosphorylation inhibition or in their ubiquinitylation and degradation by proteosomes.
SOCS-1 and -3 can promote insulin resistance through the sterol regulatory element binding protein-1c (SREBP-1c) activation (Ueki *et al.*, 2004). Interestingly, it has been recently reported, in a murine model, that SOCS-1 expression protects against systemic inflammation and hepatic insulin resistance in hematopoietic cells (Sachithanandan *et al.*, 2011).
NO is an endogenous molecule produced by inducible nitric oxide synthase (iNOS) that can be activated by different inflammatory cytokines. NO leads to IRS-1 degradation, because iNOS determines the reduction of AKT activity which is the main mediator of the IRS cascade.
There have been identified further alterations able to establish insulin resistance in the effector system. There is a defective action of the glucose transporter system

mediated by GLUT-4 which can be traced back to different mechanisms, such as GLUT-4 intracellular content reduction, its defective translocation on the cellular membrane, or its functional activity reduction (Xu *et al*., 2015).

1.4.4 Metabolic consequences of insulin resistance

One of the main insulin resistance metabolic consequences is hyperglycemia, resulting from insulin inability to suppress hepatic glucose production and to promote glucose absorption and metabolism by peripheral tissues. β-pancreatic cells respond to plasmatic glucose excess by secreting more insulin in order to overcome the insulin resistance effects and to maintain the normal plasma glucose concentration. When insulin resistance progresses and the β cells are no longer able to maintain adequate insulin secretion, plasma glucose levels increase and type 2 diabetes develops (Cerf *et al*., 2013). Skeletal muscles, which determine 80-90% of insulin-stimulated glucose clearance (Kanda *et al.,* 2006), have been implicated as the main insulin resistance site in the context of type 2 diabetes (Inouye *et al*., 2007; Weisberg *et al.,* 2006). Among the most serious insulin resistance metabolic consequences, there is the increase in FFA plasma concentrations, resulting in adipose tissue lipolysis and FFA release. The increase in FFA levels alters the insulin signalling pathway and promotes the FFA oxidation in muscle cells (Todoric *et al*., 2006; Lumeng *et al.,* 2007). This efficiently decreases glucose oxidation and glycogen synthesis and leads to reduced glucose uptake and progressive insulin responsiveness loss in the muscles. Moreover, the plasma FFA excess presence provides an abundant substrate for triglycerides synthesis in the liver and stimulates gluconeogenesis, which results in VLDL increased secretion and hepatic glucose production (Lumeng *et al*., 2007; Clement *et al*., 2004).

Increased plasma FFA concentrations, which are toxic to pancreatic cells, accelerate dysfunction and lead to β-cells death (Cani *et al*., 2007). Therefore, the FFA excess in plasma interferes with insulin actions in the main target insulin tissues and contributes to T2DM and to complications cluster including hyperglycemia and dyslipidemia. Despite the remarkable evidences linking the FFA excess with the insulin systemic resistance induction and progression, the mechanisms by which the plasma FFA levels increase in individuals with T2DM

are less clear. The FFA plasmatic concentrations are in fact determined by the relationship between FFA production and use; in addition, lipolysis in adipocytes and triglyceride-rich lipoproteins are the main sources of plasma FFA. Several studies have also suggested that plasma FFA concentrations are linearly related to FFA release rate by adipose tissue, and this inhibition by insulin effectively suppresses FFA plasma levels (Ebbert *et al*., 2013) (**Fig. 16**).

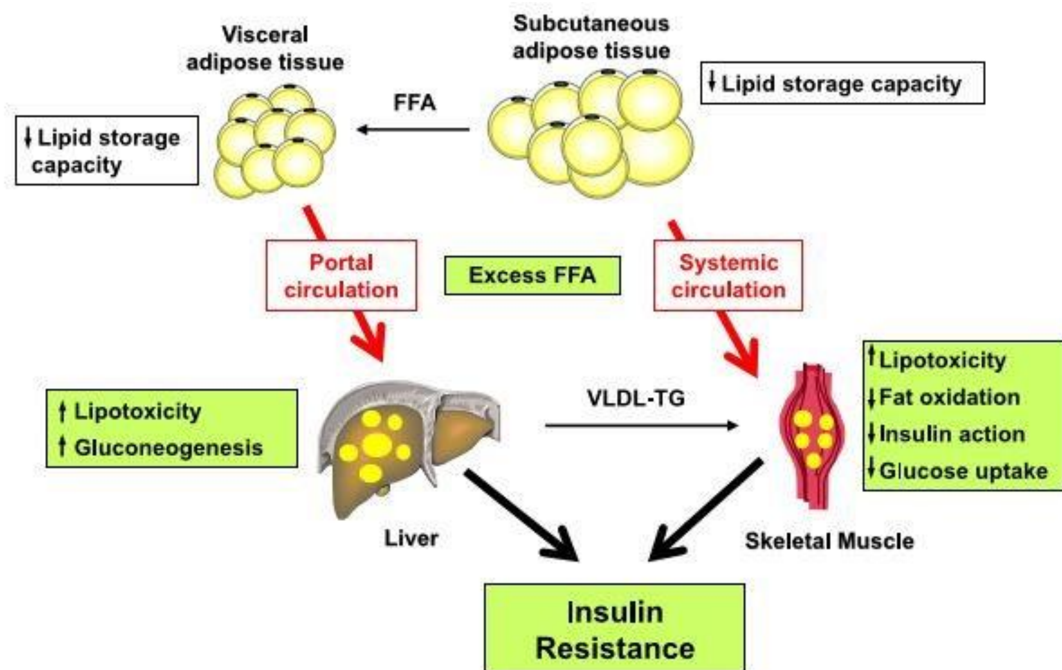


Fig. 16: Consequences of insulin resistance (Galgani *et al.,* 2008).
Altered lipid storage capacity into subcutaneous and visceral adipose tissue leads to ectopic lipid deposition into visceral fat and insulin-sensitive tissues such as liver and skeletal muscles. These tissues progressively develop a state of lipotoxicity, altering insulin signalling and action and contributing to insulin resistance and impaired glucose tolerance.

On the other hand, the increase in the FFA use also results into a decrease in FFA plasma levels. The FFA plasma clearance increases with insulin and fructose administration, presumably due to the increased glycerol-3-phosphate availability, the main substrate for the triglycerides synthesis in the main insulin target tissues. Moreover, the glycerol-3-phosphate acyltransferase or 1-acyl-glycerol-3-phosphate acyltransferase (AGAT) overexpression, which catalyze the first and second steps of glycerol synthesis, increase FFA absorption and their

incorporation into triglycerides (Ruan *et al.*, 2001; Igal *et al.*, 2001). The forced expression of AGAT in 3T3-L1 adipocytes thus suppresses FFA basal release stimulated by isoproterenol and promotes the insulin-stimulated glucose conversion to triglycerides (Ruan *et al.*, 2001). Therefore, elevated FFA plasma concentrations may result both from lipolysis increase and adipogenesis reduction. Thus, adipose tissue is emerging as a primary mediator of systemic insulin resistance because it is an important FFA plasma source. Lipolysis induction in adipocytes by TNF-α and other hormones, such as AdipoQ, may be a significant contributor to the systemic insulin resistance development.

1.4.5 Role of macrophages in the genesis of insulin resistance

The main source of TNF-α in adipose tissue is represented by infiltrating macrophages. In high adiposity condition, factors derived from adipocytes induce macrophage activation and infiltration. These active macrophages initiate a vicious circle, secreting cytokines that can compromise insulin sensitivity in adipocytes and stimulating further activation and infiltration of peripheral monocytes and macrophages in adipose tissue. The communication between adipocytes and macrophages thus appears to be one of the key factors in the systemic insulin resistance onset (Sárvári *et al.*, 2015).

The identification of the factors released by macrophages is therefore necessary to clarify the mechanisms by which there is an alteration of insulin resistance in adipocytes (**Fig. 17**).

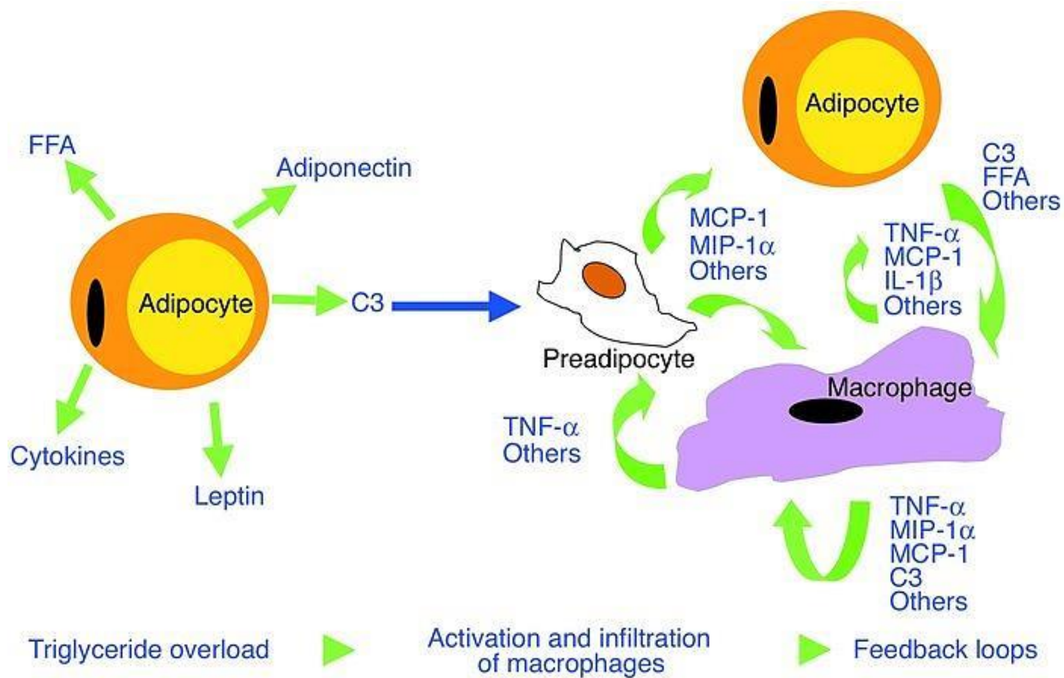


Fig. 17: Hypothetical model of chronic inflammation and insulin resistance in adipocytes (Xu *et al*., 2003).
In obesity, factors derived from adipocytes induce macrophage activation and infiltration. Activated macrophages secrete cytokines that stimulate further activation and infiltration of monocytes and macrophages into fat which can impair adipocyte insulin sensitivity and eventually cause systemic insulin resistance.

Macrophages infiltration into adipose tissue determines NO formation, lipolysis and TLR4 complex activation (Suganami *et al*., 2010; Sun *et al.,* 2011). Macrophages, in particular, play a key role in the onset of obesity-related insulin resistance, compromising insulin-stimulated PI3K/Akt activation (Lauterbach *et al.,* 2017). They express the iNOS, which catalyzes the NO synthesis responsible for the insulin signal alteration. Murine models with iNOS deficiency are protected from diet-induced insulin resistance (Charbonneau *et al*., 2010).

NO role in the crosstalk between macrophages and adipocytes in the insulin signalling regulation is complex. High NO concentrations can inhibit glucose intake, interferring with the GLUT membrane receptors. Recent studies, supported by *in vitro* coculture systems, have shown that, as a result of the interaction between adipocytes and macrophages, alterations of the normal insulin signalling and downregulation of AdipoQ and GLUT-4 gene expression occurs (Olefsky *et al*., 2010; Ieronymaki *et al.,* 2015). Moreover, the high levels of NO synthesized

by iNOS into macrophages reacts very quickly with superoxide free radicals causing DNA damage and strong protein modifications with consequent significant homeostatic alterations.

CHAPTER 2
Anthocyanins

2.1 Introduction

Anthocyanins (AC) are water-soluble pigments, belonging to the flavonoid family (Khoo *et al.,* 2017). They are widely present in various parts of higher-order plants and are mainly found in fruits and inflorescences, but they can also be found on leaves and roots. These substances are widely used as natural dyes giving a typical color, ranging from red-orange to blue, to different foods of vegetable origin. Moreover, interest in these molecules has also increased due to their well-known ability to protect human health when introduced through the diet. The main AC feature is represented by their ability to act as "radical scavenger" and their antioxidant activity (Prior and Wu, 2006; Speciale *et al.*, 2014). These compounds are believed to play an important role in many diseases such as heart disease, obesity and diabetes, and may contribute to brain and visual functions improvement. These substances also posses antibacterial activity and protection against capillary fragility. AC are widely used in therapy because they protect against various cellular aging processes triggered by oxygen, including inflammatory processes (Hou DX. *et al.,* 2003).

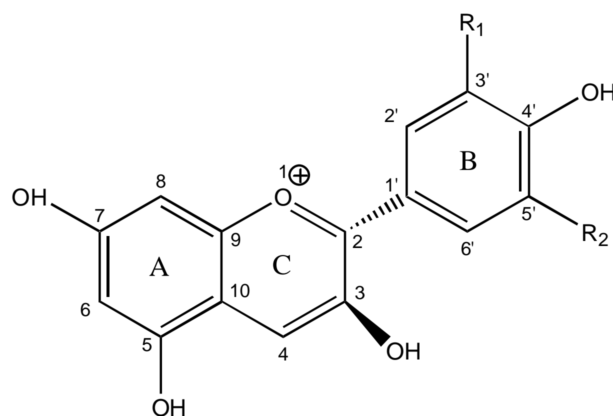
However, the AC beneficial role in different human pathological conditions cannot be explained exclusively on the basis of their antioxidant power. Recently, many studies reported the involvement of other important mechanisms beyond this activity (Domitrovic, 2011; Huang *et al.,* 2013; Speciale *et al.,* 2014). AC seem to be able to modulate different signaling and gene regulation pathways (Nothlings *et al*., 2008; De Pascual Teresa *et al.,* 2010; Cimino *et al*., 2013), thanks to the inhibition of the NF-κB pathway and, in particular, to the activation of NF-E2-related factor-2 (Nrf2), which mediates an adaptive cellular response inducing the expression of various cellular factors, responsible for an ormetic effect (Forman *et al*., 2014).

2.2 Chemical structure of anthocyanins

AC, usually present in nature as glycosides, consist of a sugar and a non-sugar part called aglycone (anthocyanidin), linked by a glycosidic bond. They posses a chemical structure common to all, represented by the 2-phenylbenzopirilium or

flavylium nucleus. It consists of two benzenic rings (A and B) interconnected by a heterocyclic ring (C) containing an oxygen atom (Tanaka *et al.,* 2008).

The structural variations of AC involve the number and position of hydroxyl groups, the methylation of the OH-groups, the nature and number of sugars attached to the aglycone, as well as the nature and number of aliphatic or aromatic acids linked to sugars (Castañeda-Ovando *et al*., 2009). To date, have been described more than 500 different anthocyanins compounds. However only six of these pigments are commonly found in fruits and vegetables: pelargonidine (Pg), Cyanidin (Cy), Delfinidine (Dp), Petunidine (Pt), Peonidine (Pn) and Malvidine (MV) (**Table 1**). Their distribution in plants, commonly consumed in the western diet, is roughly: Cy 50%, Dp 12%, Pg 12%, Pn 12%, Pt 7% and Mv 7% (Laleh *et al.,* 2006; Harborne and Williams, 2000; De Pascual-Teresa *et al.,* 2000).



Flavylium nucleus

Antocyanins	R_1	R_2
Pelargonidine	H	H
Cyanidine	OH	H
Delfinidine	OH	OH
Peonidine	OCH_3	H
Petunidine	OCH_3	OH
Malvidine	OCH_3	OCH_3

Table 1: Chemical structure of anthocyanins (from: *Speciale A, Virgili F., Saija A. Cimino F "Anthochyanins in cardiovascular disease" In: pholyphenolos in human health and dideases first edition, ed. Watson, Preedy, Zibaldi cap.72*)

AC are water-soluble molecules. They are not usually found in their aglycone form but, in the cell vacuole, they are linked to sugars (such as glucose, ramnose, xylose and galactose) more frequently as 3-monoglycosides, 3-diglycosides, 3,5-diglycosides, and 3-diglycosides-5-monoglycosides. These structures provide water stability and solubility (Pati *et al.,* 2009). The non-methylated anthocyanins glycoside derivatives (Cy, Dp, and Pg) are, however, the most common in nature. It has also been found that the frequency of the 3-glucoside derivatives is about 2.5 times higher than 3,5-diglucoside derivatives and, among them, the most common is the cyanidin-3-*O*-glucoside (C3G) (Kong *et al*., 2003).

The AC stability is influenced by several factors such as: pH, storage temperature, chemical structure, concentration, light, oxygen, solvents, presence of enzymes, proteins and metal ions (Khoo *et al.,* 2017). AC show colour variation as a function of pH (**Fig. 18**), since they possess hydroxyl phenolic acid groups and basic oxygen sites. The chromatic tonality of these substances may change according to the different substituents present on the B ring and, in particular, the colour saturation increases with the hydroxyl groups increase and it decreases with the methoxyl groups introduction. In 1939, the Nobel prize Linus Pauling was the first to suggest that the colour intensity exhibited by these pigments is caused by the flavilic ion resonance structure (Wrolstad *et al*., 2005). In acidic conditions (pH 1-3) the stable flavylium cation is the predominant species responsible for the red colouring of these compounds; at pH 4-5 the colourless carbinol is formed; at pH 6-7 the blue-violet quinoidal species are prevalent and at pH 7-8 there is the calcon, which is also practically colourless. In the aqueous phase these four chemical forms are in balance with each other; the prevailing form depends on the pH of the solution (**Fig. 18**) (Faria *et al*., 2013; Torskangerpoll *et al*., 2005).

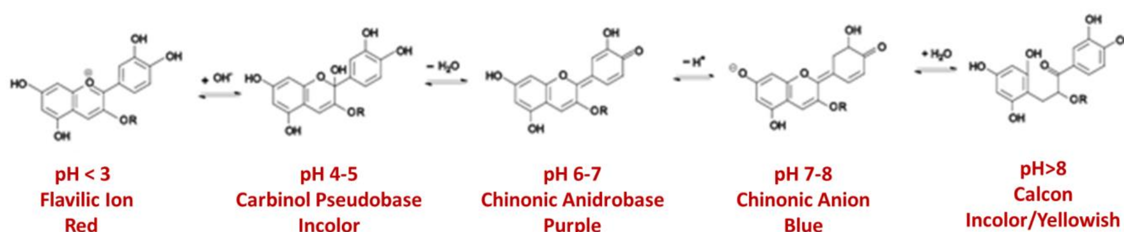


Fig. 18: AC colour variation as a function of pH (Faria *et al*., 2013).

The strong colouring power and the lack of toxicity make these heterosides natural dyes for alimentary use, able to replace the synthetic dyes used in drinks, jams and sweets, even if a certain instability limits sometimes their use. These compounds are found in food of vegetal origin such as cereals, tubers, roots, legumes, fruits and their relative abundance varies with the harvest period. Extensive research has been carried out regarding the AC distribution in nature and the cyanidins, AC presenting two hydroxyl groups on the B ring, were the most abundant, followed by delphinidins (Wu *et al*., 2006; Ogawa *et al*., 2008; De Pascural Teresa *et al*., 2010).

2.3 Anthocyanins consumption through diet

In recent years, the growing interest in AC has been concentrated in their potential beneficial effects on human health, and in the food sector, not only as natural alternatives to synthetic dyes but also as new functional or nutraceutical foods formulations. While it is known that fruits (e.g. berries and grapes), cereals and vegetables, are good AC sources, on the other hand there is considerable individual variability in the daily consumption of these substances (Patras *et al*., 2010; Chun *et al.,* 2007). The AC daily intake is estimated to be between 3 and 215 mg/day (Wang, Stoner, 2008). These numbers are significantly higher than those reported for other food flavonoids such as genistein and quercetin (estimated range: 20-25 mg/day) (Hertog *et al.,* 1993). However, the geographical, seasonal, social and cultural diversity of the populations examined can also explain the wide range of AC consumption (De Pascual-Teresa and Sanchez-Ballesta, 2008; Clifford, 2000; Manach *et al*., 2005). Recent studies of the European Prospective Investigation into Cancer and Nutrition (EPIC) involving 36,037 subjects between 35 and 74 years old distributed on ten European countries, have estimated the intake influence of food containing AC and their derivatives, with the aim to find a potential correlation between AC consumption and different lifestyles and social factors, such as sex, age, BMI, smoking, level of education and physical activity (Zamora-Ros *et al*., 2011). Interestingly, a real intake “gradient" from the south to the north has been observed. Cyanidins and Malvidins are the most used anthocyanins, especially among non-smokers, elder non-obese women, subjects with higher education

levels and those with active or moderate physical activity (Zamora-Ros *et al.,* 2011).

It has also been shown that the plant food consumption rich in AC is more "biologically effective" than an AC extract taken individually, probably due to synergistic effect that is achieved with the other bioactive components contained in them (Titta *et al.,* 2010).

2.4 Anthocyanin bioavailability

Bioactive compounds derived from plant sources must be pharmacologically bioavailable in order to exert their effects. Therefore, the knowledge of their pharmacokinetics is fundamental to understand the real impact of daily AC intake on human health protection (Speciale *et al.,* 2014). High blood AC levels are necessary in order to exert relevant biological effects on target organs and tissues. However, the studies reported in literature showed considerable discrepancies. All this can be explained by the chemical structure differences and by the interindividual differences affecting xenobiotics metabolism. For example, different polymorphisms at phase II biotransformation reactions, differences in the microflora intestinal composition and pH can occur (Miller *et al*, 2011; Lampe, 2007; Iwuchukwu *et al.,* 2009; Del Rio *et al*., 2010). AC are phenolic compounds which, due to their structure, are hydrolysed and subsequently reabsorbed in intestine. They therefore enter in epithelial cells by passive diffusion or thanks to specific carriers (Crozier *et al.,* 2009). Following intestinal uptake, these substances are further metabolized and eliminated in bile and urine with a different concentration according to the agliconic portion structure and the different sugar residue (Ichiyanagi *et al*., 2006). However, these substances are not transported in the bloodstream because the intestinal microflora is able to degrade them in intestine (McGhie and Walton, 2007). AC can be hydrolysed by specific glycosidases present in the gastrointestinal tract producing the aglycons which posses a better bioavailability, but also a lower stability. Nevertheless, studies have reported that, in rats and humans, the glycosidic portion can be uptaken in intact form (Khoo *et al.,* 2017). It was also found that in humans the AC maximum concentration in the blood, after gastric absorption, is reached within 3 h, but the bioavailability is $< 2\%$ because a rapid hepatic removal occurs

(Passamonti *et al.,* 2003; Ichiyanagi *et al.,* 2006; Stoner *et al.,* 2005). Thus, the AC percentage absorption and excretion in the urine is very low compared to the dose taken (McGhie and Walton, 2007). It was hypothesized that they could undergo to a strong first-pass metabolism (Manach *et al.,* 2005; Kroon *et al.,* 2004; Nemeth *et al.,* 2003). Therefore, the apparently low bioavailability of some AC could be due to the extensive presystemic metabolism and not to their reduced absorption from the gastrointestinal lumen. In intestine, the neutral pH and the intestinal microflora can induce AC degradation (Mc Ghie and Walton, 2007). However, there are several factors that can be responsible for the low bioavailability of these compounds. Recent studies have shown that AC have a greater bioavailability than previously supposed, and that their metabolites are still circulating up to 48h after ingestion. The differences in the AC chemical structure, both in terms of aglyonic and sugar portion, clearly influence absorption, metabolism, bioavailability and elimination of these substances (Ichiyanagi *et al*., 2006; Milbury *et al*., 2002; Milbury *et al*., 2010).

In humans, a single oral intake of a Cy-3-glycosides mixture (721 mg) leads to a cumulative serum concentrations of 377 nmol/L per hour for AC and their metabolites, with a maximum concentration of 96 nmol/l at 2.8 hours (Kay *et al*., 2005). Recent studies (Jeon *et al.,* 2012) have analyzed the C3G pharmacokinetics after 2 weeks of black beans (Phaseolus vulgaris) intake. The results obtained have confirmed that a significant amount of C3G is absorbed after ingestion of this extract. In the same way, the intake of 20 g of a BlackBerry extract to healthy volunteers led to a significant increase of AC and their metabolites levels in serum (197-986 nmol/L within 2 hours) and urine (11-13 nmol/L 24 h after administration) (Kay *et al*., 2004). In another study, AC plasma concentrations in patients with coronary heart disease, reached a maximum of 1.5 hours after administration of cranberry juice (480 ml corresponding to 95 mg of AC) with bioavailability range of 0,61-1,82%. (Milbury *et al*., 2010). The amount detected in urine was around 5% of the total dose initially assumed (Felgines *et al.,* 2003; Carkeet *et al*., 2008).

Regarding the AC metabolism, Kay and colleagues demonstrated that the Cy-3-glycosides, when rapidly absorbed, are extensively metabolized in the body to glucuronate and methylated derivatives. While glucuronate derivatives are present

in the circulation up to 5 hours after administration, the methylated derivatives are able to persist from 6 to 24 h (Mazza and Kay, 2008; Kay *et al.,* 2005).

2.5 Biological anthocyanins activities

Despite the reduced bioavailability, plasma AC levels appear to be sufficient to exert their biological activity; it seems that intracellular signalling pathways and gene regulation activities are significantly modulated by AC and their derivatives (Domitrovic, 2011). Recently dr. Kay (Kay *et al*., 2012) and dr. Cassidy (Cassidy *et al*., 2013) groups have evaluated the AC impact, as well as other flavonoids, on diet, cardiovascular function and health, highlighting a key role in comparison with other classes of flavonoids, in the protection against cardiovascular diseases, including hypertension and myocardial infarction. AC play an important role in prevention of these diseases through different mechanisms related to their main biological activities. In addition to cardiovascular protection, many studies have demonstrated an AC active role in anti-inflammatory, antioxidant, detoxifying, anti-proliferative, anti-ageing and anti-tumour activities (Domitrovic, 2011). Flavonoids and AC stimulate the immune system response against pathogens and reduce inflammation modulating cellular mechanisms that cause it: they reduce, for example, cytokines and proinflammatory molecules release, such as NO, by immune system cells (Rossi *et al*., 2003). The antioxidant power of AC also helps to counteract oxidative stress, which is among the main causes of cellular aging, and to reduce the carcinogenic phenomena developing risk, thanks to their antinflammatory activity. In fact, it is known the relationship between chronic inflammation and increased probability of developing cancer (Chen *et al*., 2013; Prasad *et al.,* 2010). In particular the orthodihydroxyphenyl structure present on the B ring seems to be responsible for their important biological properties.

These compounds thus could represent promising drugs that can be used in the treatment of various diseases. They could, in fact, represent a new class of chemotherapeutic or anti-inflammatory drugs, given the recent evidence that show a higher efficacy than some conventional drugs (Domitrovic, 2011). However, most data currently demonstrate the beneficial effect of AC in *in vitro* and/or *in vivo* studies, but not on humans; it appears therefore necessary to carry out several clinical studies to validate the applicability of these bio-active compounds in

humans, given their low stability and bioavailability (Domitrovic, 2011). **Figure 19** shows AC targets, the mechanisms of action through modulation of different signalling pathways and their final effects on health. Briefly they act on skeletal muscles, liver, pancreas and adipose tissue which produce various inflammatory cytokines, metabolic enzymes and signalling pathways that exert anti-inflammatory, antioxidant and metabolic-stabilizing action. These mechanisms are associated with obesity stabilization and diabetes, positive effects on blood pressure and lipid profiles, decrease in atherosclerotic development and improvement of vascular function.

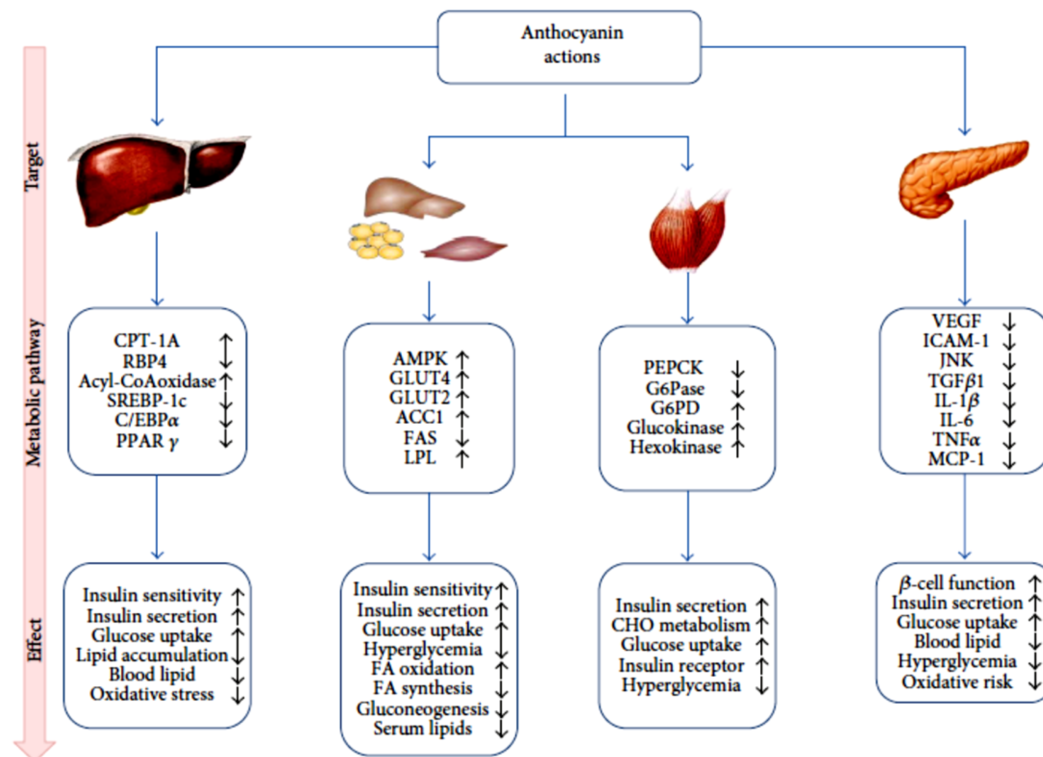


Fig. 19: Biological activities of anthocyanins and their molecular targets (Azzini et al., 2017)

Molecular targets of AC, their mechanisms of action through different pathways, and their final effects on human health. CPT1A: Carnitine palmitoyltransferase-1a; RBP4: Protein binding retinol 4; SREBP1: Sterol regulatory element-binding transcription factor 1; C/ebpα: CCAAT enhancer binding protein-α; PPAR-γ: peroxisomal proliferation receptor-γ; AMPK: Adenosine monophosphate-activated protein kinase; GLUT4: Glucose transporter-4; GLUT2: Glucose transporter-2; ACC1: acetyl-CoA carboxylase; FAS: fatty acid synthase; LPL: Lipoprotein lipase; PEPCK: phosphoenolpyruvate carboxykinase; G6pase: Glucose-6-phosphatase; G6PDH: glucose-6-phosphate dehydrogenase; VEGF: Vascular endothelial growth factor; ICAM-1: intercellular adhesion molecule-1; JNK: C-Jun N-terminal kinase; Tgfβ1: Transforming growth factor β; IL-6: Interleukin-6; TNF-α: tumor necrosis factor alpha; MCP-1: monocyte chemoattractant protein-1.

2.6 Role of anthocyanins in obesity

In recent years, because of the increase in global obesity incidence and the consequent increased prevalence of T2DM, new strategies are under development in order to fight the growing expansion in these metabolic diseases. Among the factors involved in the origin of these pathological conditions, diet seems to cover a central role. In particular, recent studies on the obesity treatment and its complications and co-morbidities have focused on different plant products potential role, such as AC, which can have a positive effect on mechanisms involved in this disease, going to reverse and to prevent pathogenic processes associated with these metabolic conditions. Indeed, several epidemiological studies show that an increase in food rich in anthocyanins consumption, in particular strawberries, blueberries and grapes, greatly reduces hypertension and T2DM risk associated with obesity (Muraki *et al.*, 2013; Cassidy *et al.*, 2011; Khoo *et al.*, 2017).

2.6.1. Modulation of oxidative stress

As suggested by many *in vitro* and *in vivo* studies, AC increase intracellular antioxidant power and, therefore, they are able to protect cells and tissues against oxidative damage (Speciale *et al.*, 2011). In fact, in obesity, chronically elevated insulin levels determine a shift in the cellular redox state resulting in increased ROS generation (Matsuda *et al.*, 2013). Prolonged oxidative stress conditions cause intense homeostatic alterations following glucose uptake impairment in muscle and adipose tissue with a consequent decrease in insulin secretion from pancreatic β cells and an increase in the T2DM incidence (Evans *et al.*, 2005).

For many years, AC have been considered as the most potent antioxidants *in vitro*, defined as "scavengers" compounds able to delay, inhibit or prevent substrates oxidation from free radicals and to decrease oxidative stress (Hu, 2011). Their powerful antioxidant activities have been attributed to their molecular structure. The hydroxyl phenol or acyl groups presence in AC structure determines, in fact, a strong ability to chelate metal ions and to form stable anthocyanin-metal complexes (Sarma *et al.*, 1997).

Recently *in vitro* and *in vivo* studies have shown that human HepG2 cells treatment with C3G, the most abundant anthocyanin in nature, leads to an increase in glutamate-cysteine ligase expression, which in turn mediates a reduction in ROS levels (Zhu *et al*., 2012). The same authors then reported that the C3G uptake (200 mg/kg diet), results in increased glutathione (GSH) synthesis in db/db diabetic mice liver (Zhu *et al.,* 2012). These observations therefore suggest that AC can modulate antioxidant defenses by activating anti-oxidative enzymes and promoting GSH synthesis. In particular has been demonstrated that AC are able to induce Nrf2 transcription factor in many cell types. Nrf2 is a redox-sensitive transcription factor able to binds specific DNA sequences, called Antioxidant Responsive Element (ARE), located in the promoter area of genes encoding many phase II detoxifying or antioxidant enzymes such as glutathione S-transferase (GST), NQO1, HO-1, glutathione peroxidase (GPx), glutamate cysteine ligase (GCL), and peroxiredoxin I (Prx I). These enzymes play key roles in cellular defence by enhancing the removal of cytotoxic electrophiles or ROS (Speciale *et al*., 2011). Induction of these enzymes through the Nrf2/ARE signaling provide an effective mechanism of cellular protection against ROS and inflammatory disorders.

2.6.2 Inflammatory response regulation

The inflammatory condition is a key component of metabolic disorders related to obesity. Altered ROS intracellular levels causes alterations in metabolic responses, with consequent oxidative stress and cellular redox imbalance. Therefore, this condition determines the sensitive redox signal molecules activation, including NF-κB and MAPKs, resulting in higher expression of inflammatory mediators, such as TNF-α, IL -6, IL-8 and MCP-1, which further aggravate the pro-inflammatory state. Chronic inflammation is, in fact, the consequence of a prolonged imbalance between pro- and anti-inflammatory mediators (He *et al.,* 2010; Van de Voorde *et al.,* 2013). Several studies have shown that AC can influence pro- and anti-inflammatory molecules *in vitro*.

IL-6 production was suppressed following the bovine arterial endothelial cells stimulated with peroxynitrite and treated with malvidin-3-glucoside (25 μM) (Paixao *et al*., 2012). Furthermore, it has been shown that, 3T3-L1 differentiated

adipocytes treatment with a purple sweet potato extract (1, 2 and 3 mg/ml) for 24 hours, IL-6 expression decreased in a dose-dependent manner (Ju *et al*., 2011). IL-8 concentrations were significantly decreased in human gastric epithelial cells, infected with Helicobacter pylori, after pretreatment with black soybean anthocyanin extract (cyanidin-3-glucoside [C3G] 72%, delphinidin-3-glucoside 20% and petunidine-3 glucoside 6%) for 24 hours (concentration of anthocyanins > 25 μg/ml) (Kim *et al*., 2012).

NF-κb is a redox-sensitive transcription factor, with a key role in the induction of genes involved in various processes, such as inflammation, immune response, apoptosis, cell development and growth. The main stimuli that activate NF-κB are the redox state alteration (oxidative stress), proinflammatory cytokines (such as TNF-α, IL-6, IL-1 and IFN-γ), hyperglycemia, toxic metals, ultraviolet radiation, alcohol and benzopyrene (Hoesel *et al*., 2013). In turn, NF-κB pathway activation increases the proinflammatory cytokines (such as TNF-α, IL-1α, IL-1β and IL-6), chemokines (IL-8, monocytic chemoattractiveness 3 protein, macrophages 1α inflammatory protein), adhesion molecules (ICAM and VCAM-1), iNOS, cyclooxygenase 2 (COX-2) and cytosolic phospholipase 2 expression (Li *et al*., 2013). Thus, the NF-κB inhibition is a potentially effective way to reduce the inflammatory condition. Numerous studies, carried out *in vitro* on human cell lines, reported that AC, and especially C3G, are able to modulate the NF-κB signalling pathway in several steps (Zhang *et al.,* 2010; Speciale *et al*., 2010). The black soybean anthocyanin extract inhibited the IκB-α phosphorylation and degradation, as well as the NF-κB/p65 nuclear translocation and activation in human gastric epithelial cells infected with H. pylori (Kim *et al*., 2013), and in human keratinocytes (HaCaT) exposed to ultraviolet B (UV-B) (Tsoyi *et al*., 2008).

The C3G, in human endothelial cells exposed to TNF-α, significantly inhibited NF-κB activation but not its nuclear translocation. NF-κB/p65 nuclear translocation was attenuated by blueberry, blackberry or blackcurrant anthocyanin fractions in LPS-activated macrophages and this effect was found to be independent of Nrf2 pathway.

In an *in vitro* model of murine monocytes, AC isolated from cranberries and blackcurrants effectively led to suppression of NF-κB-induced LPS secretion

(Karlsen *et al.,* 2007). The same authors have also observed a down-regulation of inflammatory mediators induced NF-κB, including IL-8, IL-6 and interferon-α.
The pomegranate extract rich in AC inhibited IκB-α phosphorylation, IKK-α activation and nuclear NF-κB translocation in human epidermal keratinocytes exposed to UV-B (Afaq *et al.*, 2005). Examining more closely the cellular mechanisms, Jeong and colleagues have shown that AC act by preventing nuclear NF-κB translocation and by inhibiting the JNK and p38 mitogen-activated protein kinase phosphorylation (Jeong *et al.*, 2013). AC anti-inflammatory properties have also been demonstrated in several *in vivo* studies that support most of the effects described, including the AC ability to reduce inflammatory cytokines expression and concentrations in different tissues, the expression and stress-induced iNOS and COX-2 activity and the ability to influence NF-κB and MAPK pathways. In mice with high fat diet (HFD)-induced obesity, the blueberry powder supplementation (2.7% of total energy) was associated with a reduction of ~ 50% in the dead adipocytes frequency and with inflammatory genes downregulation (TNF-α, IL-6, MCP-1, iNOS, etc.) in adipose tissue (Vendrame *et al.*, 2016).
Further studies on rats fed with a high fructose diet, 10 and 20 mg/kg of AC daily intake, significantly reduced gene expression of inflammatory cytokines such as IL-1β, IL-6 and TNFα, and increased zinc finger protein 36 (ZFP36) gene expression in epididymal adipose tissue (Qin *et al.,* 2012). To clarify however whether this effect was due exclusively to AC or to other polyphenols contained in the extracts, HFD obese and db/db diabetic mice were fed with a pure C3G extract (200 mg/kg diet) for 5 weeks. Data revealed that C3G determined a significant GLUT-4 upregulation and RBP4, MCP-1, IL-6 and TNF-α downregulation in adipose tissue (Sasaki *et al.,* 2007).
In an experiment performed on C57BL/6J male mice, fed with a purple corn-enriched diet containing C3G (2g of C3G for kilogram of diet) for 12 weeks, an upregulation of AdipoQ gene expression in WAT was detected (Tsuda *et al.,* 2004). The administration of an anthocyanin-rich chokeberry extract (100 mg/kg and 200 mg/kg body weight) for 6 weeks in Wistar rats, fed with a high-fructose diet in order to induce insulin resistance, was able to increase plasma adiponectin in a dose-dependent manner and to decrease TNF-α and IL-6 concentrations (Qin *et al.,* 2012). Moreover, from the WAT examination, it was noted that in mice fed

with C3G, cytokine mRNA levels and macrophage infiltration were reduced (Guo *et al.,* 2012).

Some studies have also shown that AC possess anti-inflammatory activity through the COX-2 inhibition and iNOS modulation respectlively in LPS-activated RAW264.7 and J774 murine macrophages (Hou *et al*., 2005; Hämäläinen *et al*., 2007). Some authors have reported the COX-2 inhibition by cyanidin and glucosides present in cherries and berries (Seeram *et al*., 2001), although other authors suggested that the anthocyanidin delphinidine is more potent in COX-2 inhibition, both at mRNA and protein levels, being able to suppress the activation of LPS-stimulated transcription factors, including C/EBP, AP-1 and NF-κB (Hou *et al*., 2005). Another study reported that an AC mixture from wild mulberry and C3G reduced mRNA COX-2 levels. All these observations suggest that AC are able to modulate, both *in vitro* and *in vivo,* the inflammatory responses induced by a variety of stress factors, especially by inhibiting NF-κB pathway and COX-2 activation and attenuating proinflammatory cytokines gene and protein expression.

2.6.3 Insulin signal regulation

As previously mentioned, obesity is strongly associated with insulin resistance and the improvement of this condition is important to prevent T2DM development. The AC efficacy, in the prevention of this pathological condition, has been demonstrated in different animal models. Treatment of HFD mice with 1g/kg of AC from cherries, resulted in decreased weight gain and improved glucose tolerance compared to untreated HFD-fed controls (Jayaprakasam *et al*., 2006). A recent study in rats, fed with a fructose-rich diet, has compared the effects of treatment with AC from a black rice extract (5 g/kg diet), with the pioglitazone (270 mg/kg diet), an insulin-sensitizing drug and has found that both treatments were able to improve glucose intolerance and hyperlipidemia. However, only pioglitazone was able to reverse fructose-induced hyperinsulinemia (Guo *et al*., 2007). In another study, in which murine models of obesity induced by HFD and genetically diabetic mice db/db had been subjected to C3G intake (2 g/kg diet) for 5 weeks, it was found that the C3G was able to lower fasting glucose levels resulting in insulin sensitivity improvement compared

to non-integrated controls (Guo *et al.,* 2012). In a study performed on KK-Ay diabetic mice, C3G treatment (2g/kg) showed up-regulate significantly GLUT-4 and down-regulate RBP4 in WAT, thereby improving hyperglycemia and insulin resistance (Sasaki *et al*., 2007). A severe metabolic defect associated with insulin resistance is failure of the peripheral tissues to properly use glucose, resulting in chronic hyperglycemia. GLUT-4 is the primary insulin-dependent glucose transporter and is present predominantly in skeletal muscles, myocardium and adipose tissue. Some studies have shown that AC exert their hypoglycemic effects by promoting GLUT-4-dependent glucose absorption in peripheral cells and tissues.

Nizamutdinova and colleagues reported that the black soy anthocyanins intake in diabetic rats increases GLUT-4 translocation to cardiac and skeletal muscles tissues, resulting in reduced fasting glucose levels and improved cardiac hemodynamic function (Nizamutdinova *et al.,* 2009). Similarly, in an *in vitro* experimental model of L6 myotubes, C3G treatment significantly increased GLUT-4 expression protein in the cell membrane without affecting protein expression, thus demonstrating a increased GLUT-4 localization in the cytoplasmic membrane (Kurimoto *et al*., 2013).

In an experimental model of insulin resistance on 3T3-L1 adipocytes exposed to H_2O_2 or TNF-α, C3G showed a reduction in intracellular ROS levels and increased insulin-stimulated glucose uptake in a dose-dependent manner. This therefore confirms that the AC use could be a crucial target for preventing insulin resistance (Guo *et al*., 2008). Tsuda and colleagues also showed that, following the C3G treatment of primary human and murine adipocytes, there was an increase in adiponectin and leptin levels (Tsuda *et al*., 2004 and 2006). Recently, some studies have focused on the PPAR-γ which plays an essential role in lipid and glucose metabolism and in the transcription of proteins involved in glucose and fatty acids absorption (Floyd *et al*., 2012). To examine the mechanism underlying the C3G insulin-sensitizing effect, Scazzocchio and colleagues evaluated the PPAR gene expression in murine adipocytes. The C3G has thus shown to mediate the glucose absorption by a greater GLUT-4 translocation in the cell membrane and a higher adiponectin secretion, also following an increase in PPAR activity (Scazzocchio *et al*., 2011).

AMPK plays a key role in lipid metabolism, glucose homeostasis and insulin sensitivity regulation. High glucose levels can inhibit the AMPK phosphorylation and activity, compromising its downstream signalling pathway resulting in glycolysis and lipolisis that worse diabetic disease (Ruderman *et al.,* 2013). Several studies have suggested that AC affect metabolic homeostasis by acting on AMPK. The AMPK activation has resulted in glucose use increase and gluconeogenesis suppression in response to insulin (Kurimoto *et al*., 2013). *In vitro* data showed that incubation with C3G increased the AMPK activity and provided significant protection against glucose-induced lipolysis in totally differentiated 3T3-L1 adipocytes (Guo *et al.,* 2012).
In addition, AC treatment of HepG2 hepatocytes resulted in increased AMPK and acetyl-coenzyme A carboxylase (ACC) phosphorylation levels, leading to an increase in carnitine palmitoyl transferase 1 (CPT-1) expression and in fatty acids oxidation (Guo *et al*., 2012; Hwang *et al.,* 2013). These observations therefore suggest that AC can improve diabetes-associated diseases by activating AMPK/ with AMPK activation.

2.7 Results obtained with anthocyanins according to my research experience

During my laboratory experience at UniMe, I had the opportunity to study in deep the biological activities of AC using of different cellular models *in vitro* and I was able to confirm how these compunds play an essential role in different pathological conditions prevention.

2.7.1 Effects of AC on insulin resistance in endothelial cells

During my period at UniMe, I tried to demonstrate the molecular mechanisms involved in the protective effects of C3G on palmitate-induced endothelial dysfunction and insulin resistance in human umbilical vein endothelial cells (HUVECs). It has been demonstrated that elevated free fatty acids (FFAs) plasma levels, including PA, promote lipotoxicity and cause oxidative stress, inflammation, and insulin resistance in endothelium (Fratantonio *et al.,* 2017).

Endothelial dysfunction is an alteration of the normal organ function that involves the loss of some structural and functional endothelium characteristics such as the regulation of the vessel tone and the acquisition of proinflammatory (through increased chemotactic factors and cytokines such as TNF-α, IL-8 and IL-6) or proaterogenic properties (through expression of cellular adhesion molecules and vasoconstrictive peptides such as endothelin-1 (ET-1) (Itani *et al*., 2002; Libby *et al*., 2007). Insulin, a part from playing a key role in the glucose and lipid homeostasis in adipose tissue, skeletal muscles and liver, exerts important beneficial effects on the endothelial function maintenance. It acts through the binding to its receptor present on endothelial cells and through intracellular protein IRS-1 activation, which triggers the initiation of cascade reactions culminating in the NO release. Under insulin resistant conditions, endothelial dysfunction is characterized thus by insulin-mediated vasodilator NO loss production, increased ET-1 and PAI-1, with consequently development of prothrombotic state (Potenza *et al*., 2005).

Lipotoxicity conditions and inflammation, which contribute to the insulin-resistance onset, also affect multiple independent and interdependent mechanisms in endothelium, interfering on the physiological insulin signal pathways.

In my project I have evaluated, in the presence of a lipotoxicity condition induced in endothelial cells with PA, the C3G effect on the main signalling pathway (IRS-1/PI3K/Akt/eNOS) activated by insulin and on the levels of NO and ET-1 in HUVECs. Pathway-specific damage in PI3K-dependent insulin signalling contributes to the relationships between insulin resistance and endothelial dysfunction that in turn promote the clustering of metabolic and cardiovascular diseases in insulin-resistant state.

For this aim data obtained with HUVECs treated for 24h with C3G 20 μM, exposed for 3 h to PA 100 μM and then with insulin 100nM or not for 15 min, confirmed that, in PA-exposed cells, insulin resistance was induced by the specific impairment of insulin IRS-1/PI3K/Akt signalling pathway and the downstream reduction of endothelial nitric oxide synthase (eNOS) and then of NO release. We observed that these effects were exerted by changes on the phosphorylation of IRS-1 on specific serine and tyrosine residues. HUVECs exposure to PA is associated in fact to an increased IRS-1 serine phosphorylation

with consequent negative feedback control of insulin signalling. Insulin-induced IRS-1 tyrosine phosphorylation was instead negatively affected by PA exposure. Furthermore, PA increased vasocostrictor ET-1 mRNA expression in HUVECs contributing to insulin resistance by impairing insulin hemodynamic effects. Interestingly, C3G pretreatment effectively reversed the effects of PA on IRS-1/PI3K/Akt axis. This effect is associated to the inhibition of IRS-1 serine phosphorylation exerted by IKKβ and JNK after PA exposure. In particular, we observed that C3G restored IRS-1 tyrosine phosphorylation, eNOS expression, NO release and ET-1 mRNA levels altered by PA.

Therefore these researches have helped to demonstrate that C3G improve endothelial dysfunction implicated in insulin resistance induced by PA so suggesting new strategies for C3G in the prevention and treatment of vascular pathologies linked to lipotoxicity conditions.

2.7.2 Anti-inflammatory effects of C3G on intestinal epithelial cells

During my period at UniMe laboratory, I followed a project on an *in vitro* model of inflammation on intestinal epithelial cells Caco-2. In particular the aim of the project was to demonstrate the C3G efficacy in the Inflammatory Bowel disease (IBDs) prevention and treatment (Ferrari *et al.,* 2016). IBDs include several pathologies such as ulcerative colitis and Crohn's disease and they are widespread in western countries. The main characteristic of these pathological conditions is the presence of chronic inflammation in the intestinal mucosa, which has an intermittent course and can cause severe complications. The IBD pathogenesis is multifactorial and certainly contributes not only to environmental elements but also to family history and dietary factors (Bernstein *et al.*, 2006).

Currently, the elective IBDs treatment is based on the use of anti-inflammatory drugs, which often involve considerable side effects with a variable therapeutic efficacy from patient to patient. For this reason, the search for new therapies and nutritional strategies, useful in the treatment of these pathologies, is a current and necessary need. For this purpose, the project was oriente to evaluate the C3G anti-inflammatory efficacy in the prevention of these pathological conditions (Domitrovic, 2011).

Our research activity was therefore focused on identifying the potential beneficial effects induced by C3G, with regard to alterations induced by TNF-α in totally differentiated Caco-2, in order to clarify the molecular mechanisms. We choose Caco-2 cells because this is a well established cell line able to differentiate in mature enterocytes. Caco-2 presents the structural, morphological and functional characteristics of the small intestine cells (Pinto *et al.,* 1983; Peters and Roelofs, 1989; Youakin *et al*., 1989; Ferrec *et al*., 2001), and are widely used as an *in vitro* model for intestinal absorption, metabolism, differentiation and toxicity studies (Artursson, 1990; Artursson and Magnusson, 1990; Baker *et al.,* 1995; Meunier *et al.,* 1995; Artursson *et al.,* 2001).

In our experimental model the Caco-2 have been exposed to TNF-α in order to generate structural and functional alterations of the intestinal barrier. Several studies have shown, in fact, that TNF-α plays a central role in intestinal inflammation, as evidenced by the presence of markedly elevated levels of this cytokine in the intestinal tissue and in the serum of patients with Crohn's disease (Ye *et al.* , 2006). In particular, Caco-2 were treated with two different concentrations of C3G (20 and 40 μM) for 24h and subsequently exposed to TNF-α for 3 and 6 hours, in order to reproduce the mechanisms involved in the inflammatory processes pathogenesis. Following interaction with its membrane receptors, TNF-α initiated the inflammatory process by NF-κB pathway activation (Pasparakis, 2009; Vermeulen *et al*., 2003), and stimulation of the ROS production. The focus was therefore on the study of NF-κB activation status and the evaluation of the protein expression of the main components involved in this inflammatory pathway (p65, p-IκB α and p-IKK α/β). Exposure to TNF-α is able to activate the IKKα/β kinase and the subsequent phosphorylation/ubiquitination of IκBα in our experimental model, while pre-treatment with C3G inhibits the phosphorylation (activation) of IKKα/β, induced by TNF-α, thus preventing the IkBα degradation and the NF-κB/p65 nuclear translocation. In addition, the results showed that NF-κB pathway activation, induced by TNF-α, has the effect of increasing both the IL-6 and COX-2 expression and the release of PGE_2 and TXB_2, main mediators involved in the inflammatory cascade. These values are kept at lower levels in the cells pretreated with C3G, demonstrating the protective effect of this anthocyanin.

The Caco-2 exposure to TNF-α also reduces the intracellular levels of GSH and TAA (markers of intracellular redox status) compared to control cells, thus indicating that this cytokine is able to induce an imbalance in the cellular redox state. Pretreatment with C3G (20 and 40 μM), instead, increased intracellular antioxidant capacity in cells exposed to TNF-α, thus showing a protective effect against oxidative damage exerted by TNF-α.

Many phytochemicals, including anthocyanins, are able to exert indirect antioxidant activity modulating the expression of antioxidant enzymes and/or cytoprotective proteins, such as NAD(P)H-quinone reductase-oxide-1 (NQO-1), superoxide dismutase, glutathione S-transferase, glutathione peroxidase, hemeoxygenase (HO-1), catalase, and thioredoxin, which are essential for cell protection (Pan and Ho, 2008; Dinkova-Kostova and Talalay, 2008). Increased expression of such molecules induced by anthocyanins is controlled by Nrf2, a member of NF-E2 family (transcription factors with basic leucine zipper domains).

The function of Nrf2 and its target genes is important for protection against oxidative stress- or chemicalinduced cellular damage. This protein is sequestered in the cytoplasm by Keap1; phase II enzyme inducers and prooxidants can cause its modification and disrupt the Nrf2-Keap1 complex, leading to Nrf2 translocation into the nucleus where it binds to a cis-acting enhancer element known as Antioxidant Responsive Element (ARE) and stimulates gene expression (Lee and Johnson, 2004).

Our results demonstrated that TNF-α does not affect the activation of Nrf2 pathway. Conversely, treatment with 20 and 40 μM C3G alone for 24 h was able to increase Nrf2 translocation, in a dose-dependent manner, and this effect was maintained also in cells treated with TNF-α.

In conclusion our findings show that C3G exhibited protective effects through the inhibition of NF-κB signalling in Caco-2 cells and these beneficial effects appear to be due to its ability to activate cellular protective responses modulated by Nrf2. In fact, previous data supported the hypothesis of a crosstalk between Nrf2 and NF-κB. Moreover, the activation of the antioxidant adaptive response should represent a fundamental molecular target for anthocyanins, as already shown also in previous studies carried out in different in vitro experimental models.

With the IBD progression, cytokine-mediated inflammation and epithelial cell apoptosis lead to intestinal barrier alteration, causing an intense inflammatory response (Jiang et al., 2004), and simultaneously causing increased microvascular endothelial permeability (Oshima et al., 2001). Therefore, we have also developed an *in vitro* experimental model with the aim of studying the crosstalk between activated intestinal epithelium and endothelium through the use of Caco-2 cells and HUVEC endothelial cells coculture (Ferrari *et al.*, 2017).
For this purpose, we evaluated the activation of adhesion molecules as markers of endothelial dysfunction. The results showed that C3G is able to inhibit the NF-κB activation (epithelium-endothelium cross-talk regulator) in Caco-2 exposed to TNF-α and to reduce the adhesion molecules gene expression in HUVEC endothelial cells cocultured with Caco-2.

2.8 Conclusion and Prospects

Increasing evidence thus demonstrates that AC can positively influence obesity and T2DM markers with consequent lower risk of incidence of all those problems known as metabolic syndrome.
Despite the promising results published up to now, their effect is still uncertain. Nowadays, there are still few clinical studies carried out on humans demonstrating a real reduction in the consequences associated with the metabolic syndrome.
Therefore, further large-scale and long-term studies are needed to verify the real obesity and diabetes management and prevention.
In the clinical practice it is necessary to highlight some problems. There are, in fact, discrepancies in the dosages applied in clinical trials compared to those on animals.
Epidemiological studies indicate that the 22-35 mg/day AC intake can be easily associated with less risk of T2DM. However, the subjects examined were exposed to a much higher AC dose (50 – 320 mg/d). Therefore, further tests are necessary to verify the optimal dose necessary to reduce DMT2 risk. Another key issue concerns safety which is particularly important when AC high doses are given for long time. The data obtained in many studies showed that there was no adverse

effect during the entire intervention period. Therefore, these results provide strong support for conventional anthocyanin extracts safety.

However, another issue could help to generate confusion in the field, is the difference between prevention and therapy: prevention implies low doses, but long duration of treatments (years), while the therapy is associated with higher doses (and potentially side effects) but at a shorter time of administration. It is also risky to hypothesize that AC can prevent obesity, based on studies of already obese mice that have received pharmacological doses of these substances. It is therefore necessary to establish new and appropriate cellular and animal models which, in turn, can allow the design of more efficient and prevention-oriented clinical trials.

Results obtained from my previous in vitro experiments confirm the potential role of C3G in the prevention of inflammation and insulin resistance providing clarifications about the possible molecular mechanisms underlying the protective effects of anthocyanins against pathological conditions associated with inflammation and oxidative stress.

PART 2: EXPERIMENTAL

3.1 Introduction

In recent decades, obesity has been considered one of the main health problems, especially in industrialized countries, and its prevalence is constantly and worryingly increasing. It is a metabolic disease of multifactorial origin that develops from the interaction of environmental, metabolic, cellular, and molecular factors, as well as social factors, with a consequent reduced quality of life and high morbidity and mortality rates (Bluer, 2013).

Obesity predisposes to the metabolic syndrome and is characterized by excess adipose tissue, altered levels of circulating proinflammatory adipokines, imbalances of the adaptive immune system, and local and systemic chronic inflammation (McLaughlin *et al.*, 2014). Lipotoxicity, due to an increase in circulating lipids typically present in obesity, determines alteration of tissue metabolism and the establishment of an inflammatory condition, both local and peripheral, which contribute to the development of important diseases, such as cardiovascular diseases (Hotamisligil *et al.,* 2006). In fact, alterations of serum concentrations of the proinflammatory cytokines released from adipose tissue determine a condition that is defined as "chronic low-grade inflammation", which seems to have an important pathophysiological role in the development and progression of complications related to obesity.

The pathological condition frequently associated with obesity is insulin resistance, a reduced ability of insulin to promote the use of glucose, with consequent inadequate response of organs and tissues to the physiological effects of insulin. The relationship between obesity, inflammation, and insulin resistance is based on some endocrine and metabolic functions typical of adipocytes. In fact, it has been observed that the increase in free fatty acids (FFA) levels, typical of obese individuals, induces a state of cellular stress with consequent activation of inflammatory signalling pathways, such as those modulated by the toll like receptors (TLRs), which, through the involvement of specific kinases, lead to the activation of the pro-inflammatory NF-κB pathway. NF-κB is a transcription factor that, once activated, translocates into the nucleus and modulates the expression of genes encoding for pro-inflammatory cytokines, such as IL-6, IL-8, TNF-α, and MCP-1. At the same time, the establishment of an inflammatory condition in the adipose tissue determines a lower production of anti-

inflammatory molecules, such as adiponectin, which plays important insulin-sensitizing effects and vascular protective activities (Martins *et al.,* 2014; Lihn *et al.,* 2005).

Although many of the inflammatory and insulin resistance molecular mechanisms in obese patients have recently been clarified, the pathogenesis of obesity-induced insulin resistance still remains to be elucidated. However, elevated FFA levels in obesity cause an alteration of the insulin signalling pathway (Poynten *et al*., 2005) and represent one of the main markers of insulin resistance in obese subjects (Engin, 2017). In fact, it has been observed that increased FFA levels are associated with alterations in glucose uptake even in obese patients without diabetes, whereas in obese patients with type 2 diabetes, normal plasma FFA levels are correlated to a better sensitivity to the action and effects of insulin (Engin, 2017).

At a molecular level, insulin receptor substrate-1 (IRS-1) protein phosphorylation represents an important step for the insulin signal transduction. In physiological conditions, the binding of insulin to its membrane receptor (IR) triggers the autophosphorylation of tyrosine residues in the activation loop. This in turn activates IRS-1 (via tyrosine phosphorylation) leading to a phosphorylation cascade involving the activation of the PI3K/Akt pathway. Activation of Akt also plays a fundamental role in the metabolic effects of insulin, resulting in an increase in the expression of glucose transporters GLUT-4 and GLUT-1, and in an inhibition of the lipolysis process (Gutiérrez-Rodelo *et al*., 2017). The presence of external stimuli, such as plasma FFA increase, may instead lead to an alteration of specific proteins of the insulin signal pathway, such as the inhibition of IRS-1 phosphorylation, with consequent drastic changes at the homeostatic level.

In recent years there has been a growing interest in many phytochemical compounds, found in common dietary items, with protective activities against various diseases. In the present study, in particular, the anthocyanin cyanidin-3-*O*-glucoside (C3G) was examined. Anthocyanins, a class of flavonoids widely present in coloured fruits and vegetables, are known for their antioxidant and radical scavengers properties, an activity in general shared by all molecules having a phenolic structure characterized by hydroxyl groups and a system of conjugated double bonds (Prior and Wu., 2006). However, recently, several

studies have shown the involvement of other mechanisms of action beyond the antioxidant activity, demonstrating that anthocyanins are able to modulate important cellular signalling pathways exerting protective effects (Nothlings *et al*., 2008; De Pascual- Teresa *et al*., 2010; Cimino *et al.,* 2013). Several epidemiological studies reported that an increase in the consumption of foods rich in anthocyanins, in particular strawberries, blueberries, and grapes, can reduce the risk of hypertension and type 2 diabetes mellitus associated with obesity (Muraki *et al*., 2013; Cassidy *et al*., 2011).

In vitro and *in vivo* studies demonstrated that C3G possesses not only a high antioxidant and anti-inflammatory activity, but also a marked anti-obesity and insulin sensitizing effect (Cassidy *et al.,* 2011). Therefore, the aim of this work was to evaluate the potential beneficial effects of C3G in counteracting inflammaton and insulin resistance induced by high concentrations of palmitic acid (PA) at the adipose tissue level.

At this aim we used several *in vitro* experimental models of murine or human adipocytes. At first we employed a murine preadipocyte cell line (3T3-L1) in a well-established experimental model for studying adipocyte biology, focussing on main molecular pathways involved in adipogenesis (PPAR-γ pathway), inflammatory process (NF-κB pathway), and insulin resistance (IRS-1/PI3K/Akt pathway) in order to study PA lipotoxicity and C3G protective effects.

However, most of the information on the molecular mechanisms altered in obesity is mainly based on *in vitro* studies performed on mouse models. There are strong limitations on these *in vitro* models due to the mouse and human species differences in adipokines protein expression and in adipogenesis process. To confirm the protective effect of C3G in experimental conditions closer to human condition, the same experimental model used for 3T3-L1 was replicated in human SGBS preadipocytes focussing on transcriptional pattern of genes coding for proinflammatory and insulin-regulated proteins.

SGBS cells, derived from the vascular stromal fraction of the subcutaneous adipose tissue of babies with Simpson-Golabi-Behmel syndrome (SGBS), are able to differentiate into mature adipocytes and represent a more physiological model for studies concerning the adipogenesis process (Rosenow *et al.,* 2010). In particular, in order to characterize the effect of PA on inflammatory process and

insulin resistance at a molecular level, and to demonstrate the protective effect of C3G in such conditions, we evaluated the mRNA levels of: 1) the main cytokines modulated by NF-κB (TNF-α, IL-6, IL-8, and MCP-1); 2) GLUT-1, GLUT-4, hexokinase, and adiponectin, as markers of insulin sensitivity.

The data reported in this thesis show that C3G can represent an interesting natural compound able to exert significant protective effects on adipose tissue cells in experimental conditions mimicking a low-grade inflammatory process, similar to that typically present in obese patients.

3.2 Materials and Methods

3.2.1 Reagents

Cyanidin-3-*O*-glucoside (C3G) was supplied from Polyphenols AS, Sandnes, Norway, and was of HPLC grade. Methanol and ethanol were obtained from Carlo Erba Reagent (Milan, Italy) in their highest commercially available purity grade.

The dimethylsulfoxide sterile hybri-max (DMSO), Dulbecco's Modified Eagles Medium (DMEM), Dulbecco's Modified Eagle medium/nutrient mixture F-12 (DMEM/F12), Dulbecco's phosphate-buffered saline solution (DPBS), tripsin-EDTA, HEPES buffer, fetal bovine serum (FBS), bovine calf serum (FCS), L-glutamine, penicillin/streptomycin solution, insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), rosiglitazone, biotin, pantothenic acid, cortisol, triiodothyronine (T3), palmitic acid (PA), sodium salicylate, sodium chloride, Bradford reagent, bovine serum albumin (BSA fraction V), Oil-Red-O, ponceau S, tween 20, dithiothreitol (DTT), hydrochloric acid, sodium hydroxide, SYBR green JumpStart Taq Ready Mix and all other reagents, if not differently specified, were purchased from Sigma Aldrich (Milan, Italy).

Acrylamide/Bis-Acrylamide was bought from Fisher BioReagents.

The nitrocellulose membrane Hybond-P PVDF, the blocking agent (non-fat milk powder) and the ECL plus detection kit system were bought from Amersham Biosciences (Milan, Italy).

The primary antibodies anti-NF-κB p65, anti-PPAR-γ, anti-pIRS ser307 were all bought from Santa Cruz Biotechnology. The antibodies anti- pIKK α/β, anti-pIRS tyr895, anti-pAkt ser473, anti-PI3K, anti-GLUT-1, anti-β-Actin, anti-Lamin B and secondary antibodies HRP-labeled goat anti-rabbit Ig and anti-Mouse IgM were all purchased from Cell Signaling Technology.

The E.Z.N.A. Total RNA Kit was bought from OMEGA bio-tek (VWR). The Quanti-IT RNA assay was purchased from Invitrogen GIBCO (Milan, Italy).

3.2.2 Preparation of albumin-bound palmitic acid

Lipid-containing media was prepared by conjugation of PA to BSA according to the method described by Tripathy and coll. (Tripathy *et al.,* 2003) with slight modifications. Briefly, PA was dissolved in ethanol at 200 mM as stock solution and further diluted, before use, to 4 mM in medium with 10% FFA-free bovine serum albumin (BSA) at 60°C. The mixture was gently agitated to dissolve the fatty acid and pH was finally adjusted to 7.4 with 1N NaOH.

The fatty acid-albumin molar ratio was kept at < 3 to ensure that the fatty acid was bound to albumin.

3.2.3 Cell culture and treatment

3.2.3.1 3T3-L1 cells

The preadipocyte cell line 3T3-L1 was originally developed by clonal expansion from murine Swiss 3T3 cells. Because of its potential to differentiate from fibroblasts to adipocytes, the cell line has widely been used in more than 5.000 published articles to establish the underlying molecular mechanisms of adipogenesis and to evaluate the biochemistry of adipocytes and the potential application of various compounds in obesity treatment (Poulos *et al*., 2010).

One of the main advantages of this cell line is that it is easier to culture and less costly to use than freshly isolated cells, such as mature adipocytes. However, the 3T3-L1 *in vitro* model has the limitation that the adipogenic differentiation takes at least two weeks.

3T3-L1 pre-adipocytes, obtained from the American Tissue Culture Collection (ATCC), were grown in DMEM supplemented, for the first week of culture, with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin/streptomycin solution and, subsequently, with 10% FBS, 4 mM L-glutamine and 100 U/ml penicillin/streptomycin solution. Cells were maintained at 37 °C in a humidified atmosphere with 95% air and 5% CO_2. To prepare 3T3-L1 monolayers, cells were plated at 13×10^4 cells per cm^2 and cultured for 14 days post-confluence to obtain fully differentiated cells.

To convert 3T3-L1 cells from their fibroblastic phenotype to adipocytes, they have been treated after growth arrest for 7 days with the following

prodifferentiative agents: 5 μM insulin, 1 μM DEX, 0.5 mM IBMX, and 2 μM rosiglitazone. Roughly 4 days after adding the agents, cells start to accumulate lipids in the form of lipid droplets that grow in number and size over cultivation time. After this week the medium is further changed and cells are cultured until complete differentiation in medium supplemented with only 5 μM insulin following the scheme in figure 1 (**Fig.1**).

In all experiments cells were used within the 20th passage since we have found that the differentiation efficiency declines rapidly with higher passage number.

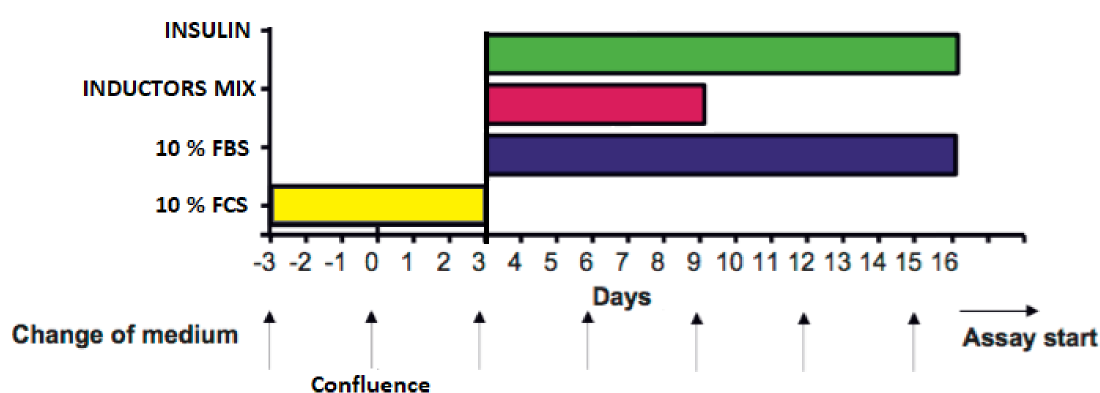


Fig. 1: Differentiation pattern for optimal differentiation of 3T3-L1 cells. Until three days post-confluence cells are maintained in DMEM supplemented with FCS, L-Glut and penicillin/streptomycin solution. Differentiation is started by incubating cells in DMEM supplemented with FBS and the inductor mix (5 μM insulin, 1 μM DEX, 0.5 mM IBMX, and 2 μM rosiglitazone). Subsequently the cells are cultured until complete differentiation in medium with FBS and 5 μM insulin.

For all experiments fully differentiated 3T3-L1 adipocytes were pretreated with different concentrations of C3G (5-10 μM) for 24 h. C3G was always freshly dissolved in DMSO and immediately used. The final concentration of DMSO in the culture medium during the different treatments was < 0.025% (v/v). The control cells were treated only with 0.025% DMSO.

The C3G concentrations used in the present study are consistent and lower with those employed in many other studies concerning the protective effect of anthocyanins in cultured cells and also successfully used in our previous studies (Speciale *et al.*, 2010; Lazzè *et al.*, 2003 and 2004; Russo *et al.*, 2005). These

concentrations are within a range physiologically reachable with a pharmacological intervention (Speciale *et al*., 2014).

After this incubation time, cells were washed with DPBS under sterile conditions and then starved for 24 h in serum-free medium containing 10% BSA in the presence of albumin-bound PA (1 mM), prepared as described above, in order to activate pro-inflammatory pathways. To evaluate insulin resistance condition, cells were subsequently treated with 100 nM insulin for 15 min for protein expression evaluation, or 3 h for estimating gene expression (**Fig. 2**).

Cells treated with C3G vehicle (DMSO 0.025%) and then exposed only to 10 % FFA-free BSA medium (containing ethanol 0.05% v/v), followed by insulin exposure (100 nM insulin) or not (Control) were used as controls. At the end of the exposure time, cells were immediately processed and/or preserved at -80°C until analysis as expected for each test.

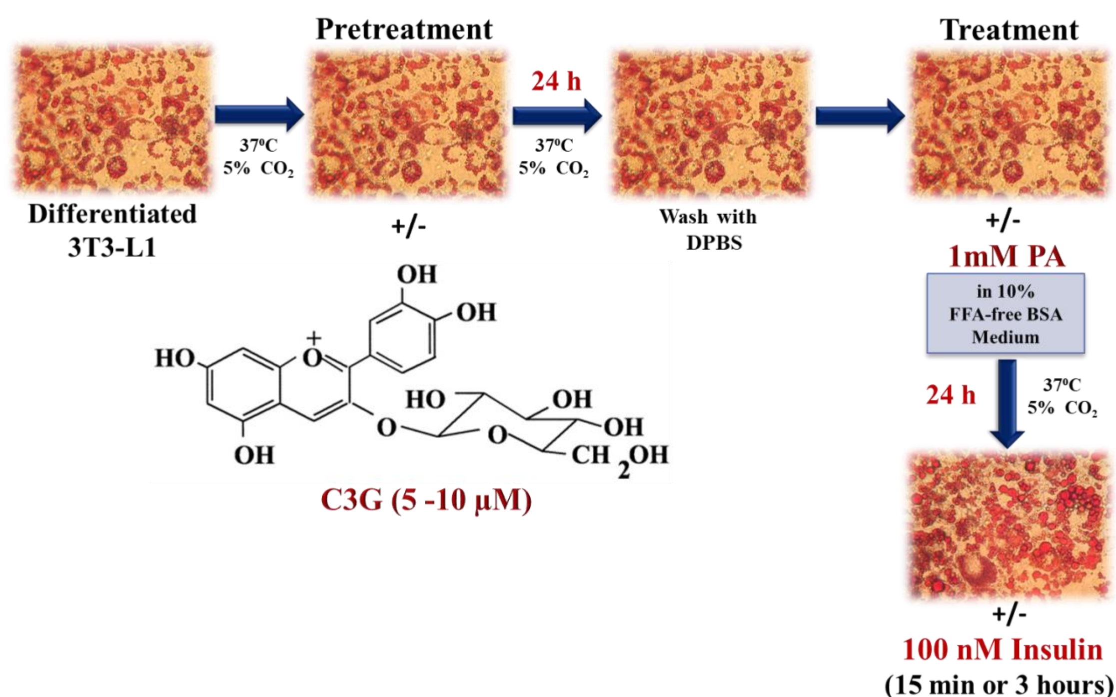


Fig: 2. Pattern of treatments and exposure times of 3T3-L1 cells.

3.2.3.2 SGBS cells

The human Simpson-Golabi-Behmel syndrome (SGBS) cell strain originates from an adipose tissue specimen of a patient with Simpson-Golabi-Behmel syndrome and has been named accordingly. This cell line was kindly donated by Professor Martin Wabitsch (Wabitsch *et al*., 1996, 1997, 2001; Fischer-Posovszky *et al*., 2008). The SGBS syndrome is an X-linked congenital overgrowth syndrome with features that include macroglossia, macrosomia, and renal and skeletal abnormalities, as well as an increased risk of embryonal cancers (Wabitsch *et al*., 2001; Fischer-Posovszky *et al*., 2008). The cell strain is characterized by a high capacity for adipogenic differentiation, and the cells retain their ability for adipogenic differentiation up to generation 50 (Wabitsch *et al*., 2001). Until now, the cells have been used for a number of studies on adipose differentiation, adipocyte glucose uptake, lipolysis, apoptosis, regulation of expression of adipokines, and protein translocation. The cells were plated at 18 x 10^4 per cm^2 and cultured for 14 days postconfluence to obtain fully differentiated cells.

For the differentiation, SGBS cells were grown in DMEM/F12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 33 µM biotin and 17 µM pantothenic acid. After 4 days, medium was changed and cell were cultured for other 7 days in medium DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 33 µM biotin, 17 µM pantothenic acid, 25 nM dexamethasone, 0.5 mM IBMX, 2 µM rosiglitazone, 20 nM insulin, 100 nM cortisol, and 200 pM triiodothyronine (T3). After this step, cells were cultured until complete differentiation in medium supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 33 µM biotin, 17 µM pantothenic acid, 20 nM insulin, 100 nM cortisol, and 200 pM T3 (**Fig. 3**).

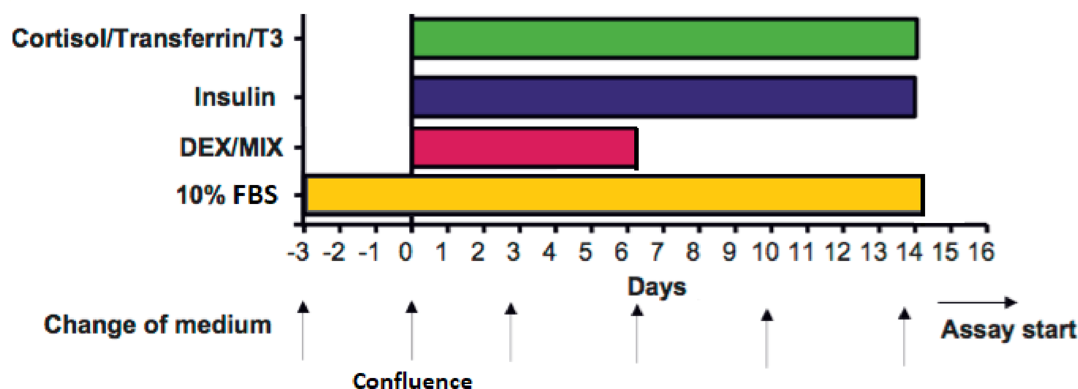


Fig. 3: Differentiation pattern for optimal differentiation of SGBS cells. Cells are always maintained in DMEM supplemented with FBS, L-Glut, penicillin/streptomycin solution, 17 µM pantothenic acid, and 33 µM biotin. At confluence the differentiation is started by incubating cells in DMEM supplemented with the inductor mix (25 nM dexamethasone, 0.5 mM IBMX, 2 µM rosiglitazone, 20 nM insulin, 100 nM cortisol, and 200 pM T3). Subsequently,cells are cultured until complete differentiation in medium with 10% FBS, 33 µM biotin, 17 µM pantothenic acid, 20 nM insulin, 100 nM cortisol, and 200 pM T3.

For all experiments, fully differentiated SGBS adipocytes were pretreated with different concentrations of C3G (1-10-20 µM) for 24 h. C3G was always freshly dissolved in DMSO and immediately used. The final concentration of DMSO in the culture medium during treatments was <0.05% (v/v). The control cells were treated only with 0.05% DMSO.

The C3G concentrations used in the present study are consistent and lower with those employed in many other studies concerning the protective effect of anthocyanins in cultured cells and also successfully used in our previous studies (Speciale *et al.*, 2010; Lazzè *et al.*, 2003 and 2004; Russo *et al.*, 2005). These concentrations are within a range physiologically reachable with a pharmacological intervention (Speciale *et al.*, 2014).

After this incubation time, cells were washed with PBS under sterile conditions and then starved for 24 h in serum-free medium containing 10% BSA in the presence of albumin-bound PA (500 µM), prepared as above described, in order to activate pro-inflammatory pathways. Control cells were not exposed to PA. To

evaluate the insulin resistance condition, cells were subsequently treated with 1 μM insulin for 6 h (**Fig. 4**).

Cells treated with C3G vehicle (DMSO 0.05%) and then exposed only in 10 % FFA-free BSA medium (containing ethanol 0.05 % v/v), followed insulin exposure (100 nM insulin) or not (Control) were used as controls.

At the end of the exposure time, cells were immediately processed and/or preserved at -80°C until analysis as expected for each test.

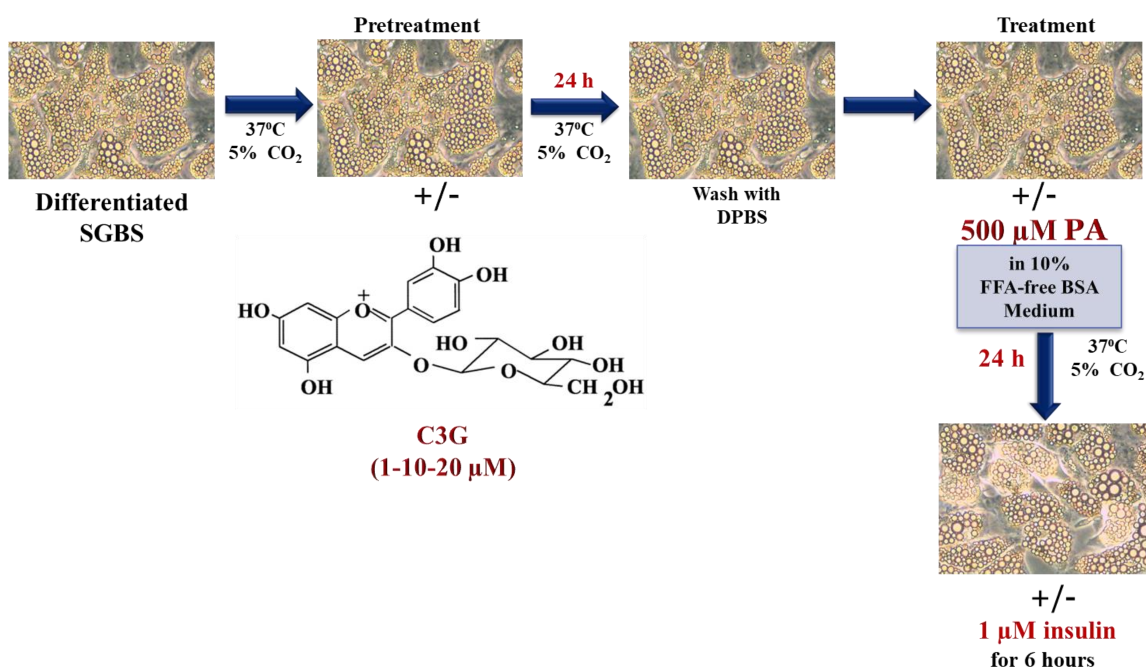


Fig. 4: Pattern of treatments and exposure times of SGBS cells.

3.2.4 Oil Red Assay

Oil Red O staining was performed according to the method described by Ramírez-Zacarías and coworkers (1992) with slight modifications. In brief, an Oil Red O stock solution was prepared by stirring 0.3% Oil Red O (Sigma–Aldrich, Taufkirchen, Germany) in isopropanol overnight. After warming at 37°C to enhance dissolution, the solution was filtered through a 0.45 μm filter with a syringe, and stored at 4°C. Fresh Oil Red O working solution was prepared by mixing stock solution with distilled water (3:2) followed by another filtration. Cells were washed twice with PBS and fixed with 4% formaldehyde (no. 28908, Perbio Science, Bonn, Germany) in PBS for 1 h at room temperature.

Subsequently, cells were washed 2 times with PBS, washed 1 time with 60% isopropanol, and dried. Oil Red O working solution (2 ml/dish) was added for 1 h. Dishes were then washed extensively with distilled water, dried, and photographed.

3.2.5 Evaluation of gene expression

3.2.5.1 RNA extraction

RNA was extracted by using the E.Z.N.A. Total RNA Kit I (OMEGA bio-tek VWR) following the manufacturer's instructions. At the end of the extraction RNA was solubilized in an appropriate volume of nuclease free water. To facilitate the dissolution, the solution was mixed with a micropipette and heated at 55°C for 15 min.

3.2.5.2 RNA integrity

After isolation, total RNA concentration was measured using the Quant-iT TM RNA assay kit with the Qubit^{TM} flourimeter (Invitrogen, Milano). The quality of RNA was evaluated by verifying its integrity by electrophoresis on denaturant agarose gel. The integrity of RNA was evaluated on ribosomal RNA (28S and 18S rRNA), the only double strand RNA detectable with ethidium bromide. Total RNA (3 µg) was taken and diluted with *Loading Buffer* 2X (consisting of deionized formamide, MOPS, formaldehyde, glycerol, bromophenol blue, and water) in a 1:1 ratio. The sample was heated to 65°C for 10 min and then allowed to cool on ice for 5 min.

Then a 1% agarose gel containing 0.66 M formaldehyde was prepared to perform the RNA electrophoretic run. The RNA samples were loaded and run at 100 V for about 1 h. At the end of the run the RNA was verified through a transilluminator to highlight the 28S and 18S rRNA bands.

3.2.5.3 Preparation of cDNA

After verifying the integrity of the RNA, to obtain the corresponding cDNA, reverse transcription was carried out following the manufacturer’s instructions (Sigma, Product Code M1302).

At this aim, the following components were added in an RNase and DNase free tube:

- 1 µL oligo$(dT)_{23}$ primer (final concentration 70 µM)
- mRNA template
- Nuclease-free water up to 12.5 µL of total volume

Then the tube was centrifuged to collect all the components on the bottom of the tube. The sample was heated to 75°C for 5 min to denature the RNA and then allowed to cool on ice for 5 min. At this point the remaining components were added:

- 5 µL 10X M-MLV reverse transcriptase buffer
- 0.5 µL M-MLV reverse transcriptase
- 1.25 µL 10 mM dNTP mix
- 0,5 µL RNase inhibitor (40 units/ml)
- 5.25 µL nuclease-free water

- 25 µL of final volume

The tube was incubated at 42°C for 30 min to activate the enzyme. Then the reaction tube was heated at 95°C for 5 min to denature the M-MLV reverse transcriptase.

3.2.6 Quantitative RT-PCR

3.2.6.1 3T3-L1 experiments

mRNA levels were determined by Real-Time qPCR (Applied Biosystems 7300 Real-Time PGR System, GA, USA) with SYBR green chemistry (SYBR green JumpStart Taq Ready Mix - Sigma). Each cDNA sample (1 µL) was subjected to amplification using primer pairs specific for individual genes (Table 1 and 2). To check the possible contamination of the reagents, a sample containing all the reagents except the cDNA was included (NTC - No Template Control). All real-

time PCR reactions were performed in triplicate and normalized with ROX. The ROX dye is used as a reference. Normal pipetting errors do not affect the determination of the Ct values in real-time PCR instrument using a reference dye. A constant concentration of the reference dye along the plate provides a signal proportional to the real volume in the wells. The instrument software normalizes the differences in volume of the wells by normalizing the signals of the SYBR Green dye with the reference. Tools that do not use a reference dye cannot correct the pipetting errors.

In the PCR tubes the following reagents were added:

- 12.5 µL SYBR Green JumpStart Taq ReadyMix
- 1X ROX
- Forward Primer 0.2 µM
- Reverse Primer 0.2 µM
- H_2O
- 1 µL of cDNA sample previously obtained by reverse transcription

Then the tubes were vortexed and finally centrifuged.

		Primer	Amplicon Leight	Amplicon Tm	Reference
FABP4	Forward	5'-AAG GTG AAG AGC ATC ATA ACC CT-3'	133 bp	61 °C	Zhang *et al.*, 2016 Chen *et al.*, 2017
	Reverse	5'-TCA CGC CTT TCA TAA CAC ATT CC-3'			
IL-6	Forward	5'-GAT GGA TGC TAC CAA ACT GGA T-3'	85 bp	57°C	Tsujimura *et al.*, 2011
	Reverse	5'-CCA GGT AGC TAT GGT ACT CCA GA-3'			
AdipoQ	Forward	5'-TGT TCC TCT TAA TCC TGC CCA-3'	104 bp	55 °C	Zhang *et al.*, 2016
	Reverse	5'-CCA ACC TGC ACA AGT TCC CTT-3'			
18S	Forward	5'-GTA ACC CGT TGA ACC CCA TT-3'	151 bp	55° C	Lood *et al.*, 2016
	Reverse	5'-CCA TCC AAT CGG TAG TAG CG-3'			

Table 1. Sequences of the primers used for PCR analysis in 3T3-L1 cells.

The following parameters were used for amplification.40 cycles each consisting of the following steps:

- Denaturation: 94 ° C for 15 seconds
- Annealing and extension: 60 ° C for 1 minute

Before carrying out 40 cycles requires a step of 2 minutes at 94°C so as to activate the TAQ polymerase.

Finally, at the end of the amplification cycles, a step of dissociation has been carried out, to assess the presence of other amplified products and to determine the melting temperature (Tm) of our amplified product. The Tm was compared with that calculated using the DNA Star program (Madison, USA). The melting temperature, is specific for each amplified product and corresponds to the temperature at which 50% of the DNA is denatured, it depends on the percentage of G and C bases present.

3.2.6.2 SGBS experiments

RNA and DNA were extracted using an affinity column-based method (Machery-Nagel). Reverse transcription was performed using random hexamer primers (Applied Biosystems) and 0.5µg RNA. Polymerase chain reaction (PCR) was conducted in an ABI/Prism-7700 Sequence Detector System using 25ml of reaction mixture containing 1µl of cDNA, 12.5µl of TaqMan Universal PCR Master Mix, 250 nM probes and 900 nM primers (TaqMan; Applied Biosystems).

3.2.6.3 Post-analysis Elaboration

The data were processed using the SDS 1.3.1 software (Apphed Biosystems, Foster City, CA, USA) and expressed as threshold cycle (Ct), which indicates the cycle at which the detected fluorescence exceeds the threshold line. The Ct values for each target gene and for the reference gene were obtained, and then the difference (ΔCt) vas calculated. The efficiency of the primers for the genes tested was corresponding to that of the primers for 18S (reference gene). The purpose of the reference gene is to normalize the PCR for the amount of RNA added in the reactions of reverse transcription. For a valid calculation of ΔΔCt, the amplification efficiency of the target and the reference must be approximately equal. The fold increase in mRNA expression compared with the control cells not treated and not exposed to PA was determined using the $2^{-\Delta\Delta Ct}$ method (Livak *et al.*, 2008). The $2^{-\Delta\Delta Ct}$ method is a convenient method for analyzing the results of gene expression obtained with qPCR experiments. Data analyses of gene expression that used the $2^{-\Delta\Delta Ct}$ method have been reported in the literature.

3.2.7 Western blot analysis

3.2.7.1 Extraction of cellular protein

3.2.7.1.1 Nuclear and cytoplasmic proteins

The preparation of nuclear and cytoplasmic fractions was carried out as follows. After the appropriate treatments, the cells were washed with DPBS, harvested with a scraper, resuspended in a hypotonic buffer (10mM Hepes, 10mM KCl, 1.5 mM $MgCl_2$, 5% glycerol, pH 7.8), containing a cocktail of protease inhibitors (2 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 mM benzamidine) and 1 mM DTT; kept on ice for 10 min, and then 0.65% Igepal (Sigma) for 5 min was added. Following centrifugation at 1300 rpm for 1 min at 4°C, the supernatant containing the cytoplasmic proteins was isolated. The precipitate, containing the nuclei, was lysed in a hypertonic buffer (20 mM Hepes, 400 mM NaCl, 1 mM MgCl2, 0.1 mM EDTA, 1mM EGTA, 10% glycerol, pH 7.8) also containing a cocktail of protease inhibitors (2 µg/mL aprotinine, 1 µg/mL leupeptine, and 1 mM benzamidine), and 1 mM DTT to obtain nuclear proteins.

3.2.7.1.2 Total proteins

At the end of the treatments, the cells were washed with DPBS, harvested with a scraper, and 50 µL of lysis buffer was added. Following sonication for 30 sec, the extracted total proteins were crushed with a thin needle and stored at -20°C. Before quantifying the protein content, samples were centrifuged at 4°C for 10 min at 14000 rpm. All the protein fractions were stored at -80°C until use. Protein concentration in lysates was determined using Bradford reagent.

3.2.7.2 Determination of protein content

The protein content was quantified by the colorimetric assay of Bradford (Bradford, 1976) using bovine serum albumin as a standard. The Bradford assay exploits the change of the absorption peak of the dye Coomassie Brillant Blue when it binds to arginine and to the hydrophobic amino acid residues present in proteins. In anionic form, the linked dye is blue and has an absorbance maximum at 595 nm. In its cationic form, the unbound dye is green and red. Briefly, 5 min after adding the dye to the samples, absorbance was red at 595 nm. The increase in absorbance is proportional to the amount of dye bound to protein. All analyses were performed in triplicate.

3.2.7.3 Immunoblotting

For immunoblot analyses, 40 μg of protein lysates per sample were denatured in 4X SDS-PAGE reducing sample buffer and subjected to SDS-PAGE on 10% acrilamide/bisacrilamide gels. Separated proteins were transferred to nitrocellulose membrane (Hybond-P PVDF, Amersham Bioscience). Residual binding sites on the membrane were blocked with 5% (w/v in TBST- 10 mM Tris, 100 mM NaCl, 0.1% Tween 20) non-fat milk overnight at 4°C. Membranes were then probed with specific primary antibodies: rabbit anti- NF-κB p65 monoclonal antibody (Santa Cruz Biotechnology) (1:1000); rabbit anti-Phospho-IKK α/β (Cell Signaling Technology) (1:1000); mouse anti-β-Actin monoclonal antibody (Cell Signaling Technology) (1:6000), mouse anti-Lamin-B monoclonal antibody (Cell Signaling Technology) (1:1500), rabbit anti pIRSser307 monoclonal antibody (Santa Cruz Biotechnology) (1:1000), rabbit anti pIRStyr895 monoclonal antibody (Cell Signaling Technology) (1:1000), rabbit anti PI3K monoclonal antibody (Cell Signaling Technology) (1:1000), rabbit anti pAkt ser473 monoclonal antibody (Cell Signaling Technology) (1:1000), mouse anti-PPAR-γ monoclonal antibody (Santa Cruz Biotechnology) (1:1500), rabbit anti-GLUT-1 monoclonal antibody (Cell Signaling Technology) (1:1000), followed by peroxidase-conjugated secondary antibody HRP labeled goat anti-rabbit Ig (Cell Signaling Technology) (1:6000), Goat anti-Mouse IgM Secondary Antibody, HRP conjugate (Cell Signaling Technology) (1:10000) and visualized with an ECL plus detection system (Amersham Biosciences). Quantitative analysis was performed by densitometry. The equivalent loading of proteins in each well was confirmed by Ponceau staining and actin control.

Statistical analysis

All the experiments were performed in triplicate and repeated three times. Results are expressed as mean ± SD from three experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD, using the statistical software ezANOVA (http://www.sph.sc.edu/comd/rorden/ezanova/home.html). Differences in groups and treatments were considered significant for $P < 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 STUDIES ON 3T3-L1 ADIPOCYTES EXPOSED TO PALMITIC ACID

3.3.1.1 Protective effect of C3G against PA-induced hypertrophy

Obesity is a condition characterized by an increase of adipose tissue mass in order to store excess fat. Adipocyte hypertrophy (increased cell size) is an adaptive response to excessive nutrient supply, thus protecting other tissues from lipotoxicity. In particular, the expression of key transcription factors in the adipogenesis process, such as LXRα, C/EBPα, PPAR-γ, and SREBP-1c, is involved (Lee *et al*., 2011; Hwang *et al*., 2011). In fact, their activation increases lipids accumulation through the upregulation of lipogenic genes resulting in adipocyte hypertrophy.

In our experimental conditions, adipocyte hypertrophy was induced following exposure of 3T3-L1 cells to high concentrations of PA, as the main circulating saturated fatty acid. The accumulation of intracellular fat was evaluated by the histological technique of Oil Red O staining (**Fig. 5**) (Zebisch *et al*., 2012). The results obtained showed that, as a result of treatment with 1 mM PA for 24 h, there is an increase in the number and size of lipid deposits compared to control cells, while cells pretreatment with C3G at both the tested concentrations (5-10 μM) reduces hypertrophic condition induced by PA in a dose dependent way.

These data are confirmed by several studies on animals and humans that have demonstrated the antiobesity effect of anthocyanins through the decrease of lipogenesis (Xie *et al*., 2017).

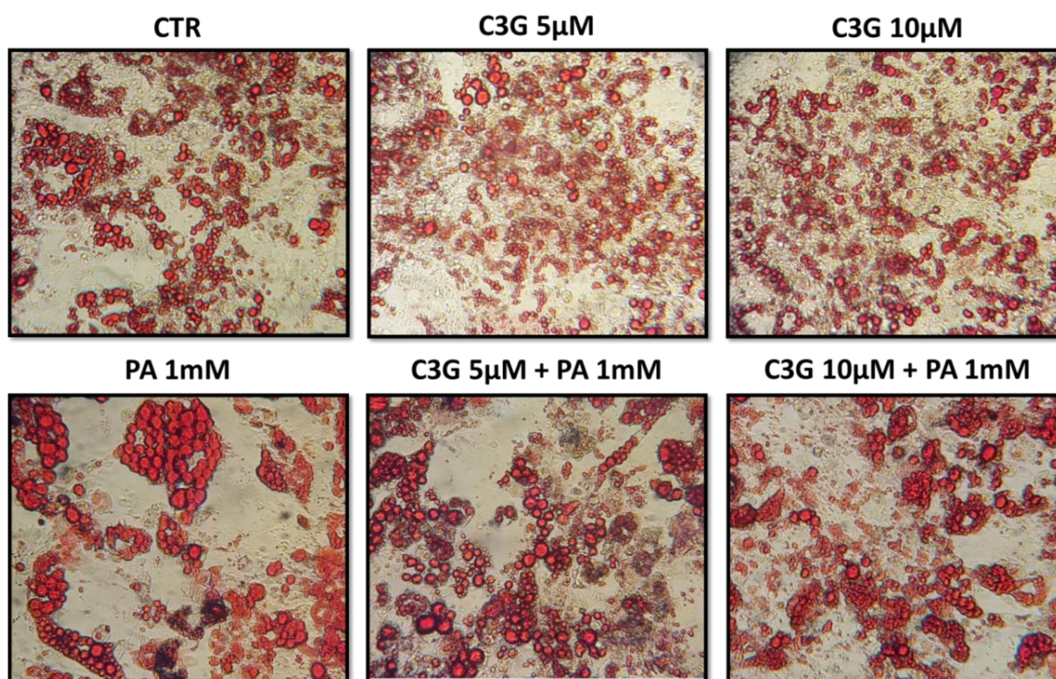


Fig. 5: Protective effect of C3G against PA-induced hypertrophy in 3T3-L1 adipocytes - Oil Red O staining - Totally differentiated 3T3-L1 adipocytes were pretreated or not for 24 h with C3G (5-10 μM) and exposed for 24 h to 1 mM PA. Cells treated with the vehicle alone (0.025% DMSO v/v) were used as controls (CTR). The images are representative of three independent experiments with 40X magnification.

3.3.1.2 Effect of C3G on PPAR-γ expression

In order to explore the mechanisms underlying the effects of PA and C3G on adipogenesis, we have studied, by means of western blot analysis, the expression of the transcription factor PPAR-γ, the main modulator of adipogenesis. PPAR-γ, a protein of the nuclear receptor superfamily, once activated, modulates the expression of genes involved in adipogenesis and lipogenesis through the binding of a specific DNA sequence (PPRE). In fact, some studies have shown how PPAR-γ silencing, in mature 3T3-L1 adipocytes, induces a de-differentiation with a consequent decrease in the expression of typical adipocyte markers (Rosen *et al.,* 2006).

The results obtained show an increase in PPAR-γ levels following exposure for 24 h to 1 mM PA, confirming the activation of the adipogenesis process. Pretreatment for 24 h with C3G (5-10 μM) reduced the expression of PPAR-γ with values below those of cells treated with PA, even if the statistical significance is reached only with the highest concentration. These results therefore indicate that C3G is able to reduce the adipogenic process, and therefore the accumulation of lipids, induced by high PA concentrations through the modulation of the PPAR-γ transcription factor (**Fig. 6**).

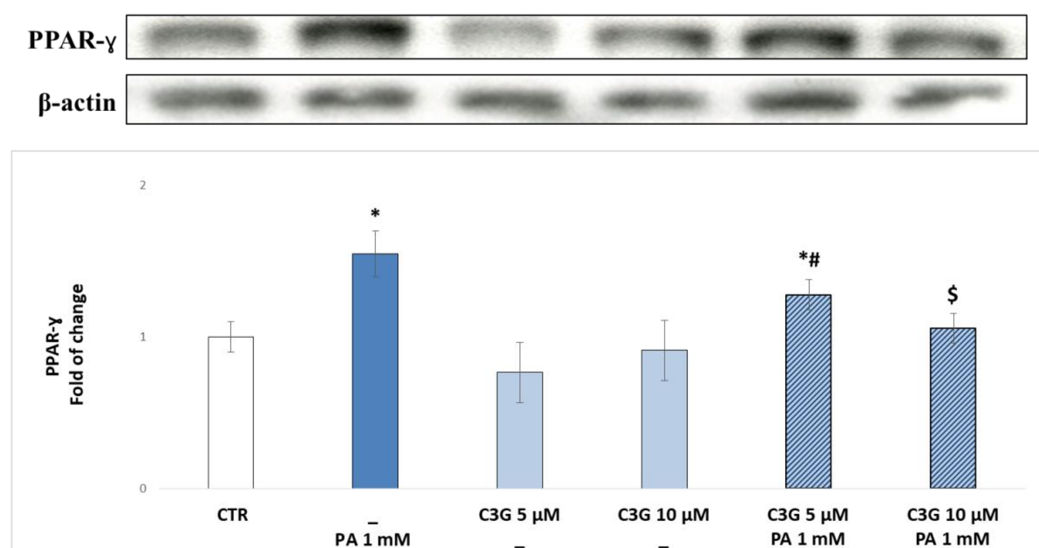


Fig. 6: Effect of C3G on PPAR-γ expression induced by PA - 3T3-L1 cells were pretreated or not with C3G 5-10 μM for 24 h and subsequently exposed to 1 mM PA for further 24 h. Cells treated with the vehicle alone (0.025% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments. The densitometry results are reported as fold change compared to the controls, expressed as mean ± SD of three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. The intensity values of the PPAR-γ protein were normalized to the corresponding value of β-actin. *$p < 0.05$ vs CTR; $^{\$}p < 0.05$ vs PA 1 mM; $^{\#}p < 0.05$ vs C3G 5μM.

3.3.1.3 Effect of C3G on FABP4 gene expression

The fatty acid binding protein 4 (FABP4) is a cytosolic protein widely expressed during the differentiation process from preadipocytes to adipocytes. This protein has been identified as a key regulator of fatty acid uptake and lipid accumulation during the adipogenic process. Furthermore, a correlation between the increase in this protein and certain disorders of lipid metabolism, diabetes, and obesity has been demonstrated (Poulos *et al*., 2010). The expression of this protein is induced by the activation of PPAR-γ, thus making FABP4 a good marker of adipogenesis.

To confirm the increase in the hypertrophic condition of murine 3T3-L1 adipocytes following PA exposure, FABP4 gene expression was then evaluated by real-time PCR. The data obtained showed a strong increase in mRNA levels of FABP4 following treatment with PA. In contrast, these levels were significantly reduced in adipocytes pretreated with C3G. These results confirm the ability of C3G to inhibit adipocytes hypertrophy induced by high PA concentrations, modulating the signaling pathway of PPAR-γ and thus protecting cells from an excessive accumulation of fatty acids (**Fig. 7**).

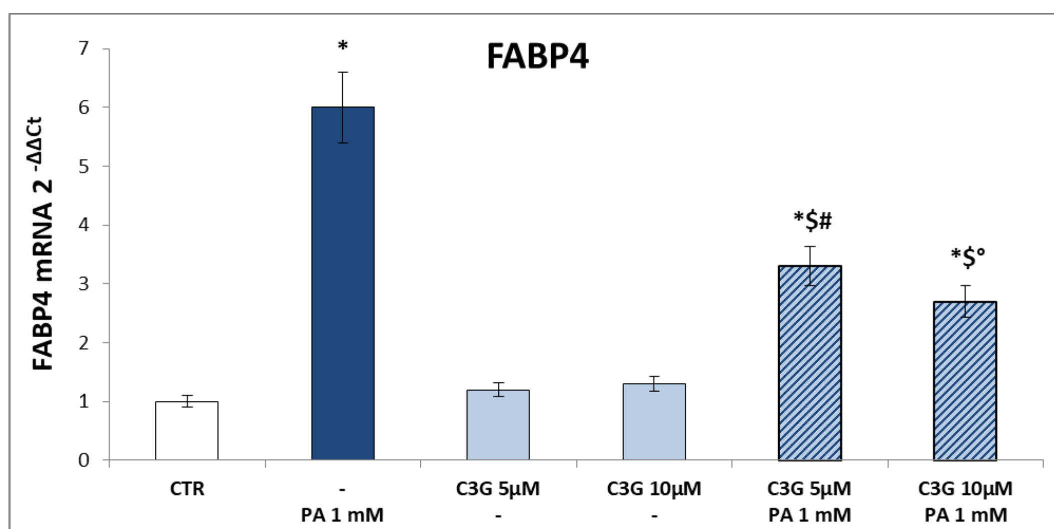


Fig. 7: Effect of C3G on PA-induced FABP4 gene expression. 3T3-L1 cells were pretreated or not with C3G for 24 h and subsequently exposed to 1 mM PA for 24 h. Cells treated with the vehicle alone (0.025% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. FAPB4 mRNA expression was analysed by real-time PCR and data are expressed as $2^{-\Delta\Delta Ct}$ and normalized to CTR. 18S rRNA was used as housekeeping gene. *p <0.05 vs CTR; $^{\$}$p <0.05 vs PA 1mM; $^{\#}$p <0.05 vs C3G 5µM; °p <0.05 vs C3G 10µM.

3.3.1.4 Protective effect of C3G on PA-induced NF-κB *pathway*

Obesity is a pathological condition characterized by chronic low-grade inflammation in adipose tissue. It is related to the chronic activation of different pro-inflammatory cell signaling pathways. The hypertrophic growth of adipocytes, typical in obesity, causes alteration of cells functionality resulting in the release of inflammatory adipokines, oxidative stress, infiltration, and activation of macrophages, that strongly reinforce the inflammatory process. Furthermore, it has been demonstrated that exposure of adipocytes to high concentrations of fatty acids activates toll-like receptors (TLRs), such as TLR2 and TLR4, which induce a series of kinases leading to translocation of the κB factor (NF-κB) into the nucleus.

NF-κB is the most important transcription factor involved in the activation of the inflammatory process (Hayden *et al.,* 2014). It is a ubiquitous factor that resides in the cytoplasm of unstimulated cells in the form of an inactive complex, associated with inhibitory IκB protein. Following various proinflammatory stimuli, such as TNF-α, LPS, IL-1, and other cytokines, NF-κB is activated, dissociates from its inhibitor and migrates into the nucleus where it promotes the transcription of several genes encoding for important pro-inflammatory mediators such as TNF-α, MCP-1, COX-2, and IL-6 (Sultana *et al.,* 2016).

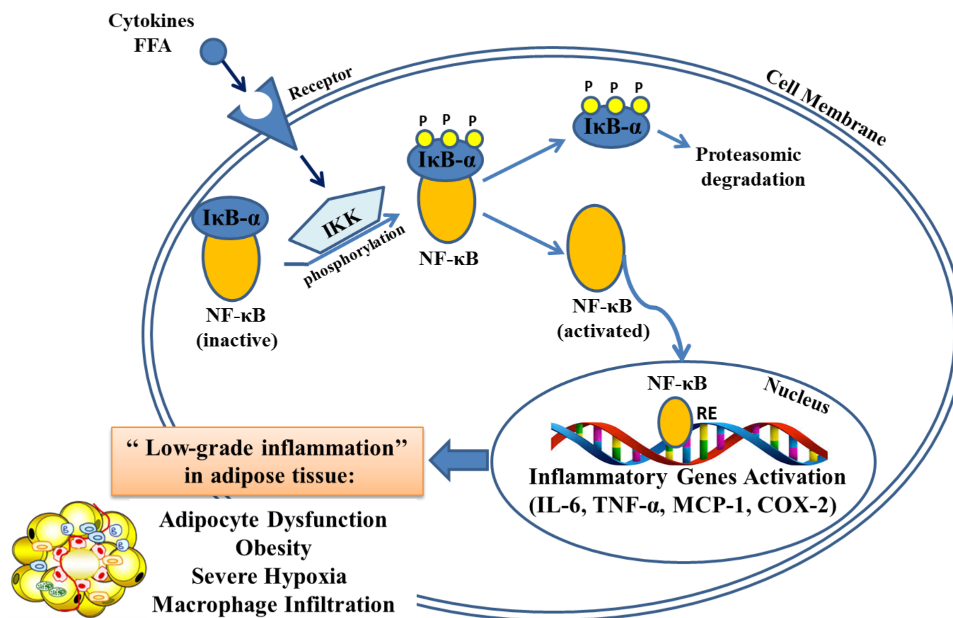


Fig. 8: Pathway of NF-κB and its effects in adipose tissue.

Therefore, in order to study the protective effect of C3G on PA-induced inflammatory response, the attention was focused on the activation of the NF-κB inflammatory pathway. At this aim we evaluated both the nuclear NF-κB p65 levels and cytoplasmic IKK kinase levels in differentiated 3T3-L1 cells.

Results obtained show that PA activates the nuclear translocation of NF-κB (demonstrated by higher p65 nuclear levels) compared to the untreated and unexposed control. In contrast, activation of NF-κB is significantly inhibited by pretreatment with C3G and subsequent exposure to PA. Treatment with C3G alone do not show any effect on nuclear NF-κB levels (**Fig. 9**).

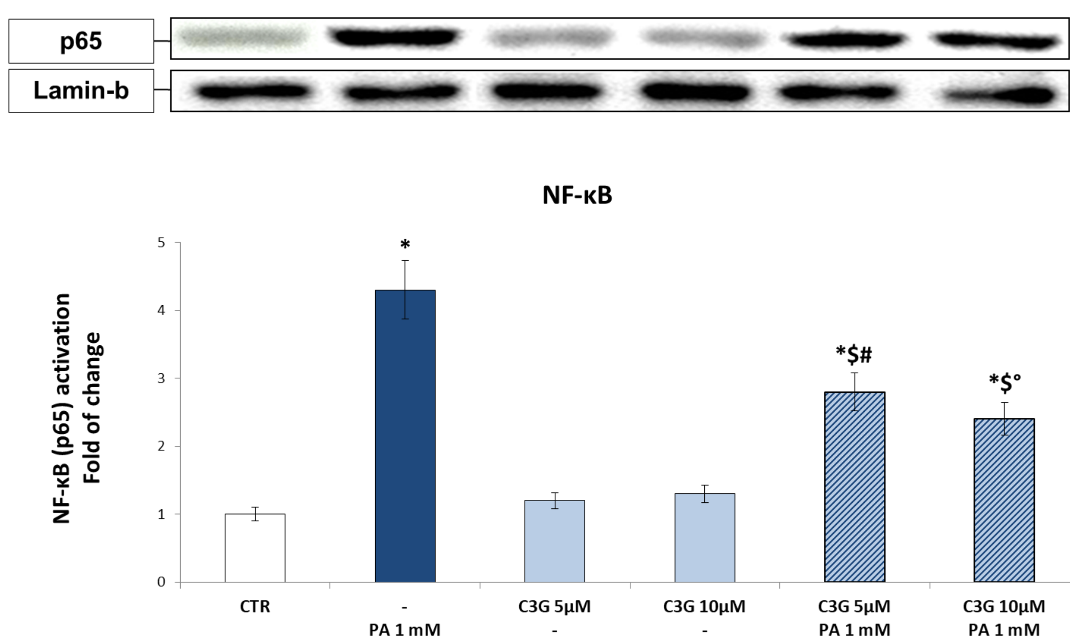


Fig. 9: Effect of C3G on NF-κB p65 nuclear levels increase induced by PA - 3T3-L1 cells were pretreated or not with C3G 5-10 µM for 24 h and subsequently exposed to 1 mM PA for further 24 h. Cells treated with the vehicle alone (0.025% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments. The densitometry results are reported as fold change compared to the controls, expressed as mean ± SD of three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. The intensity values of the NF-κB /p65 protein were normalized to the corresponding value of lamin-b. *p <0.05 vs CTR; $^{\$}$p <0.05 vs PA 1mM; $^{\#}$p <0.05 vs C3G 5µM; °p <0.05 vs C3G 10µM.

The mechanisms involved in C3G protection against the activation of the NF-κB pathway, were further verified by IKK evaluation. In fact, in the absence of an inflammatory stimulus, NF-κB is located into the cytoplasm, complexed with an inhibitory protein called IκB (Inhibitor of NF-κB). When an extracellular stimulus triggers the pathway of NF-κB, IKKα/β is activated and phosphorylates IκB at two serine residues in the N-terminal domain. This induces the release and translocation of NF-κB into the nucleus, thus activating transcription of target genes (Hayden and Ghosh, 2014).

We then investigated if the modulatory effects of C3G on nuclear accumulation of NF-κB p65 related to an inhibition of IKK. In our experimental model, PA exposure is able to activate (phosphorylate) IKK. Instead, pretreatment with C3G at both the tested concentrations, and in a dose dependent way, is able to inhibit the phosphorylation of IKK α/β induced by the PA, thus leading to a consequent decrease in the nuclear accumulation of NF-κB p65 shown above (**Fig. 10**).

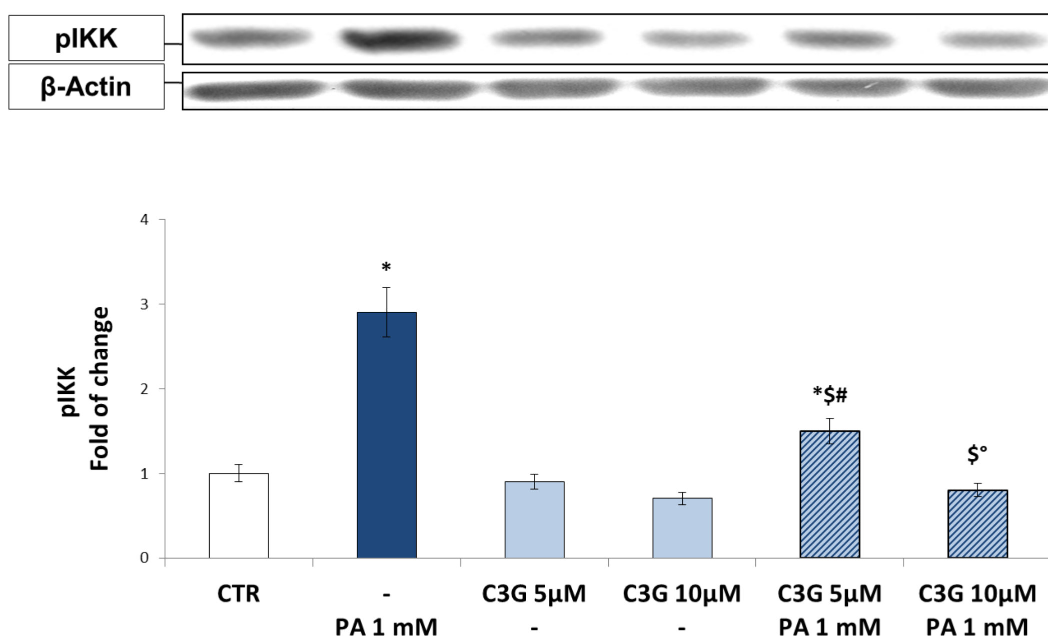


Fig. 10: Effect of C3G on IKK phosphorylation induced by PA - 3T3-L1 cells were pretreated or not with C3G 5-10 µM for 24 h and subsequently exposed to 1 mM PA for further 24 h. Cells treated with the vehicle alone (0.025% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments. The densitometry results are reported as fold change compared to the controls, expressed as mean ± SD of three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. The intensity values of the phosphorylated IKK protein were normalized to the corresponding value of β-actin. *$p < 0.05$ vs CTR; $^{\$}p < 0.05$ vs PA 1mM; $^{\#}p < 0.05$ vs C3G 5 µM; °$p < 0.05$ vs C3G 5 µM + PA 1 mM.

As previously described, NF-κB regulates the expression of a large variety of genes encoding pro-inflammatory proteins. To confirm the transcriptional activity of NF-κB, IL-6 gene expression was evaluated by real time PCR.

IL-6 is the most representative of the pro-inflammatory cytokines released by adipose tissue. It is a NF-κB-dependent cytokine involved in the activation of various local and peripheral inflammatory responses. Elevated circulating levels of IL-6 are present in obese patients, and are considered as predisposing factors to the metabolic syndrome (Lastra *et al.,* 2006).

Results demonstrated that PA exposure is able to activate IL-6 gene expression in a statistically significant manner. On the other hand, C3G pretreatment, at both the tested concentrations, statistically reduces cytokine mRNA levels. These results demonstrate a protective effect of C3G and confirm the inhibitory activity on NF-κB pathway (**Fig.11**).

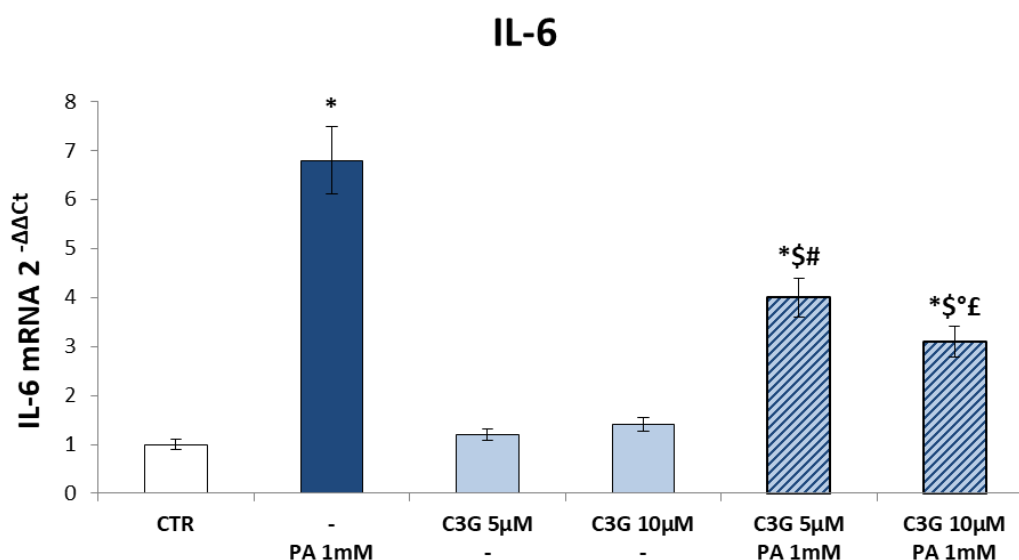


Fig. 11: Effect of C3G on PA-induced IL-6 gene expression. 3T3-L1 cells were pretreated or not with C3G for 24 h and subsequently exposed to 1 mM PA for 24 h. Cells treated with the vehicle alone (0.025% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. IL-6 mRNA expression was analysed by real time PCR and data are expressed as $2^{-\Delta\Delta Ct}$ and normalized to CTR. 18S rRNA was used as housekeeping gene. *p <0.05 vs CTR; $^{\$}$p <0.05 vs PA 1mM; $^{\#}$p <0.05 vs C3G 5 μM; °p <0.05 vs C3G 10μM; $^{£}$p <0.05 vs C3G 5μM + PA 1 mM .

3.3.1.5 Effect of C3G on PA-induced insulin resistance

Several scientific evidences have shown that obesity-associated inflammation contributes to the development of insulin resistance and precedes the onset of type 2 diabetes mellitus (Martins *et al*., 2014). At a molecular level, insulin resistance is due to changes in insulin signal pathway so reducing the sensitivity of cells to this hormone (Dei Cas *et al*., 2010).

IRS-1 protein phosphorylation is a determining factor for the insulin signal transduction. In physiological conditions, the binding of insulin to its membrane receptor (IR) triggers the autophosphorylation of tyrosine residues and leads to a series of phosphorylation cascade involving the activation of the IRS/PI3K/Akt pathway (**Fig. 12**). Finally, Akt activation plays a fundamental role in the metabolic effects of insulin, leading to an increase in the expression of GLUT-4 and GLUT-1 glucose transporters in the cell membrane and inhibition of the lipolysis process (Gutiérrez-Rodelo *et al*., 2017). High levels of circulating cytokines or FFA cause drastic changes in insulin pathway and in particular induce IRS-1 phosphorylation at the serine site and inhibit IRS-1 tyrosine phosphorylation, thus blocking the PI3K/Akt pathway with subsequent insulin resistance (Gual *et al*., 2005).

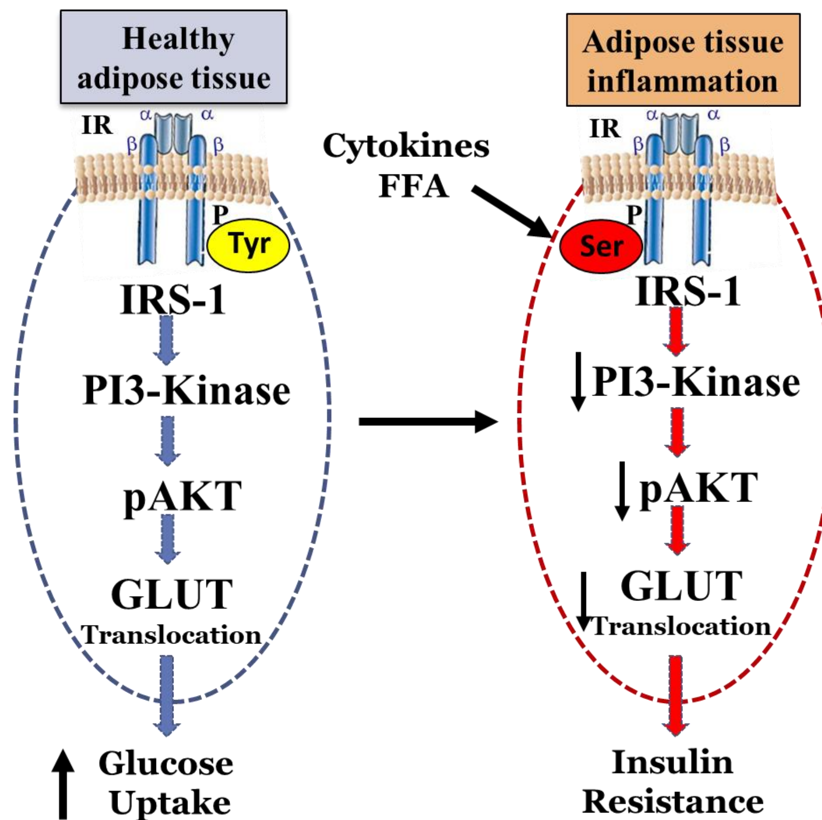


Fig. 12: IRS/PI3K/Akt pathway role at the level of adipose tissue - In homeostatic conditions, the phosphorylation of the tyrosine site of the IRS-1 leads to the initiation of a phosphorylation cascade process culminating in a greater glucose uptake. In an inflammatory condition, due to increase of serum FFAs and proinflammatory cytokines, serine phosphorylation occurs with consequent insulin-resistance (adapted from Kim *et al*., 2005).

Therefore, in our study we focused on the effects exerted by the PA on the IRS-1/PI3K/Akt signaling pathway modulated by insulin and the potential protective effect of C3G pretreatment. Results show an increase in phosphorylation at Ser307 site of IRS following PA exposure for 24 h and a consequent reduction of Tyr895 phosphorylation. Pretreatment with C3G, at both the tested concentrations (5-10 µM), was able to reduce phosphorylation at the serine site, restore insulin sensitivity, and increase activation at the tyrosine site (**Fig. 13**).

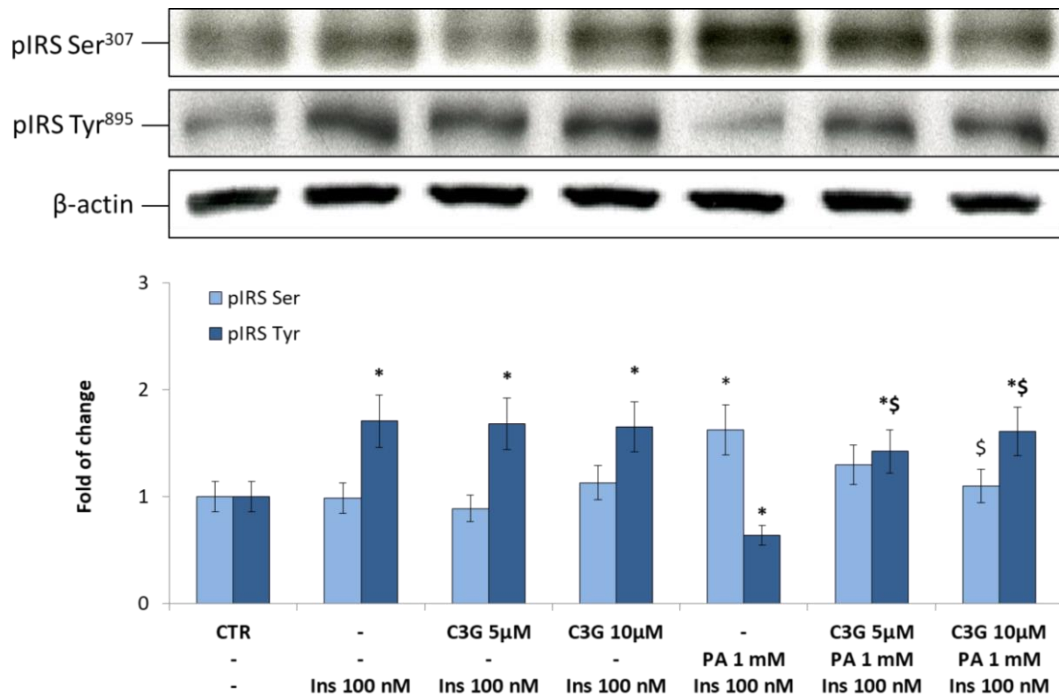


Fig. 13: Effect of C3G on serine 307 and tyrosine 895 phosphorylation of IRS-1 modulated by PA - 3T3-L1 cells were pretreated or not with C3G 5-10 µM for 24 h and subsequently exposed to 1 mM PA for further 24 h. Cells treated with the vehicle alone (0.025% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments. The densitometry results are reported as fold change compared to the controls, expressed as mean ± SD of three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. The intensity values of the phosphorylated proteins were normalized to the corresponding value of β-actin. *p <0.05 vs CTR; $^{\$}$p <0.05 vs PA 1 mM + ins 100 nM.

The study of the effects of PA on insulin resistance was further analysed by evaluating phosphorylation levels of IRS-1 downstream kinases. PA reduces PI3K (**Fig. 14 A**) and Akt (**Fig. 14 B**) phosphorylation at levels considerably lower than those of cells exposed to insulin alone. Also in this case, C3G pretreatment of 3T3-L1 at both the tested concentrations (5-10 µM) restores the PI3K/Akt signaling pathway. In fact, in cells exposed to insulin and palmitic acid, C3G induces phosphorylation and activation of PI3K and Akt (even more than those observed in cells exposed only to insulin); these effects were statistically significant. C3G pretreatment alone did not affect phosphorylation levels.

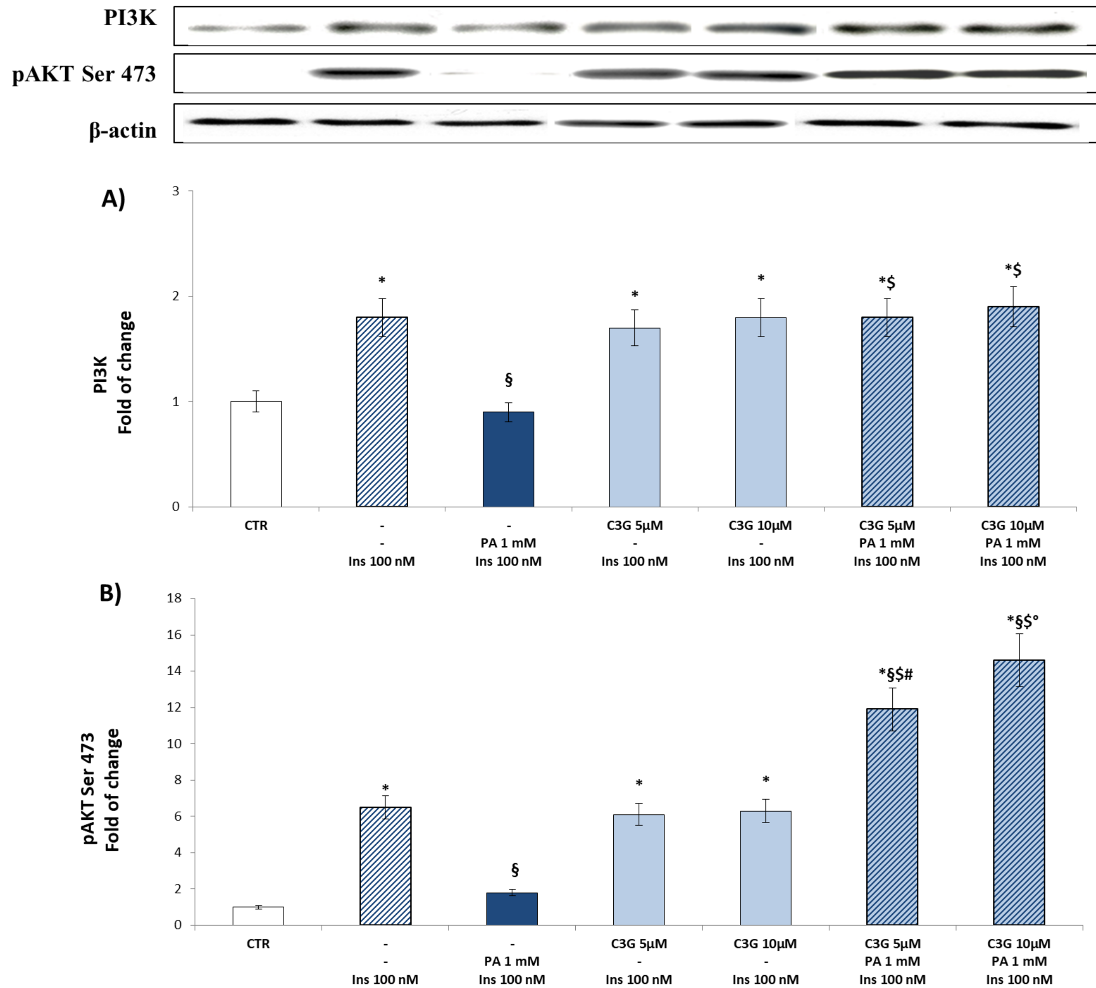


Fig. 14: Effect of C3G on PI3K and Akt phosphorylation modulated by PA - 3T3-L1 cells were pretreated or not with C3G 5-10 µM for 24 h and subsequently exposed to 1 mM PA for further 24 h. Cells treated with the vehicle alone (0.025% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments. The densitometry results are reported as fold change compared to the controls, expressed as mean ± SD of three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. The intensity values of PI3K and pAkt proteins were normalized to the corresponding value of beta-actin. *p <0.05 vs CTR; §p <0.05 vs Ins 100 nM; $^{\$}$p <0.05 vs PA 1 mM + Ins 100 nM ; $^{\#}$p <0.05 vs C3G 5µM + Ins 100 nM; °p <0.05 vs C3G 10µM + Ins 100 nM.

The modulation of the insulin pathway was finally confirmed by evaluating GLUT-1 glucose transporter protein levels. GLUT-1 belongs to a family of integral membrane glucose transport proteins that promote glucose uptake from the extracellular compartments. In our experimental model, PA exposure of 3T3-L1 cells results in a reduction in GLUT-1 levels, thus demonstrating a decrease in insulin sensitivity. Pretreatment with C3G restores GLUT-1 levels induced by insulin (bringing them to values above those measured in control) and modulated by PA. The effects of C3G pretratment is dose-dependent (**Fig. 15**).

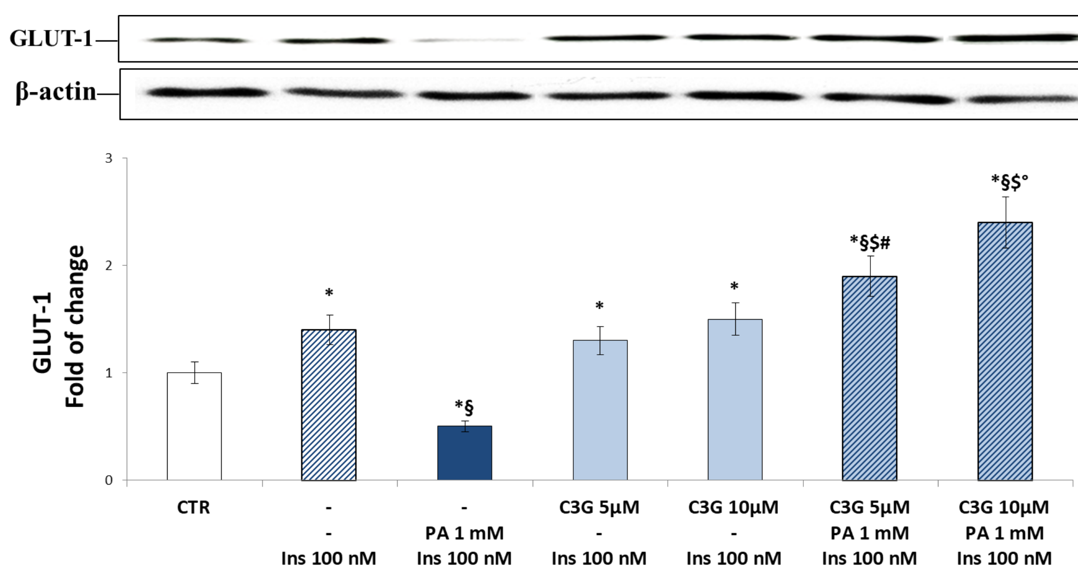


Fig. 15:Effect of C3G on GLUT-1 protein expression - 3T3-L1 cells were pretreated or not with C3G 5-10 μM for 24 h and subsequently exposed to 1 mM PA for further 24 h. Cells treated with the vehicle alone (0.025% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments. The densitometry results are reported as fold change compared to the controls, expressed as mean ± SD of three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. The intensity values of GLUT-1 protein were normalized to the corresponding value of β-actin. *p <0.05 vs CTR; §p <0.05 vs Ins 100 nM; $^{\$}$p <0.05 vs PA 1mM + Ins 100 nM; $^{\#}$p <0.05 vs C3G 5μM + Ins 100 nM; °p <0.05 vs C3G 10μM + Ins 100 nM.

3.3.1.6 Effect of C3G on Adiponectin gene expression

Adiponectin is the most important anti-inflammatory adipokine expressed in the adipose tissue (Nigro *et al*., 2014). It is involved in energy metabolism, is able to increase insulin sensitivity at the systemic level, and has shown important antiatherogenic effects. In physiological conditions it is present in the blood circulation at high concentrations (5-10 µg/ml); in obesity and type 2 diabetes mellitus conditions, the circulating levels of this protein are however considerably reduced. Proinflammatory factors such as FFAs, TNF-α, IL-6, reactive oxygen species and hypoxia reduce its expression in adipocytes (Li *et al*., 2009) with important consequences for human health (Nigro *et al*., 2014).

For this purpose, in order to further study the influence of PA on the insulin pathway and the protective effects of C3G, adiponectin (AdipoQ) gene expression was examined using real time PCR. Results show that insulin treatment induce AdipoQ gene expression. Furthermore, PA exposure determines, in our experimental conditions, a reduction of AdipoQ levels with values below the control exposed to insulin, thus confirming a lower response to the hormone induced by the PA. Data obtained with C3G pretreatment, at both the tested doses (5-10 µM), confirm the protective effect of this anthocyanin, by increasing AdipoQ levels reduced by PA (**Fig. 16**).

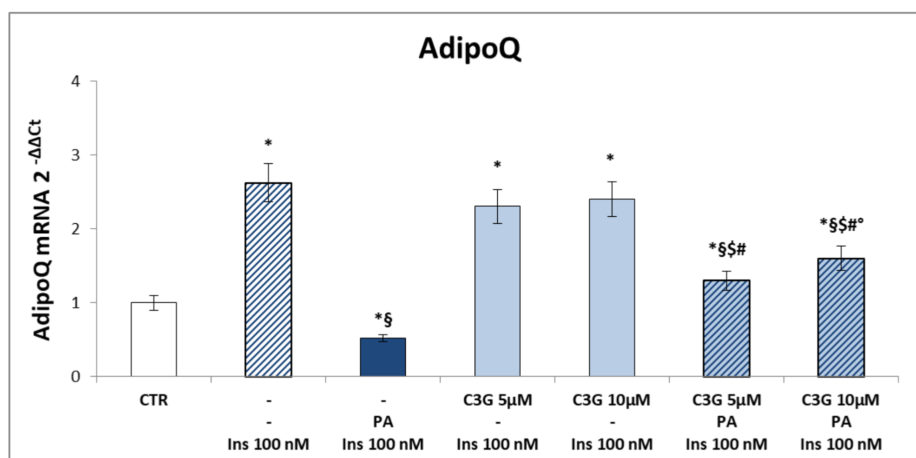


Fig. 16: Effect of C3G on AdipoQ gene expression. 3T3-L1 cells were pretreated or not with C3G for 24 h and subsequently exposed to PA (1mM) for 24 h. Cells treated with the vehicle alone (0.025% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. AdipoQ mRNA expression was analysed by real time PCR and data are expressed as $2^{-\Delta\Delta Ct}$ and normalized to CTR. 18S rRNA was used as housekeeping gene. *p <0.05 vs CTR; §p <0.05 vs Ins 100 nM; $^{\$}$p <0.05 vs PA + Ins 100 nM; $^{\#}$p <0.05 vs C3G 5 µM + Ins 100 nM; °p <0.05 vs C3G 10µM + Ins 100 nM

3.3.2 STUDIES ON HUMAN SGBS ADIPOCYTES EXPOSED TO PALMITIC ACID

3.3.2.1 Effects of C3G on the overexpression of proinflammatory genes induced by PA

The knowledge regarding obesity and related diseases, nowadays is mainly based on studies carried out on mouse models. The 3T3-L1 murine adipocytes are, in fact, the most popular *in vitro* model to study many biological and pathological aspects of adipose tissue cells thanks to their easy handling, simple culture conditions and high replicative capacity and differentiation in mature adipocytes. However, there are strong limitations due to the numerous species differences concerning the expression of proteins and adipokines that play key roles in the adipogenesis process. Furthermore, 3T3-L1 are aneuploid and behave differently than human cells in some respects (Gregoire *et al.,* 1998). For example, 3T3-L1 cells and human-derived SGBS cells show a different sensitivity to apoptosis: the first are sensitive to apoptosis induced by serum deprivation (Magun *et al.,* 1998), while the latter and primary adipocytes are characterized by increased resistance in the absence of serum (Fischer-Posovszky *et al*., 2008).

In addition it has been demonstrated that SGBS cells show highest levels of differentiation and of expression of a range of adipocyte markers so that nearly 100% of differentiated adipocytes accumulate lipids and are more functionally active with respect to other adipocytes cell lines such as murine one (Felicity *et al.,* 2006)

To confirm the protective effects observed on murine cells, the same experimental model used for 3T3-L1 was applied on a line of human SGBS preadipocytes. SGBS cells are derived from the stromal vascular fraction of the subcutaneous adipose tissue of infants affected by Simpson-Golabi-Behmel syndrome (SGBS), a rare congenital syndrome linked to the X chromosome (Rosenow *et al.*, 2010). Like 3T3-L1, SGBS show a high differentiation capacity (up to 90%) following exposure to specific differentiation inducers, 18-21 days after seeding. These cells maintain this characteristic for at least 30 generations, being morphologically, biochemically, and functionally similar to primary human preadipocytes. SGBS represent therefore a valid model for the studies of the adipogenesis process (Rosenow *et al.,* 2010), the adipokine secretion profile of adipocytes (specific

adipose biomarkers such as adiponectin and leptin), and glucose and fatty acids metabolism in the adipose tissue (Lasa *et al*., 2011).

In particular, this study focused on the molecular mechanisms involved in the inflammatory process induced by exposure to high concentrations of PA. After an initial setup, performed in order to confirm the experimental conditions, the PA concentration chosen for the SGBS experiments was 500 μM, the lowest concentration able to induce the proinflammatory genes (data not shown). These cells are therefore more sensitive than 3T3-L1 to PA-induced lipotoxicity condition probably due to higher triglycerides accumulation and differentiation level (Yeo *et al.,* 2017; Felicity *et al.,* 2006). To study the inflammatory process induced by PA, the levels of TNF-α, IL-6, IL-8, and MCP-1, chemokines known to be expressed and released by adipocytes, were evaluated at the transcriptional level (Tilg *et al.,* 2010).

The protective effect of C3G was assessed by pretreating SGBS cells with increasing anthocyanin concentrations (1, 10, and 20 μM) for 24 h (**Fig. 17**).

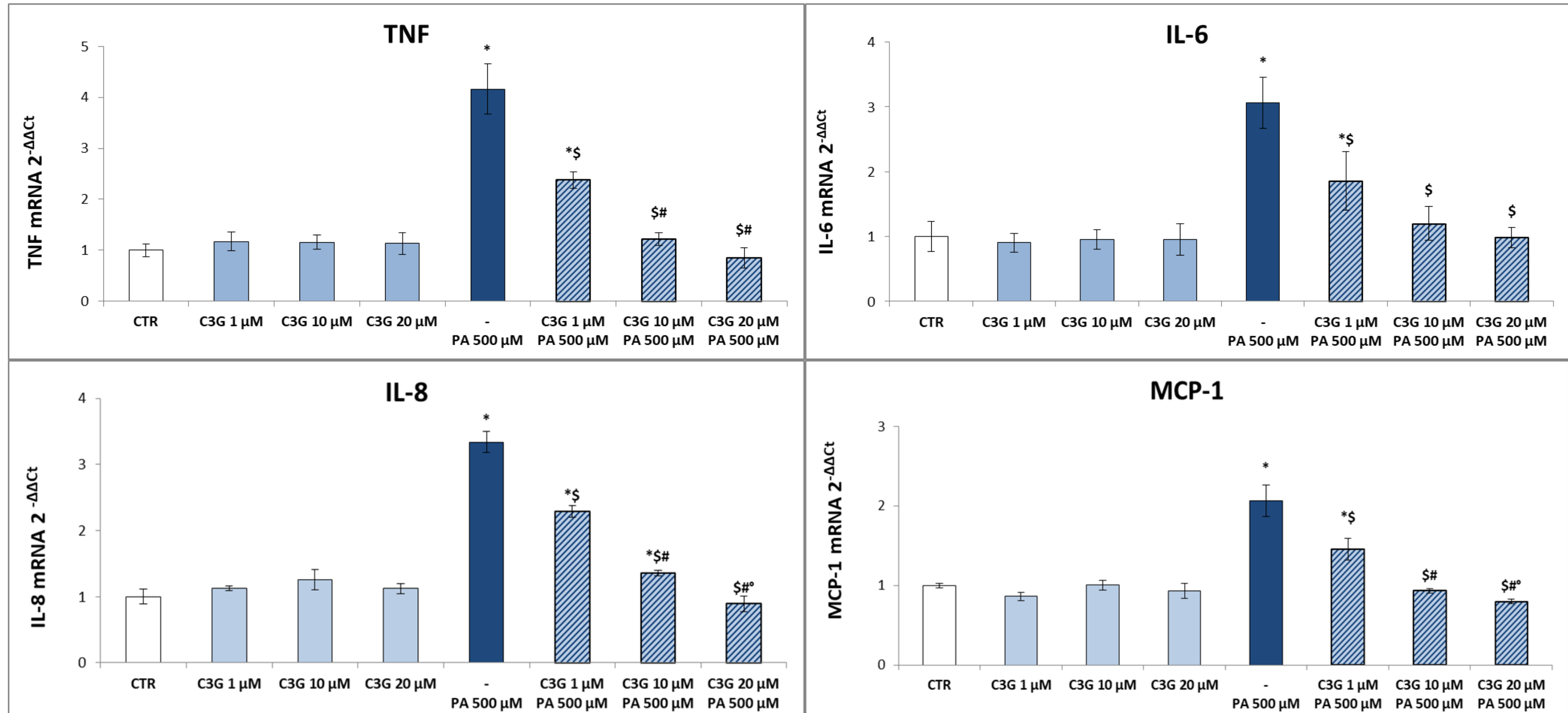


Fig. 17: Effect of C3G on PA-induced TNF, IL-6, IL-8, MCP-1 gene expression - SGBS cells were pretreated or not with C3G for 24 h and subsequently exposed to PA (500µM) for 24 h. Cells treated with the vehicle alone (0.05% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. mRNA expression levels were analyzed by real time PCR and data were expressed as $2^{-\Delta\Delta Ct}$ and normalized to CTR. 18S rRNA was used as housekeeping gene. *$p < 0.05$ vs CTR; $^{\$}p < 0.05$ vs PA 500 µM; $^{\#}p < 0.05$ vs C3G 1µM + PA 500 µM; °$p < 0.05$ vs C3G 10µM + PA 500 µM.

In our experimental model, SGBS cells exposed to PA have significantly higher levels of cytokines mRNA expression than control cells. The activation of gene expression induced by PA is completely inhibited by the pretreatment with C3G at all the tested concentrations and in a dose-dependent way. In particular, the results show that C3G 1 μM, considered a physiological concentration, easily reachable after appropriate supplementation, is already able to statistically reduce the expression of all the cytokines compared to the cells exposed to the PA. This different sensitivity between human and murine adipocytes seems to be common for polyphenols compounds as reported elsewhere for resveratrol (Li *et al.,* 2016). Unfortunately, the mechanisms underlying this different behaviour remain unknown to date. Treatment with C3G alone has no effect on the baseline expression levels of all the cytokines tested.

3.3.2.2.Effect of C3G on insulin resistance induced by PA

In our study we also focused on the effects of C3G on the modulation of GLUT-1 and GLUT-4 glucose transporters and hexokinase, altered by PA in order to confirm the data obtained on murine cells. In fact, glucose transporters and hexokinase, which catalyzes glucose phosphorylation and participates to the first reaction of glycolysis, are under the direct control of insulin (Roberts *et al.,* 2015). Results demonstrate how the lipotoxicity induced by PA determines the onset of insulin resistance. PA, in fact, causes a reduction of the gene expression of GLUT-1 and GLUT-4 which will result in a lower glucose uptake. Hexokinase levels are also reduced by exposure to PA. However, C3G pretreatment restores the normal activities of GLUT transporters and hexokinase at all the tested concentrations in a statistically significant and dose-dependent manner. Also this data show that the lower concentration of C3G (1 μM) is already able to exert a protective and statistically significant effect; while at the intermediate concentration of C3G (10 μM) mRNA levels are similar to those of the insulin control with the achievement of a roof effect at higher concentrations. Furthermore, treatment with C3G has no effect on the baseline gene expression levels of GLUT-1, GLUT-4, and HK-II (**Fig. 18**).

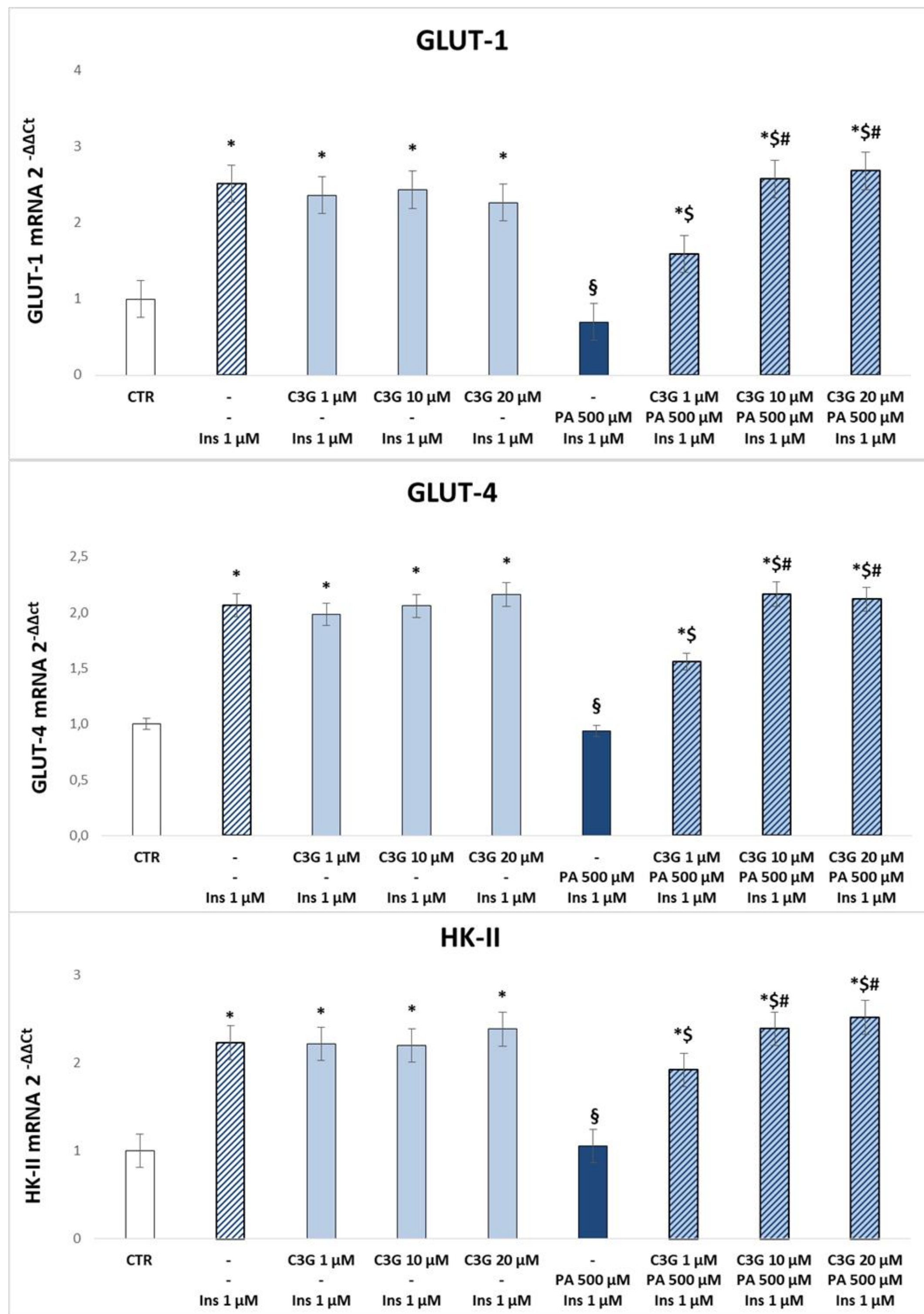


Fig. 18: Effect of C3G on GLUT-1, GLUT-4 and HK-II gene expression modulated by PA - SGBS cells were pretreated or not with C3G for 24 h and subsequently exposed to PA (500µM) for 24 h. Cells treated with the vehicle alone (0.05% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. mRNA expression levels were analyzed by RT-PCR and data were expressed as $2^{-\Delta\Delta Ct}$ and normalized to CTR. 18S rRNA was used as housekeeping gene. *p <0.05 vs CTR; §p <0.05 vs Ins 1 µM; $p <0.05 vs PA 500 µM + Ins 1 µM; #p <0.05 vs C3G 1µM + PA 500 µM + Ins 1 µM.

3.3.2.3 Effect of C3G on the Adiponectin gene expression

Among adipokines expressed in adipose tissue, adiponectin plays a central role in the regulation of glucose and lipid metabolism. It is secreted by differentiated adipocytes and possesses regulatory effects on energy homeostasis, glucose metabolism, and inflammation (Sikaris *et al.,* 2004). Reduced circulating levels of adiponectin, during obesity, negatively affect insulin sensitivity by inducing insulin resistance (Kadowaki *et al*., 2006).

Data obtained show that, in our experimental conditions, insulin treatment induces activation of the AdipoQ gene in SGBS cells. On the contrary, exposure to PA led to a reduction of AdipoQ levels with values below the control cells without insulin, thus confirming the reduction in insulin sensitivity induced by PA. Furthermore, C3G pretreatment at all the tested doses confirms that the insulin sensitivity is restored, with consequent reduction of insulin resistance.The treatment with C3G only did not affect AdipoQ gene expression respect to control cells (**Fig. 19**).

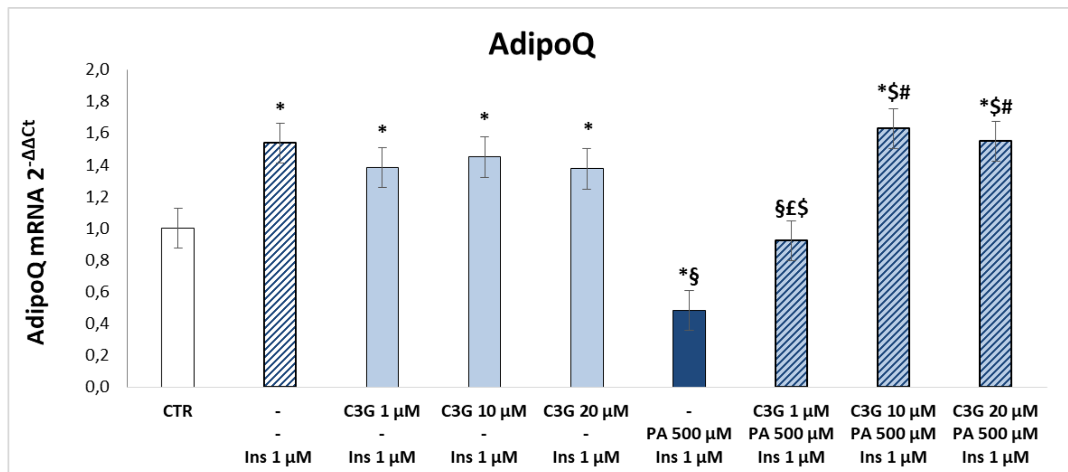


Fig. 19: Effect of C3G on AdipoQ gene expression reduced by PA - SGBS cells were pretreated or not with C3G for 24 h and subsequently exposed to PA (500µM) for 24 h. Cells treated with the vehicle alone (0.05% DMSO v/v) were used as controls (CTR). The image is representative from three independent experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD. mRNA expression levels were analyzed by real time PCR and data were expressed as $2^{-\Delta\Delta Ct}$ and normalized to CTR. 18S rRNA was used as housekeeping gene. *p <0.05 vs CTR; §p <0.05 vs Ins 1 µM; £p <0.05 vs C3G 1µM + Ins 1 µM; $p <0.05 vs PA 500 µM + Ins 1 µM; #p <0.05 vs C3G 1µM + PA 500 µM + Ins 1 µM.

3.4 Conclusions

Obesity is an important health problem and is associated to an increase in morbidity and mortality for many chronic diseases. Among these, the most important are type 2 diabetes mellitus and cardiovascular diseases (Vachharajani *et al.,* 2009). For this reason, increasing interest has been dedicated to the knowledge related to adipose tissue cells and their possible pathophysiological role in the development of associated diseases.

For many years, adipose tissue was merely considered a deposit of energy and a protection from thermal and mechanical stresses (Kershaw *et al.,* 2004). More recently it has been recognized as a complex endocrine organ able to secrete numerous molecules, called adipokines, with multiple actions at local and systemic level (Zoico *et al*., 2011). Alterations in adipokines secretion results in adipocyte hypertrophy and increased infiltration of immune cells, such as macrophages and lymphocytes, thus triggering the inflammatory process (Unamuno *et al.,* 2018). In particular, obesity is associated with a state of "chronic low-grade inflammation", which plays an important pathophysiological role in the development and progression of obesity-related complications, such as insulin resistance, endothelial dysfunction, and dyslipidemia (Unamuno *et al*., 2018). Furthermore, lipotoxicity, common in the adipose tissue, contributes to further exacerbate the problems associated with these pathological conditions. FFAs, mainly from lipolysis processes of adipose tissue, are considered among the main causes of the onset of inflammation and insulin resistance in the adipose tissue (Hotamisligil *et al.,* 2006). Several *in vitro* and *in vivo* studies have shown that high levels of intracellular FFAs determine, in fact, greater expression of inflammatory adipokines with consequent activation of inflammatory signaling pathways and alteration of the insulin receptor activity.

In recent years, epidemiological evidences have shown that different molecules of nutritional interest are able to reduce the incidence of various diseases such as cardiovascular, diabetes mellitus and cancer. In the present study, anthocyanins, a class of flavonoids widely distributed in fruits and vegetables used in Mediterranean diet, were examined. These substances have been extensively studied for their potential beneficial effects on human health and for their ability

to exercise positive modulatory activity on endothelial function, and lipid and glucose metabolism (Wallace *et al.,* 2011).

However, although evidences of the pleiotropic properties of anthocyanins, such as the suppressive effect on body fat accumulation and anti-diabetic action, continue to accumulate, only a limited number of studies have identified the specific molecular structures of anthocyanins responsible for their health benefits. Usually these effects seem to be due to their interaction with different cellular molecular pathways, thus modulating oxidative damage and the expression of pro-inflammatory genes (Speciale *et al*., 2011).

In our study we demonstrated, through the use of *in vitro* experimental models on murine (3T3-L1) and human (SGBS) adipocytes, the anti-inflammatory and insulin-sensitizing effects of C3G, and elucidated the potential molecular mechanisms involved in these beneficial effects.

From the results obtained on 3T3-L1 it is possible to confirm that C3G (5 and 10 μM) is able to reduce adipocyte hypertrophy induced by high concentrations of PA. Reduced accumulation of intracellular lipids, demonstrated by histochemical staining with Oil Red O, was associated with the ability of C3G to modulate, in a dose-dependent manner, the expression of the transcription factor PPAR-γ, the main activator of the process of adipogenesis. The transcriptional activity of PPAR-γ was also confirmed by evaluating the gene expression of FABP4, a gene that codes for a fatty acid carrier protein. Pretreatment with C3G, in fact, significantly reduced the expression of FABP4 induced by PA in a statistically significant manner. These data provide a mechanistic support for the effects observed in *in vivo* experimental animal models. In fact, it has been shown that anthocyanins are able to reduce the accumulation of fat in laboratory animals fed with a high fat intake diet (Prior *et al*., 2008; Prior *et al*., 2009; Johnson *et al*., 2016).

C3G has also been shown to inhibit the NF-κB -modulated inflammatory pathway. NF-κB nuclear levels are, in fact, considerably lower following C3G pretreatment compared with those of the cells exposed to PA. The same trend has been observed for the activation of IKK, a kinase that activates upstream the NF-κB factor. Furthermore, the results show that activation of PA-induced NF-κB pathway leads to increased gene expression of IL-6, the main cytokine involved in

the NF-κB inflammatory pathway. All these values are maintained at lower levels in cells pretreated with C3G in a dose-dependent way, thus demonstrating the protective effect of this anthocyanin.
The protective effect of C3G has been confirmed by evaluating its ability to decrease insulin resistance induced by PA. For this purpose, 3T3-L1 cells were further treated with 100 nM insulin to activate the normal insulin signalling pathway. The results obtained showed that C3G is able to restore insulin sensitivity altered by PA. Exposure to high concentrations of PA, in fact, increased the phosphorylation of IRS-1 on the serine site (Ser307) at the expense of the tyrosine site (Tyr895) which mediates the physiological effects of insulin. Pretreatment with C3G, on the other hand, restored phosphorylation on the insulin-induced tyrosine site, thus reducing the effect of PA.
The study then continued examining IRS-1downstream kinases. The results obtained on PI3K and Akt confirm the inhibition of the insulin pathway exerted by PA. In fact, the increase in serine phosphorylation of IRS-1 causes a reduction in phosphorylation and activation of PI3K and Akt. Also in this case, C3G pretreatment restores the levels of these proteins with values similar to the cells treated with insulin only. The post-transcriptional changes modulated by the insulin pathway allowed us to confirm that PA reduces the expression of the GLUT-1 glucose transporter and the expression of the gene encoding adiponectin. Also in this case the results confirm the protective effect of C3G with a dose-dependent effect on the restoration of insulin sensitivity in murine adipocytes.
However, it should be pointed out that these results were obtained on an *in vitro* model of murine adipocytes, and that extrapolating data from animal to humans is not always possible. For this reason, the results obtained with 3T3-L1 murine cells have been validated by using a human cell line of preadipocytes SGBS, able to differentiate into mature adipocytes following the addition of particular inducers. Results confirm, also in this case, that PA exposure induces the inflammatory process, as shown by increased gene expression of TNF-α, IL-6, IL-8, and MCP-1, cytokines known to be expressed and released by adipocytes. Cells pretreatment with C3G results in a reduction in mRNA levels of these cytokines in a statistically significant way and at very low concentrations (1 μM). This

concentration is not effective in the 3T3-L1 cells (data not shown); in murine adipocytes, in fact, the minimum effective concentration is 5 μM.
With regard to insulin resistance experiments, PA exposure results in a reduced gene expression of the main insulin sensitivity markers, such as GLUT-1, GLUT-4, hexokinase, and adiponectin. Data obtained with C3G pretreatment on SGBS, at all the tested doses, allows us to confirm the effect of this anthocyanin on the insulin sensitivity restoration. Also in this case, the lowest dose of C3G tested (1 μM) is already effective on all the parameters tested.
In conclusion, this study provides support for potential molecular mechanisms involved in inflammation and insulin resistance induced by PA in adipose tissue, as well as the protective effect of C3G even at low concentrations. These effects are supported by the large number of papers reporting that anthocyanins possess a wide range of health-promoting properties and may have important implications in the prevention of chronic and metabolic diseases through their capability to modulate cell redox-dependent signalling pathways (Speciale et al., 2014). However, much remains to be clarified before a rational employment of these compounds for human health benefits.
Actual criticism regarding anthocyanins is represented by their relatively low bioavailability, although the dietary consumption of these pigments has been proposed to be associated with a significant protection against several human pathological conditions. Furthermore many papers focussing on *in vitro* properties of C3G, use very high non physiologically achievable concentrations (> 20μM) of this compound so that the direct transfer of these data to real life condition is very difficult to be implemented. On the contrary, in the present study, we tried to assess realistic and physiological concentrations supporting C3G bioactivity at low concentrations (1 and 5μM).
Even if more *in vivo* studies (on animals and in humans) are needed to clarify the efficacy of C3G and anthocyanins, the results discussed in this thesis allow us to hypothesize a possible application of C3G in the prevention of pathological conditions related to lipotoxicity, and more generally, to obesity and metabolic syndrome.

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References

- Afaq F., Malik A., Syed D., Maes D., Matsui M.S., & Mukhtar H. "**Pomegranate Fruit Extract Modulates UV-B–mediated Phosphorylation of Mitogen-activated Protein Kinases and Activation of Nuclear Factor Kappa B in Normal Human Epidermal Keratinocytes**." *Photochemistry and Photobiology* 81.1: 38-45, **2005**.
- Agostini M., Schoenmakers E., Mitchell C., Szatmari I., Savage D., Smith A., Rajanayagam O., Semple R., Luan J., Bath L., Zalin A., Labib M., Kumar S., Simpson H., Blom D., Marais D., Schwabe J., Barroso I., Trembath R., Wareham N., Nagy L., Gurnell M., O'Rahilly S., Chatterjee K. "**Non-DNA binding, dominant-negative, human PPAR gamma mutations cause lipodystrophic insulin resistance**" *Cell Metabolism,* 4(4):303-11*,* **2006**.
- Aguirre V., Werner E.D., Giraud J., Lee Y.H., Shoelson S.E., White M.F. "**Phosphorylation of ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action".** *J. Biol. Chem;* 277: 1531-1537*,* **2002**.
- Ahmad L.A., and Crandall J.P. "**Type 2 diabetes prevention: a review**." *Clinical Diabetes* 28.2: 53-59, **2010**.
- Akbay E., Muslu N., Nayir E., Ozhan O., and Kiykim A."**Serum retinol binding protein 4 level is related with renal function sin Type 2 diabetes**." *J. Endocrinol.Invest.* 33, 725–729. doi:10.3275/7024, **2010.**
- Aksoy E., Amraoui Z., Goriely S., Goldman M., and Willems F. **"Critical role of protein kinase C epsilon for lipopolysaccharide-induced IL-12 synthesis in monocyte-derived dendritic cell ".** *Eur. J. Immunol.* 32**:** 3040–3049, **2002**.
- Aleffi S., Petrai I., Bertolani C., Parola M., Colombatto S., Novo E. "**Upregulation of proinflammatory and proangiogenic cytokines by leptin in human hepatic stellate cells**". *Hepatology* 42, 1339–1348. doi:10.1002/hep.20965, **2005**.
- Ali A.T., Hochfeld W.E., Myburgh R., Pepper M.S. "**Adipocyte and adipogenesis**" *European Journal of Cell Biology,* 92(6-7):229-36, **2013.**
- Ameer F., Scandiuzzi L., Hasnain S., Kalbacher H., & Zaidi N. "**De novo lipogenesis in health and disease**." *Metabolism* 63.7: 895-902, **2014**.
- Anderson E.K., Gutierrez D.A., Hasty A.H. "**Adipose tissue recruitment of leukocytes"**. *Curr Opin Lipidol ;*21:172-7, **2010**.
- Antuna-Puente B., Feve B., Fellahi S., Bastard J. P. "**Adipokines: the missing link between insulin resistance and obesity**." *Diabetes & metabolism* 34.1: 2-11, **2008**.
- Anunciado-Koza R.P., Higgins D.C., Koza R.A. "**Adipose tissue Mest and Sfrp5 are concomitant with variations of adiposity among inbred mouse strains fed a non-obesogenic diet"**. *Biochimie* 124, 134-140, **2016**.
- Arner E., Westermark P.O., Spalding K.L., Britton T., Rydén M., Frisén J.,Arner P. "**Adipocyte turnover: relevance to human adipose tissue morphology**." *Diabetes*, **2009**.

- Artursson P. and Magnusson C. "**Epithelial transport of drugs in cell culture. II: Effect of extracellular calcium concentration on the paracellular transport of drugs of different lipophilicities across monolayers of intestinal epithelial (Caco-2) cells".** *J Pharm Sci*, 79 (7): 595-600, **1990.**
- Artursson P., Palm K. and Luthman K. "**Caco-2 monolayers in experimental and theoretical predictions of drug transport".** *Adv Drug Deliv Rev*, 46 (1-3): 27-43, **2001**.
- Avram M.M., Avram A.S., and James W.D. "**Subcutaneous fat in normal and diseased states: 3. Adipogenesis: from stem cell to fat cell**." *Journal of the American Academy of Dermatology* 56.3: 472-492, **2007**.
- Bai Yuntao and Qinghua Sun. "**Macrophage recruitment in obese adipose tissue**." *Obesity reviews* 16.2: 127-136, **2015**.
- Bajaj M., Suraamornkul S., Hardies L.J., Pratipanawatr T., De Fronzo R.A. **"Plasma resistin concentration, hepatic fat content, and hepatic and peripheral insulin resistance in pioglitazone-treated type II diabetic patients"**. *International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity*; 28, 783–789, **2004**.
- Baker R.D., Baker S.S. and La Rosa K. "**Polarized Caco-2 cells. Effect of reactive oxygen metabolites on enterocyte barrier function".** *Dig Dis Sci*, 40 (3): 510-518, **1995**.
- Baker R.G., Matthew S. Hayden, and Sankar Ghosh. "**NF-κB, inflammation, and metabolic disease**." *Cell metabolism* 13.1: 11-22, **2011**.
- Bendelac A., Savage P.B. and Teyton, L."**The biology of NKT cells"**. *Annu.Rev.Immunol.* 25, 297–336*,* **2007.** *doi:10.1146/annurev.immunol.25.022106.141711*
- Benomar Y., Gertler A., de Lacy P., Cre-pin D., Hamouda H.O., Riffault L., et al."**Central resistin over- exposure induces insulin resistance through toll-like receptor4".** *Diabetes* 62, 102–114, **2013**. doi:10.2337/db12-0237
- Bernstein C.N., Rawsthorne P., Cheang M., Blanchard J.F. "**A population-based case control study of potential risk factors for IBD".** *Am J Gastroenterol.* 101:993–1002, **2006**.
- Bertola A., Ciucci T., Rousseau D., Bourlier V., Duffaut C., Bonnafous S. "**Identification of adipose tissue dendritic cells correlated with obesity-associated insulin-resistance and inducing Th17 responses in mice and patients"** *Diabetes* 61, 2238–2247, **2012**. doi:10.2337/db11-1274
- Bjørbæck C., Kahn B.B. "**Leptin signaling in the central nervous system and the periphery"**. *Recent progress in hormone research*, 59, 305-331, **2004**.
- Blüher Matthias. "**Adipose tissue dysfunction contributes to obesity related metabolic diseases.**" *Best practice & research Clinical endocrinology & metabolism;* 27.2: 163-177*,* **2013**.
- Bokarewa M., Nagaev I., Dahlberg L., Smith U., and Tarkowski A. "**Resistin, an adipokine with potent proinflammatory properties"**. *J. Immunol.* 174, 5789–5795, **2005**.
- Borst Stephen E. "**The role of TNF-α in insulin resistance**." *Endocrine* 23.2-3: 177-182, **2004**.

- Brasaemle D.L. "**Thematic review series: adipocyte biology**. **The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis"**. *Journal of Lipid Research*, 48 *2547–2559,* **2007**
- Bruunsgaard H., Pedersen B.K. "**Age-related inflammatory cytokines and disease"**. *Immunol Allergy Clin North Am;* 23: 15– 39, **2003**.
- Cachofeiro V., Miana M., Martín B. "**Obesidad, inflamación y disfunción endotelial"**. *Rev. Esp. Obes.* 4, 195–204, **2006**.
- Cani P.D., Amar J., Iglesias M.A., Poggi M., Knauf C., Bastelica D., Neyrinck A.M., Fava F., Tuohy K.M., Chabo C., Waget A., Delmee E., Cousin B., Sulpice T., Chamontin B., Ferrieres J., Tanti J.F., Gibson G.R., Casteilla L., Delzenne N.M., Alessi M.C., Burcelin R. "**Metabolic endotoxemia initiates obesity and insulin resistance"**. *Diabetes 56: 1761–1772,* **2007**.
- Cannon B., and Nedergaard J.A.N. "**Brown adipose tissue: function and physiological significance."** *Physiological reviews* 84.1: 277-359, **2004**.
- Cao H., Sekiya M., Erikci Ertunc M., Burak M.F., Mayers J.R., White A., Inouye K.,Rickey L.M., Ercal B.C., Furuhashi M., et al. "**Adipocyte lipid chaperone AP2 is a secreted adipokine regulating hepatic glucose production."** *Cell Metab.* 17**:** 768–778*,* **2013.**
- Cao S., Zhang X., Edwards J. P., & Mosser D. M. **NF-κB1 (p50) homodimers differentially regulate pro-and anti-inflammatory cytokines in macrophages**. *Journal of Biological Chemistry,* **2006**.
- Capurso C., Capurso A. "**From excess adiposity to insulin resistance: The role of free fatty acids"**. *Vascul. Pharmacol* 57, 91–97, **2012**.
- Carkeet C., Clevidence B.A., and Novotny J.A. "**Anthocyanin excretion by humans increases linearly with increasing strawberry dose"**. *J Nutr* 138, 897-902, **2008**.
- Carpene C., Dray C., Attane C.,Valet P., Portillo M.P., Churruca I., et al. "**Expanding role for the apelin/APJ system in physio pathology"**. *J. Physiol.Biochem.* 63, 359–373, **2007**. doi:10.1007/BF03165767
- Carstensen-Kirberg M., Hatziagelaki E., Tsiavou A., Chounta A., Nowotny P., Pacini G. "**Sfrp5 associates with beta-cell function in humans"**. *Eur J Clin Invest* 46, 535-543*,* **2016**.
- Carstensen-Kirberg M., Kannenberg J.M., Huth C., Meisinger C., Koenig W., Heier M. "**Inverse associations between serum levels of secreted frizzled-related protein-5 (SFRP5) and multiple cardiometabolic risk factors: KORA F4 study"**. *Cardiovasc Diabetol* 16, 109*,* **2017**.
- Cassidy A., Mukamal K.J., Liu L., Franz M., Eliassen A.H., and Rimm, E.B. "**High anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women".** *Circulation* 127,188-196*,* **2013**.
- Cassidy A., O'Reilly EJ., Kay C., Sampson L., Franz M., Forman JP., et al. "**Habitual intake of flavonoid subclasses and incident hypertension in adults**". *Am J Clin Nutr;*93(2):338–47*,* **2011**.
- Castañeda-Ovando A., De Lourdes Pacheco-Hernández M., Páez-Hernández E. "**Chemical studies of anthocyanins: a review"**. *Food Chem*;113(4):859– 871, **2009**.

- Ceddia R.B., Somwar R., Maida A., Fang X., Bikopoulos G., Sweeney G. "**Globular adiponectin increases GLUT4 translocation and glucose uptake but reduces glycogen synthesis in rat skeletal muscle cells"**. *Diabetologia*, 48(1):132-9, **2005**
- Cerf Marlon E. "**Beta cell dysfunction and insulin resistance**." *Frontiers in endocrinology* 4: 37, **2013**.
- Charbonneau A., Marette A. "**Inducible nitric oxide synthase induction underlies lipid-induced hepatic insulin resistance in mice: Potential role of tyrosine nitration of insulin signaling proteins"**. Diabetes ;59:861e71, **2010**.
- Chen A.Y., Chen Y.C. "**A review of the dietary flavonoid, kaempferol on human health and cancer chemoprevention**". *Food Chem; 138, 2099−2107,* **2013**.
- Chen L., Zhao X., Liang G., Sun J., Lin Z., Hu R. "**Recombinant SFRP5 protein significantly alleviated intrahepatic inflammation of nonalcoholic steatohepatitis"**. *Nutr Metab (Lond)* 14, 56, **2017.**
- Chen W., Müller D., Richling E., Wink M. "**Anthocyanin-rich purple wheat prolongs the life span of Caenorhabditis elegans probably by activating the DAF-16/FOXO transcription factor**." *Journal of agricultural and food chemistry 61.12*: 3047-3053, **2013**.
- Chun O.K., Chung S.J., Song W.O. "**Estimated dietary flavonoid intake and major food sources of U.S. adults."** *J Nutr. 137, 1244–1252,* **2007.**
- Cimino F., Speciale A., Anwar S., Canali R., Ricciardi E., Virgili F., Trombetta D., and Saija, A. "**Anthocyanins protect human endothelial cells from mild hyperoxia damage through modulation of Nrf2 pathway**". *Genes Nutr*; 8, 391-399*,* **2013**.
- Cinti S, Mitchell G, Barbatelli G, Murano I., Ceresi E., Faloia E., Obin M.S. "**Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans**" *J Lipid Res*;46:2347-55*,* **2005**.
- Clement K., Viguerie N., Poitou C., Carette C., Pelloux V., Curat C.A., Sicard A., Rome S., Benis A., Zucker J.D., Vidal H., Laville M., Barsh G.S., Basdevant A., Stich V., Cancello R., Langin D. "**Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects".** FASEB J; 18: 1657–1669, **2004**.
- Clifford M.N. "**Anthocyanins-nature, occurrence and dietary burden"**. Journal of the Science of Food and Agriculture 80, 1063-1072, **2000**.
- Crozier A., Del Rio D., Clifford M.N. "**Bioavailability of dietary flavonoids and phenolic compounds"**. *Mol Aspects Med*. 31, 446−467, **2010.**
- Cui X.B., and Chen S.Y. "**White adipose tissue browning and obesity**." *Journal of biomedical research 31*(1), 1, **2017**.
- Curat C. A., Miranville A., Sengenès C., Diehl M., Tonus C., Busse R., & Bouloumié, A. **"From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes"**. *Diabetes,* 53(5), 1285-1292*,* **2004**.
- D'amore S., Palasciano G., and Moschetta A. "**Peroxisome proliferator-activated receptors (PPARS), metabolic sindrom and atherosclerosis**." *Giornale Italiano dell'Arteriosclerosi* 4.2: 8-22, **2013**.

- Day R.T., Cavaglieri R.C., and Feliers D. "**Apelin retards the progression of diabetic nephropathy"**. *Am.J.Physiol. Renal Physiol*. 304, F788–F800, **2013**.
- De Pascual-Teresa S., Moreno D.A., and Garcia-Viguera, C. "**Flavanols and anthocyanins in cardiovascular health: a review of current evidence**". *Int J Mol Sci;* 11, 1679-1703*,* **2010**.
- De Pascual-Teresa S., Sanchez-Ballesta M.T. "**Anthocyanins: from plant to health".** *Phytochem Rev* 7:281–299, **2008.**
- De Pascual-Teresa S., Santos-Buelga C., & Rivas-Gonzalo J. C. **"Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages"**. *Journal of Agricultural and Food Chemistry* ; 48(11), 5331-5337, **2000**.
- Dei Cas A., Spigoni V., Metra M., & Dei Cas, L. "**Insulino-resistenza e rischio cardiovascolare**." *G Ital Cardiol 10.*Suppl 1: 93-97*,* **2010**.
- Del Rio D., Rodriguez-Mateos A., Spencer J.P, Tognolini M., Borges G., Crozier A. "**Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases".** *Antioxid Redox Signal*. 18, 1818−1892, **2013.**
- Dimitriadis G., Mitrou P., Lambadiari V., Maratou E., & Raptis S. A. "**Insulin effects in muscle and adipose tissue**". *Diabetes research and clinical practice*, *93*, S52-S59, **2011**.
- Dinkova-Kostova A.T., & Talalay P. **"Direct and indirect antioxidant properties of inducers of cytoprotective proteins"**. *Molecular nutrition & food research*, 52(S1), S128-S138, **2008**.
- Diradourian C., Girard J., Pegorier J.P. "**Phosphorylation of PPARs: from molecular characterization to physiological relevance"**. *Biochimie*; 87:33–38, **2005**.
- Dominguez H., Storgaard C., Rask-Madsen et al. "**Metabolic and vascular effects of tumor necrosis factor-α blockade with etanercept in obese patients with type 2 diabetes**," *Journal of Vascular Research*, vol. 42, no. 6, pp. 517–525, **2005**.
- Domitrovic. "**The Molecular Basis for the Pharmacological Activity of Anthocyans"**. *Current Medicinal Chemistry*, 18, 4454-4469, **2011**.
- Duffaut C., Zakaroff-Girard A., Bourlier V., et al. "**Interplay between human adipocytes and T lymphocytes in obesity: CCL20 as an adipochemokine and T lymphocytes as lipogenic modulators"**. *Arterioscler Thromb Vasc Biol*; 29:1608-14, **2009**.
- Dulloo A.G., Jacquet J., Solinas G., Montani J.P., Schutz Y. "**Body composition phenotypes in pathways to obesity and the metabolic syndrome"** *Int. J. Obes; 34* (Suppl. 2), S4–S17, **2010**.
- Duncan R.E., Ahmadian M., Jaworski K., Sarkadi-Nagy E., Sul H. S. "**Regulation of lipolysis in adipocytes**." *Annu. Rev. Nutr.* 27: 79-101, **2007**.
- Ebbert J.O., and Jensen M.D. "**Fat depots, free fatty acids, and dyslipidemia**." *Nutrients* 5.2: 498-508, **2013**.
- Ehrlund A., Mejhert N., Lorente-Cebrian S., Astrom G., Dahlman I., Laurencikiene J., et al. "**Characterization of the Wnt inhibitors secreted frizzled-related proteins (SFRPs) in human adipose tissue"**. *J Clin Endocrinol Metab* 98, E503-508, **2013**.

- Engin, Ayse Basak. "**What is lipotoxicity?**" *Obesity and Lipotoxicity. Springer, Cham* . 197-22 , **2017**.
- Ertunc M.E., Hotamisligil G.S. "**Lipid signaling and lipotoxicity in metaflammation Indications for metabolic disease pathogenesis and treatment**" *J Lipid Res*. 57(12):2099-2114, **2016.**
- Esposito K., Ciotola M., Giugliano D. "**Oxidative stress in the Metabolic Syndrome"**. J. *Endocrinol. Invest.* **2006**, 29, 791–795. Int. J. Mol. Sci, 123131, **2011**.
- Evans J.L., Maddux B.A., Goldfine I.D. "**The molecular basis for oxidative stress-induced insulin resistance"**. *Antioxid Redox Signal*; 7(7–8):1040–52, **2005**.
- Fain J.N, Madan A.K., Hiller M.L., et al. "**Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans"**. *Endocrinology*;145:2273-82, **2004**.
- Fantuzzi, Giamila. "**Adipose tissue, adipokines, and inflammation**." *Journal of Allergy and Clinical Immunology* 115.5: 911-919, **2005**.
- Farese R.V., and T. C. Walther. **"Lipid droplets finally get a little R-E-S-P-E-C-T"** *Cell*. 139: 855–860, **2009**.
- Faria A., Fernandes I., Norberto S., Mateus N., Calhau C. "**Interplay between Anthocyanins and Gut Microbiota".** *J Agric Food Chem*. 23;62(29):6898-902, **2014**.
- Farjo K.M., Farjo R.A., Halsey S., Moiseyev G., and Ma J.X. "**Retinol-binding protein 4 induces inflammation in human endothelial cells by a NADPH oxidase and nuclear factor kappa b dependent and retinol independent mechanism".** *Mol.Cell.Biol*. 32, 5103–5115, **2012**.
- Felgines C., Talavera S., Gonthier M.P., Texier O. "**Strawberry anthocyanins are recovered in urine as glucuro- and sulfoconjugates in humans".** J *Nutr.* 133, 1296−1301, **2003**.
- *Felicity* S.N., Su H., Tornqvist H., Whitehead J.P., Prins J.B., Hutley L.J., Prins J.B. "**Characterization of the transcriptional and functional effects of fibroblast growth factor-1 on human preadipocyte differentiation**". *The FASEB journal*, *20*(14), 2615-2617, **2006**.
- Fernández-Sánchez A., Madrigal-Santillán E., Mirandeli B., Esquivel-Soto J., Morales-González A., Esquivel-Chirino C., Durante-Montiel I., Sánchez-Rivera G., Valadez-Vega C., and Morales-González J.A. "**Inflammation, Oxidative Stress, and Obesity"** Int J Mol Sci; 12(5): 3117–3132, **2011**.
- Ferrari D., Cimino F., Fratantonio D., Molonia M.S., Bashllari R., Busà, R., Speciale, A. "**Cyanidin-3-O-glucoside modulates the in vitro inflammatory crosstalk between intestinal epithelial and endothelial cells"** *Mediators of inflammation*, **2017**.
- Ferrari D., Speciale A., Cristani M., Fratantonio D., Molonia M.S., Ranaldi G., Cimino F. "**Cyanidin-3-O-glucoside inhibits NF-kB signalling in intestinal epithelial cells exposed to TNF-α and exerts protective effects via Nrf2 pathway activation"**. *Toxicology letters*, *264*, 51-58, **2016**.

- Ferré P. "**The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity**." Diabetes 53.suppl 1: S43-S50, **2004**.
- Fève Bruno. "**Adipogenesis: cellular and molecular aspects**." *Best Practice & Research Clinical Endocrinology & Metabolism* 19.4: 483-499, **2005**.
- Fischer-Posovszky P., Newell F. S., Wabitsch M., & Tornqvist, H. E "**Human SGBS cells–a unique tool for studies of human fat cell biology.**" *Obesity facts* 1.4: 184-189*,* **2008**.
- Floyd Z.E., Stephens J.M. "**Controlling a master switch of adipocyte development and insulin sensitivity: Covalent modifications of PPAR gamma".** *Biochim Biophys Acta***;** 1822(7):1090–5, **2012**.
- Fonseca-Alaniz M.H., Takada J., Alonso-Vale M.I., Lima F.B. "**Adipose tissue as an endocrine organ: From theory to practice"**. *J. Pediatr* 83 (Suppl. 5),S192–S203, **2007**.
- Forman H.J., Davies K.J., Ursini F. "**How do nutritional antioxidants really work: nucleophilic tone and parahormesis versus free radical scavenging in vivo"**. *Free Radic Biol Med* 66, 24-35, **2014**.
- Franckhauser S., Elias I., Rotter Sopasakis V., Ferre T., Nagaev I., Andersson C.X., Agudo J., Ruberte J., Bosch F., Smith U. "**Overexpression of IL-6 leads to hyperinsulinaemia, liver inflammation and reduced body weight in mice"**. *Diabetologia;* 51: 1306–1316, **2008**.
- Fratantonio D., Cimino F., Molonia M.S., Ferrari D., Saija A., Virgili F., Speciale A.**"Cyanidin-3-O-glucoside ameliorates palmitate-induced insulin resistance by modulating IRS-1 phosphorylation and release of endothelial derived vasoactive factors"**. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1862*(3), 351-357, **2017**.
- Frayn K.N., Karpe F., Fielding B.A., et al. "**Integrative physiology of human adipose tissue".** *Int J Obes Relat Metab Disord*; 27:875-88, **2003**.
- Fu S., Fan J., Blanco J., Gimenez-Cassina A., Danial N.N., Watkins S.M., and Hotamisligil G.S. "**Polysome profiling in liver identifies dynamic regulation of endoplasmic reticulum translatomeby obesity and fasting"**. *PLoS Genet*. 8:e1002902, **2012**.
- Fu Y., Luo N., Klein R.L., Garvey W.T. "**Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation"**. *Journal of lipid Research*, 46(7):1369-79, **2005**.
- Furuhashi M., Hotamisligil G.S."**Fatty acid-binding protein 4 (FABP4): pathophysiological insights and potent clinical biomarker of metabolic and cardiovascular diseases**." *Clinical Medicine Insights: Cardiology* 8: CMC-S17067, **2014**.
- Gabrielsen J.S., Gao Y., Simcox J.A., Huang J., Thorup D., Jones D. **Adipocyte iron regulates adiponectin and insulin sensitivity"**. *J. Clin.Invest*. 122, 3529–3540, **2012**. doi:10.1172/JCI44421
- Galgani J.E., Moro C., and Ravussin E. "**Metabolic flexibility and insulin resistance.**" *American journal of physiology-endocrinology and metabolism* 295.5: E1009-E1017, **2008**.

- Gan L., Guo K.,Cremona M.L., Mc Graw T.E.,Leibel R.L., and Zhang,Y. "**TNF-alpha up- regulates protein level and cell surface expression of the leptin receptor by stimulating its export via a PKC-dependent mechanism**". Endocrinology 153, 5821– 5833, **2012**.
- Gao Z., Zhang X., Zuberi A., Hwang D., Quon M.J., Lefevre M., Ye J. "**Inhibition of insulin sensitivity by free fatty acids requires activation of multiple serine kinases in 3T3-L1 adipocytes"**. *Mol Endocrino*; 18: 2024-2034, **2004**.
- Garcia-Tobilla P., Solorzano S.R., Salido-Guadarrama I., Gonzalez-Covarrubias V., Morales-Montor G., Diaz-Otanez C.E. "**SFRP1 repression in prostate cancer is triggered by two different epigenetic mechanisms".** *Gene* 593, 292-301, **2016**.
- Gesta S., Tseng Y-H., Kahn R. "**Developmental origin of fat: tracking obesity to its source cell"**;131:242-256, **2007**.
- Gonzalez-Gay M.A., De Matias J.M., Gonzalez-Juanatey C., Garcia-Porrua C., Sanchez-Andrade A., Martin J. "**Anti- tumor necrosis factor-alpha block and improves insulin resistance in patients with rheumatoid arthritis".** *Clin. Exp.Rheumatol*. 24, 83–86, **2006**.
- Goossens G.H., Blaak E.E., Van Baak M.A. "**Possible involvement of the adipose tissue renin-angiotensin system in the pathophysiology of obesity and obesity-related disorders"**. *Obes Rev* , 4: 43-55, **2003**.
- Graham T.E.,Yang Q., Bluher M., Hammarstedt A.,Cia- raldi T.P., Henry R.R. "**Retinol-binding protein 4 and insulin resistance in lean, obese,and diabetic subjects"** *N. Engl.J.Med*, **2006**.
- Grant R.W., Dixit V.D. "**Adipose tissue as an immunological organ**". Obesity **2015.**
- Green Howard, and Olaniyi Kehinde. "**An established preadipose cell line and its differentiation in culture II. Factors affecting the adipose conversion**." Cell 5.1: 19-27, **1975.**
- Greenberg A.S., and Martin S. Obin. "**Obesity and the role of adipose tissue in inflammation and metabolism**–." *The American journal of clinical nutrition* 83.2: 461S-465S, **2006**.
- Greene M.W., Sakaue H., Wang L., Alessi D.R., Roth R.A. "**Modulation of insulin-stimulated degradation of human insulin receptor substrate substrate-1 by Serine 312 phosphorylation"**. J *Biol Chem*; 278: 8199-8211, **2003**.
- Gregoire FM., Smas CM., Sul HS., "**Understanding adipocyte differentiation**". *Physiological Reviews,* 78: 783–809*,* **1998**.
- Gregor M.F. and Hotamisligil G.S. "**Inflammatory mechanisms in obesity**," *Annual Review of Immunology*, vol. 29, pp. 415–445, **2011.**
- Gual P., Le Marchand-Brustel Y., and Tanti JF. "**Positive and negative regulation of insulin signaling through IRS-1 phosphorylation**". *Biochimie;* 87: 99-109*,* **2005**.
- Guilherme A., Virbasius J.V., Puri V., & Czech, M. P. "**Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes**." *Nature reviews Molecular cell biology* 9.5: 367, **2008**.

- Guo H., Ling W., Wang Q., Liu C., Hu Y., Xia M. "**Effect of anthocyanin-rich extract from black rice (Oryza sativa L. indica) on hyperlipidemia and insulin resistance in fructose-fed rats**". *Plant Foods Hum Nutr*;62(1):1–6, **2007**.
- Guo H., LingW.,Wang Q., Liu C., Hu Y., Xia M. "**Cyanidin 3-glucoside protects 3T3-L1 adipocytes against H2O2- or TNF-alpha-induced insulin resistance by inhibiting c-Jun NH2-terminal kinase activation**". *Biochem Pharmacol*;75(6):1393–401, **2008**.
- Guo H., Liu G., Zhong R., Wang Y., Wang D., Xia M. "**Cyanidin-3-O beta-glucoside regulates fatty acid metabolism via an AMP-activated protein kinase-dependent signaling pathway in human HepG2 cells**". *Lipids Health Dis*;11:10, **2012**.
- Gurnell, Mark. "**Peroxisome proliferator-activated receptor γ and the regulation of adipocyte function: lessons from human genetic studies**." *Best practice & research Clinical endocrinology & metabolism* 19.4: 501-523, **2005**.
- Gutiérrez-Rodelo C., Roura-Guiberna A., and Olivares-Reyes J.A. "**Molecular mechanisms of insulin-resistance: an update.**" *Gaceta medica de Mexico;* 153.2: 214-228, **2017**.
- Hämäläinen M., Nieminen R., Vuorela P., Heinonen M., Moilanen E. "**Antiinflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT1 and NF-kB activations, whereas flavones, isorhamnetin naringenin, and pelargonidin inhibit only NF-kB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages**". *Mediators Inflamm*. 45673, **2007**.
- Harborne J.B., and Williams C.A. "**Advances in flavonoid research since 1992**." *Phytochemistry* 55.6: 481-504, **2000**.
- Harms M., Seale P. "**Brown and beige fat: development, function and therapeutic potential**". *Nat Med*, 19(10): 1252–1263, **2013**.
- Hauser S, Adelmant G., Sarraf P., Wright H.M., Mueller E., Spiegelman B.M. "**Degradation of the peroxisome proliferator-activated receptor γ is linked to liganddependent activation**". *J Biol Chem* ;275:18527–18533, **2000.**
- Hausman D.B., Di Girolamo M., Bartness T.J., Hausman G.J., Martin, R.J. "**The biology of white adipocyte proliferation**." *Obesity reviews* 2.4: 239-254, **2009**.
- Hayden M.S., Ghosh S. "**Regulation of NF-κB by TNF family cytokines**". *Semin Immunol.;* 26(3):253-66, **2014**.
- He J., Giusti M.M. "**Anthocyanins: natural colorants with health-promoting properties**". *Annu Rev Food Sci Technol*;1:163–187, **2010**.
- Herman M.A, Kahn B.B. "**Glucose transport and sensing in the maintenance of glucose homeostasis and metabolic harmony**". *The Journal of Clinical Investigation*, 116(7):1767-75, **2006.**
- Hertog M.G., Hollman P.C., Katan M.B., and Kromhout, D. "**Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands".** *Nutr Cancer* 20, 21-29, **1993**.
- Hoesel B., Schmid J.A. "**The complexity of NF-kB signaling in inflammation and cancer**". *Mol Cancer*;12:86, **2013**.

- Holland W.L., Bikman B.T., Wang L.P.,Yuguang G., Sargent K.M., S. Bulchand, T. A. Knotts, G. Shui, D. J. Clegg, M.R. Wenk. "**Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice".** *J. Clin. Invest*. 121: 1858–1870, **2011**.
- Hosogai N., Fukuhara A., Oshima K., Miyata Y., Tanaka S., Segawa K., Shimomura I. "**Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation**". *Diabetes*, *56*(4), 901-911, **2007**.
- Hotamisligil G.S. "**Inflammation and metabolic disorders**," *Nature*, vol. 444, no. 7121, pp. 860–867, **2006**.
- Hou D.X., Yanagita T., Uto T., Masuzaki S., Fujii M. "**Anthocyanidins inhibit cyclooxygenase-2 expression in LPS-evoked macrophages: structure-activity relationship and molecular mechanism involved".** *Biochem. Pharmacol.* 70:417- 425, **2005**.
- Hou, De-Xing. "**Potential mechanisms of cancer chemoprevention by anthocyanins**." *Current molecular medicine* 3.2: 149-159, **2003**.
- Hu M.H. "**Dietary Polyphenols as Antioxidants and Anticancer Agents: More Questions than Answers"**. *Chang Gung Med* J, Vol. 34 No. 5, **2011.**
- Hu W., Li L., Yang M., Luo X., Ran W., Liu D. "**Circulating Sfrp5 is a signature of obesity-related metabolic disorders and is regulated by glucose and liraglutide in humans**". *J Clin Endocrinol Metab* **98**, 290-298, **2013**.
- Huang S., Rutkowsky J. M., Snodgrass R.G., Ono-Moore K.D., Schneider D.A., Newman J.W "**Saturated fatty acids activate TLR-mediated pro-inflammatory signaling pathways**." *Journal of lipid research* : jlr-D029546, **2012**.
- Huang W.Y., Davidge S.T., and Wu J. "**Bioactive natural constituents from food sources-potential use in hypertension prevention and treatment"**. *Crit Rev Food Sci Nutr* 53, 615-630, **2013**.
- Huber J., Kiefer F.W., Zeyda M., Ludvik B., Silberhumer G.R., Prager G., Zlabinger G.J., Stulnig T.M: "**CC chemokine and CC chemokine receptor profiles in visceral and subcutaneous adipose tissue are altered in human obesity"**. *J Clin Endocrinol Metab***;** 93: 3215–3221, **2008**.
- Hui X., Lam K.S., Vanhoutte P.M., & Xu A. "**Adiponectin and cardiovascular health: an update**." *British journal of pharmacology* 165.3: 574-590,**2012**.
- Hukshorn C.J.; Lindeman J.H.; Toet K.H.; Saris W.H.; Eilers P.H.;Westerterp-Plantenga M.S.; Kooistra T. "**Leptin and the proinflammatory state associated with human obesity".** *J. Clin. Endocrinol. Metab*, 89, 1773–1778, **2004**.
- Hwang H.H., Moon P.G., Lee J.E., Kim J.G., Lee W., Ryu S.H., Baek M.C. "**Identification of the target proteins of rosiglitazone in 3T3-L1 adipocytes through proteomic analysis of cytosolic and secreted proteins**". *Mol. Cells;* 31 239–246*,* **2011**.
- Hwang Y.P., Choi J.H., Han E.H., Kim H.G., Wee J.H., Jung K.O."**Purple sweet potato anthocyanins attenuate hepatic lipid accumulation through activating adenosine monophosphateactivated protein kinase in human HepG2 cells and obese mice".** *Nutr Res*.;31(12):896–906, **2011**.

- Ichiyanagi T., Shida Y., Rahman M.M., Hatano Y., Konishi T. "**Bioavailability and tissue distribution of anthocyanins in bilberry (Vaccinium myrtillus L.) extract in rats**" J *Agric Food Chem* 54:6578–6587, **2006.**
- Ieronymaki E., Theodorakis E., Aznaourova M., Vaporidi K., Margioris A., Eliopoulos A., & Tsatsanis C. "**Insulin resistance in macrophages results in a distinct metabolic profile promoting an M2-like phenotype affecting both acute and chronic innate immune responses**" *(INM1P. 430)*, **2015.**
- Igal R.A., Wang S., Gonzalez-Baro M., Coleman R.A. "**Mitochondrial glycerol phosphate acyltransferase directs the incorporation of exogenous fatty acids into triacylglycerol".** *J Biol Chem* ;276: 42205–12, **2001**.
- Iikuni N., Lam, K., Queenie L., Lu L., Matarese G., & Cava A.L. "**Leptin and inflammation**." *Current immunology reviews* 4.2: 70-79, **2008**.
- Imai T., Takakuwa R., Marchand S., Dentz E., Bornert J.M., Messaddeq N. "**Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse".** *Proc Natl Acad Sci USA*; 101:4543-4547, **2004**.
  implications for inflammatory diseases". *Nat Rev Immunol*. 9:778-88, **2009**.
- Inouye K.E., Shi H., Howard J.K., Daly C.H., Lord G.M., Rollins B.J. Flier J.S. "**Absence of CC chemokine ligand 2 does not limit obesity-associated infiltration of macrophages into adipose tissue.**" *Diabetes* 56.9: 2242-2250, **2007**.
- Itani S.I., Ruderman N.B., Schmieder F., & Boden G. "**Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IκB-α**." Diabetes 51.7: 2005-2011., **2002**.
- Iwuchukwu O.F., Ajetunmobi J., Ung D., and Nagar S. "**Characterizing the effects of common UDP glucuronosyltransferase (UGT) 1A6 and UGTlAl polymorphisms on cis- and trans-resveratrol glucuronidation**". *Drug Metab Dispos* 37, 1726-1732, **2009.**
- Jager J., Gremeaux T., Cormont M., Le Marchand-Brustel Y., Tanti J.F: "**Interleukin-1 - induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression**". *Endocrinology*; 148: 241–251, **2007**.
- Janssen-Heininger Y.M., Poynter M.E., Baeuerle P.A. "**Recent advances towards understanding redox mechanisms in the activation of nuclear factor kB**". *Free Radic Biol Med*. 28(9):1317-27, **2000.**
- Jaworski K., Sarkadi-Nagy E., Duncan R.E., Ahmadian M., Sul H.S. "**Regulation of triglyceride metabolism. IV. Hormonal regulation of lipolysis in adipose tissue**". 132 American journal of physiology. Gastrointestinal and liver physiology, 293(1):G1-4, **2007.**
- Jayaprakasam B., Olson L.K., Schutzki R.E., Tai M.H., Nair M.G. "**Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in Cornelian cherry (Cornus mas)".** J Agric Food Chem;54(1):243–8, **2006**.
- Jeon S., Han S., Lee J., Hong T., Yim D.S. "**The safety and pharmacokinetics of cyanidin-3-glucoside after 2-week administration of black bean seed coat extract in healthy subjects**". Korean J Physiol Pharmacol 16(4):249–253, **2012**.

- Jeong J.W., Lee W.S., Shin S.C., Kim G.Y., Choi B.T., Choi Y.H. "**Anthocyanins downregulate lipopolysaccharide-induced inflammatory responses in BV2microglial cells by suppressing the NF-κB and Akt/MAPKs signaling pathways".** Int J Mol Sci;14(1):1502– 15,**2013**.
- Jianping. "**Mechanisms of insulin resistance in obesity**." *Frontiers of medicine* 7.1: 14-24,**2013**.
- Johnson M.H., Wallig M., Luna Vital D.A., De Mejia E.G. "**Alcohol-free fermented blueberry–blackberry beverage phenolic extract attenuates diet-induced obesity and blood glucose in C57BL/6J mice"**. *The Journal of nutritional biochemistry;* 31, 45-59, **2016**.
- Johnson P.R., and Francendese A.A., "**Cellular regulation of adipose tissue growth**" *Journal of Animal Science 61.Supplement_2 :* 57-75, **1985**.
- Jones D.P., Park Y., Gletsu-Miller N. Liang Y., Yu T., Accardi C. J., Ziegler T.R. "**Dietary sulfur amino acid effects on fasting plasma cysteine/cystine redox potential in humans".** *Nutrition*, vol. 27, no. 2, pp. 199–20, **2011.**
- Ju J.H., Yoon H.S., Park H.J., Kim M.Y., Shin H.K., Park K.Y. "**Anti-obesity and antioxidative effects of purple sweet potato extract in 3T3-L1 adipocytes in vitro"**. *J Med Food.*;14: 1097– 1106, **2011**.
- Kadowaki T., Yamauchi T., Kubota N., Hara K., Ueki K., & Tobe K. "**Adiponectin and adiponectin receptors in insulin-resistance, diabetes, and the metabolic syndrome**." *The Journal of clinical investigation* 116.7: 1784-1792, **2006**.
- Kahn B.B., and J.S. Flier. "**Obesity and insulin resistance**." *The Journal of clinical investigation* 106.4: 473-481, **2000**.
- Kanda H., Tateya S., Tamori Y., Kotani K., Hiasa K.I., Kitazawa R., Kitazawa S., Miyachi H., Maeda S., Egashira K., 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**.
- Karlsen A., Retterstol L., Laake P., Paur I., Bohn S.K., Sandvik L. "**Anthocyanins inhibit nuclear factor-kappaB activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults"**. J Nutr;137(8):1951–4, **2007**.
- Karpe F., Dickmann J.R., Frayn K.N. "**Fatty acids, obesity, and insulin resistance: Time for a reevaluation"**. Diabetes , 60, 2441–2449, **2011**.
- Kay C.D. "**Analysis of the bioactivity, metabolism, and pharmacokinetics of anthocyanins in humans"**. PhD thesis. University of Guelph, Ontario, Canada, pp. 1-9, **2004.**
- Kay C.D., Hooper L., Kroon P.A., Rimm E.B., Cassidy A. "**Relative impact of flavonoid composition, dose and structure on vascular function: a systematic review of randomised controlled trials of flavonoid-rich food products".** *Mol Nutr Food Res* 56(11):1605–1616, **2012.**
- Kay C.D., Mazza G., Holub B.J. "**Anthocyanins exist in the circulation primarily as metabolites in adult men"**. *J. Nutr*., 135, 2582-2588, **2005.**
- Kelesidis T., Kelesidis I., Chou S., & Mantzoros C.S. "**Narrative review: the role of leptin in human physiology: emerging clinical applications**." *Annals of internal medicine* 152.2: 93-100, **2010**.

- Kershaw E.E., Flier J.S., "**Adipose tissue as an endocrine organ**". *J Clin Endocrinol Metab ;*89:2548-56*,* **2004**.
- Khoo H.E., Azlan A., Tang S.T., & Lim S.M. "**Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits**". *Food & nutrition research*, *61*(1), 1361779, **2017**.
- Kiec-Wilk B., Dembinska-Kiec A., Olszanecka A., Bodzioch M., Kawecka-Jaszcz K. "**The selected pathophysiological aspects of PPARs activation**". J Physiol Pharmacol;56:149– 62, **2005**.
- Kiefer F.W., Zeyda M., Todoric J., Huber J., Geyeregger R., Weichhart T., Aszmann O., Ludvik B., Silberhumer G.R., Prager G., Stulnig T.M: "**Osteopontin expression in human and murine obesity: extensive local up-regulation in adipose tissue but minimal systemic alterations**".Endocrinology; 149: 1350–1357, **2008**.
- Kievit P., Howard J.K., Badman M.K., Balthasar N., Coppari R., Mori,H."**Enhanced leptin sensitivity and improved glucose homeostasis in mice lacking suppressor of cytokine signaling-3 in POMC-expressing cells**". *Cell Metab*. 4, 123–132, **2006**.
- Kim J., Bachmann R.A, and Chen J. "**Interleukin-6 and insulin resistance.**" *Vitamins & Hormones* 80: 613-633, **2009**.
- Kim J.M., Kim K.M., Park E.H., Seo J.H., Song JY., Shin S.C. "**Anthocyanins from black soybean inhibit Helicobacter pylori-induced inflammation in human gastric epithelial AGS cells**". *Microbiol Immunol*.; 57:366–373, **2013**.
- Kim Jeong-A., Kwang K. K., and Quon M.J. "**The union of vascular and metabolic actions of insulin in sickness and in health.**" 889-891*;* **2005**.
- Kim K.A., Kim J.H., Wang Y., Sul H.S. "**Pref-1 (preadipocyte factor 1) activates the MEK/extracellular signal-regulated kinase pathway to inhibit adipocyte differentiation".** *Molecular and cellular biology*, 27(6):2294-308, **2007.**
- Kim S.M., Chung M.J., Ha T.J., Choi H.N., Jang S.J., Kim S.O. "**Neuroprotective effects of black soybean anthocyanins via inactivation of ASK1-JNK/p38 pathways and mobilization of cellular sialic acids".** *Life Sci*;90:874–882, **2012.**
- Kirk E.A., Sagawa Z.K., Mc Donald T.O., O'Brien K.D., and Heinecke J.W. **"Monocyte chemo attractant protein deficiency fails to restrain macrophage infiltration into adipose tissue"** *Diabetes* 57, 1254–1261, **2008**.
- Kitade H.,Sawamoto K.,Nagashimada M., Inoue H.,Yamamoto Y.,Sai, Y. **"CCR5 plays a critical role in obesity induced adipose tissue inflammation and insulin resistance by regulating both macrophage recruitment and M1/M2 status".** *Diabetes* 61, 1680–1690, **2012**.
- Knight J.A. "**Diseases and disorders associated with excess body weight**". *Ann. Clin. Lab Sci*., 41, 107–121, **2011**.
- Kong J.M., Chia L.S., Goh N.K., Chia T.F., Brouillard R. "**Analysis and biological activities of anthocyanins".** *Phytochemistry*. 64, 923–933, **2003.**
- Kroon P.A., Clifford M.N., Crozier A., Day A.J., Donovan J.L., Manach C., Williamson G. "**How should we assess the effects of exposure to dietary polyphenols in vitro?"** *Am J Clin Nutr*. 80, 15−21, **2004.**

- Kumada M., Kihara S., Ouchi N., Kobayashi H.,Okamoto Y., Ohashi K. **“Adiponectin specifically increased tissue inhibitor of metallo proteinase-1 through interleukin-10 expression in human macrophages”**. Circulation 109, 2046–2049, **2004**.
- Kurebayashi S., Sumitani S., Kasayama S., Jette A.M., & Hirose T. "**TNF-α inhibits 3T3-L1 adipocyte differentiation without downregulating the expression of C/EBPβ and δ."** *Endocrine journal* 48.2: 249-253, **2001**.
- Kurimoto Y., Shibayama Y., Inoue S., Soga M., Takikawa M., Ito C. **“Black soybean seed coat extract ameliorates hyperglycemia and insulin sensitivity via the activation of AMP-activated protein kinase in diabetic mice”.** *J Agric Food Chem*;61(23):5558–64, **2013**.
- Kusminski C.M., Mc Ternan P.G., Kumar S. “**Role of resistin in obesity, insulin resistance and Type II diabetes”.** *Clinical science (London, England : 1979*), 109, 243–256, **2005.**
- La Cava A., Matarese G. “**The weight of leptin in immunity”.** *Nature reviews. Immunology*, 4, 371–379, **2004.**
- Laleh G.H., Frydoonfar H., Heidary R., Jameei R., & Zare S. “**The effect of light, temperature, pH and species on stability of anthocyanin pigments in four Berberis species”**. *Pak J Nutr.*;5(1):90–92, **2006**.
- Lampe, J.W. “**Diet, genetic polymorphisms, detoxification, and health risks”**. *Altern Ther Health Med* 13, S108-111, **2007**.
- Langhi C., and Baldán A. "**CIDEC/FSP27 is regulated by peroxisome proliferator-activated receptor alpha and plays a critical role in fasting-and diet-induced hepatosteatosis**." *Hepatology* 61.4: 1227-1238, **2015**.
- Large V., Peroni O., Letexier D., Ray H., Beylot M. “**Metabolism of lipids in human white adipocyte”**. *Diabetes Metab*; 30:294-309, **2004**.
- Lasa A., Churruca I., Eseberri I., Andrés-Lacueva C., & Portillo, M. P. "**Delipidating effect of resveratrol metabolites in 3T3-L1 adipocytes**." *Molecular nutrition & food research* 56.10: 1559-1568, **2012**.
- Lastra G., Manrique C.M., Hayden M.R. “**The role of beta-cell dysfunction in the cardiometabolic syndrome”**. *J. Cardiometab. Syndr*, 1, 41–46, **2006**.
- Lauterbach M.A.R., and Wunderlich F.T. "**Macrophage function in obesity-induced inflammation and insulin resistance**." *Pflügers Archiv-European Journal of Physiology* 469.3-4: 385-396, **2017**.
- Lawrence Toby. "**The nuclear factor NF-κB pathway in inflammation**." *Cold Spring Harbor perspectives in biology*: a001651, **2009**.
- Lazzè M.C., Pizzala R., Savio M., Stivala L.A., Prosperi E., Bianchi L. “**Anthocyanins protect against DNA damage induced by tert-butyl-hydroperoxide in rat smooth muscle and hepatoma cells**”. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *535*(1), 103-115, **2003**.
- Lazzè M.C., Savio M., Pizzala R., Cazzalini O., Perucca P., Scovassi A.I., Bianchi L. “**Anthocyanins induce cell cycle perturbations and apoptosis in different human cell lines**”. *Carcinogenesis*, *25*(8), 1427-1433, **2004**.
- Le Ferrec E., Chesne C., Artusson P., Brayden D., Fabre G., Gires P., Guillou F., Rousset M., Rubas W. and Scarino M.L. “**In vitro models of the intestinal**

barrier". The report and recommendations of ECVAM Workshop 46. European Centre for the Validation of Alternative methods. *Altern Lab Anim*, 29 (6): 649-668, **2001**.

- Lechleitner M. "**Obesity and the metabolic syndrome in the elderly – a mini-review"**. *Gerontology*; 54: 253–259, **2008**.
- Lee J., Finn C.E., Wrolstad R.E. "**Comparison of anthocyanin pigment and ather phenolic compounds of Vaccinium membranaceum and Vaccinium ovatum native to the Pacific Northwest of North America"**. *J. Agric. Food. Chem.* 52: 7039-7044, **2004.**
- Lee J.Y., Sohn K.H., Rhee S.H., and D. Hwang. "**Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4"**. J. *Biol.Chem*. 276: 16683–16689, **2001**.
- Lee Ji-Eun, and Kai Ge. "**Transcriptional and epigenetic regulation of PPARγ expression during adipogenesis**." *Cell & bioscience* 4.1: 29, **2014**.
- Lee K.W., Ku Y.H., Kim M., Ahn B.Y., Chung S.S., Park K.S., "**Effects of sulfonylureas on peroxisome proliferator-activated receptor c activity and on glucose uptake by thiazolidinediones**". *Diabetes Metab. J. ;* 35 340–347, **2011**.
- Lee, Jong-Min, and Jeffrey A. Johnson. "**An important role of Nrf2-ARE pathway in the cellular defense mechanism**." *BMB Reports* 37.2: 139-143, **2004**.
- Lefterova Martina I., and Mitchell A. Lazar. "N**ew developments in adipogenesis.**" *Trends in Endocrinology & Metabolism* 20.3: 107-114, **2009**.
- Lehrke M., Reilly M.P., Millington S.C., Iqbal N., Rader D.J., Lazar M.A. "**An inflammatory cascade leading to hyperresistinemia in humans"**. *PLoS medicine*, 1(2):e45, **2004.**
- Leitges M., Plomann M., Standaert M.L., Bandyopadhyay G., Sajan M.P., Kanoh Y., Farese R.V. "**Knockout of PKC enhances insulin signaling through PI3K"**. *Mol Endocrinol*; 16: 847-858, **2002.**
- Levy N., Bruce M.K., Bruce A. "**Principi di fisiologia di Berne e Levy", 2007.**
- Li J., Yin Q., Wu H. "**Structural basis of signal transduction in the TNF receptor superfamily".** *Adv Immunol*;119:135–153, **2013**.
- Li S., Bouzar C., Cottet-Rousselle C., Zagotta I., Lamarche F., Wabitsch M., Rousseau D. "**Resveratrol inhibits lipogenesis of 3T3-L1 and SGBS cells by inhibition of insulin signaling and mitochondrial mass increase**". *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1857*(6), 643-652, **2016**.
- Li Ji, Lai H., Chen S., Zhu H., & Lai S. "**Interaction of sex steroid hormones and obesity on insulin resistance and type 2 diabetes in men: the Third National Health and Nutrition Examination Survey**." *Journal of Diabetes and its Complications* 31.2: 318-327, **2017**.
- Li Jingjing, and Yi-Ping Liu. "**The roles of PPARs in human diseases**." *Nucleosides, Nucleotides and Nucleic Acids* : 1-22, **2018**.
- Li Z., Bao S., Wu Q., Wang H., Eyler C., Sathornsumetee S., et al. "**Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells**." *Cancer cell;* 15.6: 501-513, **2009**.

- Libby P. "**Inflammatory mechanisms: the molecular basis of inflammation and disease"**. Nutr Rev 65, S140-146, **2007**.
- Lihn A. S., Pedersen S. B., Richelsen B. "**Adiponectin: action, regulation and association to insulin sensitivity**". *Obesity reviews;* 6(1), 13-21, **2005**.
- Listenberger L.L., Han X., Lewis S.E., Cases S., Farese R.V, Ory D.S., and J. E. Schaffer. "**Triglyceride accumulation protects against fatty acidinduced lipotoxicity"**. *Proc. Natl. Acad*. Sci. USA. 100: 3077–3082, **2003**.
- Liu Ling-Bin, Chen X.D., Zhou X.Y., & Zhu, Q. "**The Wnt antagonist and secreted frizzled-related protein 5: implications on lipid metabolism, inflammation and type 2 diabetes mellitus**." *Bioscience reports* : BSR20180011, **2018**.
- Liu T., Zhang L., Joo D., & Sun S.C. "**NF-κB signaling in inflammation**." *Signal transduction and targeted therapy* 2: 17023, **2017**.
- Livak K.J., Schmittgen T.D. "**Analysis of relative gene expression data using realtime quantitative PCR and the 2-ΔΔCt method"**. *Methods.* 25: 402-408*,* **2001**.
- Lood C., Blanco L.P., Purmalek M.M., Carmona-Rivera C., De Ravin S.S., Smith C.K et al. "**Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease."** *Nature medicine* 22.2: 146*,* **2016**.
- Lu Y.C., Wang C.P., Hsu C.C., Chiu C.A., Yu T.H., Hung W.C. "**Circulating secreted frizzled-related protein 5 (Sfrp5) and wingless-type MMTV integration site family member 5a (Wnt5a) levels in patients with type 2 diabetes mellitus"**. *Diabetes Metab Res Rev* 29, 551-556, **2013**.
- Lumeng and Saltiel "**Inflammatory links between obesity and metabolic disease**," *The Journal of Clinical Investigation,* vol. 121, no. 6, pp. 2111–2117*,* **2011**.
- Lumeng Carey N., DeYoung S.M., Bodzin J.L., & Saltiel A.R.. "**Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity**." *Diabetes* 56.1: 16-23, **2007**.
- LuY., Zhu X., Liang G.X.,Cui R.R., Liu Y.,Wu S.S. "**Apelin APJ induces ICAM- 1, VCAM-1 and MCP-1 expression via NF-kappaB/JNK signal pathway in human umbilical vein endothelial cells".** *Amino Acids* 43, 2125–2136, **2012**.
- Lv X., Kong J., Chen W. D., Wang Y.D."**The role of the apelin/APJ system in the regulation of liver disease**." *Frontiers in pharmacology* 8: 221, **2017**.
- Lynch L.,Nowak M.,Varghese B., Clark J.,Hogan A.E.,Toxavidis V. **"Adipose tissue in variant NKTcells protect against diet- induced obesity and metabolic disorder through regulatory cytokine production"**. *Immunity* 37, 574–587, **2012**.
- Maeda N., Shimomura I., Kishida K., Nishizawa H., Matsuda M.,Nagare- tani H. "**Diet-induced insulinresistanceinmicelacking adiponectin/ACRP30"**. *Nat.Med*. 8, 731–737, **2002**.
- Maeda N., Takahashi M., Funahashi T., Kihara S., Nishizawa H., Kishida K "**PPARγ ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein**." *Diabetes* 50.9: 2094-2099, **2001**.

- Magun R., Boone D. L., Tsang B. K., & Sorisky A . "**The effect of adipocyte differentiation on the capacity of 3T3-L1 cells to undergo apoptosis in response to growth factor deprivation."** *International journal of obesity* 22.6: 567, **1998**.
- Makki Kassem, Philippe Froguel and Isabelle Wolowczuk. "**Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines**." *ISRN inflammation* **2013**.
- Manach C., Williamson G., Morand C., Scalbert A., Remesy C. "**Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies**" *Am J Clin Nutr*. 81, 230S−242S, **2005.**
- Mantell B.S., Stefanovic-Racic M., Yang X., Dedousis N.,Sipula I.J., and O'Doherty R.M."**Mice lacking NKT cells but with a complete complement fCD8C T-cells are not protected against the metabolic abnormalities of diet induced obesity"**. *PLoSONE* 6:e19831, **2011**.
- Margetic S., Gazzola C., Pegg G.G., Hill RA. "**Leptin: a review of its peripheral actions and interactions"**. *Jnt J Obes Relat Metab Disord*; 26: 1407-33, **2002**.
- Martins L.M., Oliveira A.R.S., Cruz K.J.C., Torres-Leal F.L., & Marreiro D.D.N. "**Obesity, inflammation, and insulin-resistance**". *Brazilian Journal of Pharmaceutical Sciences;* 50(4), 677-69, **2014**.
- Mathieu P., Poirier P., Pibarot P., Lemieux I., Després J.P. "**Visceral obesity: the link among inflammation, hypertension, and cardiovascular disease.**" *Hypertension* 53.4: 577-584, **2009**.
- Matsuda M., Shimomura I. "**Roles of adiponectin and oxidative stress in obesity-associated metabolic and cardiovascular diseases"**. *Rev Endocr Metab Disord*. **2013.**
- Matthews V.B., Allen T.L., Risis S., Chan M.H., Henstridge D.C., Watson N. "**Interleukin-6-deficient mice develop hepatic inflammation and systemic insulin resistance"**. Diabetologia 53, 2431–2441, **2010**.
- Mazza Giuseppe, and Colin D. Kay. "**Bioactivity, absorption, and metabolism of anthocyanins**." *Recent advances in polyphenol research* 1: 228-262, **2008**.
- Mc Ghie T.K., Walton M.C. **"The bioavailability and absorption of anthocyanins: towards a better understanding".** *Mol Nutr Food Res* 51:702–713, **2007**.
- Mead James R., Scott A. Irvine, and Dipak P. Ramji. "**Lipoprotein lipase: structure, function, regulation, and role in disease.**" *Journal of molecular medicine* 80.12: 753-769, **2002.**
- Meunier V., Bourrie M., Berger Y. and Fabre G. "**The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications"**. *Cell Biol Toxicol,* 11 (3-4): 187-194, **1995.**
- Milbury P.E., Cao G., Prior R.L., Blumberg J. "**Bioavailablility of elderberry anthocyanins"** *Mech Ageing Dev*. 123:997–100, **2002.**
- Milbury P.E., Vita J.A., Blumberg J.B. "**Anthocyanins are bioavailable in humans following an acute dose of cranberry juice"**. *J Nutr* 140:1099–1104, **2010.**

- Miller R.S., Diaczok D., Cooke D.W: “**Repression of GLUT4 expression by the endoplasmic reticulum stress response in 3T3-L1 adipocytes”**. *Biochem Biophys Res Commun*; 362: 188–192, **2007**.
- Minokoshi Yasuhiko, Chitoku Toda and Shiki Okamoto. "**Regulatory role of leptin in glucose and lipid metabolism in skeletal muscle**." *Indian journal of endocrinology and metabolism*16.Suppl 3: S562, **2012**.
- Mojiminiyi O.A., and Abdella N.A. "**Associations of resistin with inflammation and insulin resistance in patients with type 2 diabetes mellitus**." *Scandinavian journal of clinical and laboratory investigation* 67.2: 215-225, **2007**.
- Mook S., Halkes C.C., Bilecen S., Castro Cabezas M. “**In vivo regulation of plasma free fatty acids in insulin resistance”**. *Metabolism*, 53, 1197–1201, **2004.**
- Moon M., Dalamaga S.Y., Kim S.Y., Polyzos S.A., Hamnvik O.P., Magkos F. **“Leptin’s role in lipodystrophic and nonlipodystrophic insulin-resistant and diabetic individuals**” *Endocrine Reviews*, vol. 34, pp. 377–412, **2013**.
- Morigny P., M. Houssier E. Mouisel and D. Langin **. “Adipocyte lipolysis and insulin resistance”** .*Biochimie*. 125: 259–266, **2016**.
- Muenning P., Lubetkin E., Jia H., & Franks P. “**Gender and the burden of disease attributable to obesity”**. *American Journal of Public Health*, 96(9):1662–1668, **2006**.
- Muraki I., Imamura F., Manson J.E., Hu F.B., Willett W.C., Van Dam R.M., et al. “**Fruit consumption and risk of type 2 diabetes: Results from three prospective longitudinal cohort studies**”. *BMJ ;*347:f5001*,* **2013**.
- Muraki I., Imamura F., Manson J.E., Hu F.B.,WillettW.C., Van Dam R.M. “**Fruit consumption and risk of type 2 diabetes: Results from three prospective longitudinal cohort studies”**. *BMJ*.;347:f5001, **2013**.
- Murano I., Barbatelli G., Parisani V. “**Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice”**. *J Lipid Res*;49:1562-8, **2008**.
- Nakamura T., Furuhashi M., Li P., Cao H.,Tuncman G., Sonenberg N., Gorgun C.Z., and Hotamisligil G.S. “**Doublestranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis”**. Cell. 140: 338–348, **2010**.
- Neal Bruce. "**Quantifying the importance of interleukin-6 for coronary heart disease**." *PLoS medicine* 5.4: e84, **2008**.
- Nedergaard J., Bengtsson T., Cannon B. “**Unexpected evidence for active brown adipose tissue in adult humans”**. *Am J Physiol Endocrinol Metab*;293:E444–E452, **2007**.
- Nemeth K., Plumb G.W., Berrin J.G., Juge N., Jacob R., Naim H.Y., Williamson G., Swallow D. M., Kroon P.A. “**Deglycosylation by small intestinal epithelial cell β-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans”.** *Eur J Nutr*. 42, 29−42, **2003.**
- Nerlov Claus. "**The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control**." *Trends in cell biology* 17.7: 318-324, **2007**.

- Nigro E., Scudiero O., Monaco ML., Palmieri A., Mazzarella G., Costagliola C., Daniele., A. "**New Insight into Adiponectin Role in Obesity and Obesity-Related Diseases**". *BioMed Research International;* 2014:658913, **2014**.
- Nizamutdinova I.T., Jin Y.C., Chung J.I., Shin S.C., Lee S.J., Seo H.G. "**The anti-diabetic effect of anthocyanins in streptozotocin-induced diabetic rats through glucose transporter 4 regulation and prevention of insulin resistance and pancreatic apoptosis**". *Mol Nutr Food Res* ;53(11):1419–29, **2009**.
- Nothlings U., Schulze M.B., Weikert C., Boeing H., Van der Schouw Y.T., Bamia C., Benetou V., Lagiou P., Krogh V., Beulens J.W. "**Intake of vegetables, legumes, and fruit, and risk for all cause, cardiovascular, and cancer mortality in a European diabetic population**". *J Nutr* 138, 775-781, **2008**.
- Nothlings U., Schulze M.B., Weikert C., Boeing H., Van der Schouw Y.T., Bamia C., Benetou V., Lagiou P., Krogh V., Beulens J.W., et al. "**Intake of vegetables, legumes, and fruit, and risk for all cause, cardiovascular, and cancer mortality in a European diabetic population**". *J Nutr* 138, 775-781, **2008**.
- Ogawa K., Sakakibara H., Iwata R., Ishii T. "**Anthocyanin composition and antioxidant activity of the Crowberry (Empetrum nigrum) and other berries**". *J Agric Food Chem*. 56, 4457–4462, **2008**.
- Olefsky J.M., and Glass C.K. "**Macrophages, inflammation, and insulin resistance.**" *Annual review of physiology* 72 : 219-246, **2010**.
- Otto T.C., Lane M.D. **"Adipose development: from stem cell to adipocyte"**. *Critical Reviews in Biochemistry and Molecular Biology*, 40, 229-242, **2005**.
- Ouchi N., Higuchi A., Ohashi K., Oshima Y., Gokce N., Shibata R. "**Sfrp5 is an anti-inflammatory adipokine that modulates metabolic dysfunction in obesity**". *Science* 329, 454-457, **2010**.
- Ouchi N., Parker J.L., Lugus J.J., and Walsh K. "**Adipokines in inflammation andmetabolic disease**,"*Nature Reviews Immunology*, vol. 11, no. 2, pp. 85–97, **2011**.
- Ouchi N., Parker J.L., Lugus J.J., and Walsh K."**Adipokines in inflammation and metabolic disease"**. *Nat.Rev.Immunol*. 11, 85–97, **2011**.
- Ozcan L., Ergin A.S., Lu A., Chung J., Sarkar S., Nie D."**Endoplasmicreticu-lumstressplaysacentralrolein developmentofleptinresistance"**. *Cell Metab*. 9, 35–51, **2009**.
- Ozcan U., Yilmaz E., Ozcan L., Furuhashi M., Vaillancourt E., Smith R.O., Gorgun C.Z., Hotamisligil G.S: "**Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes".** *Science*; 313: 1137–1140, **2006.**
- Ozcan U., Cao Q., Yilmaz E., Lee A.H., Iwakoshi N.N., Ozdelen E., Tuncman G., Gorgun C., Glimcher L.H., Hotamisligil G.S.: "**Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes".** *Science*; 306: 457–461, **2004**.
- Paixao J., Dinis T.C., and Almeida L.M. "**Malvidin-3- glucoside protects endothelial cells up-regulating endothelial NO synthase and inhibiting**

peroxynitrite-induced NF-kB activation". *Chem Biol Interact* 199,192-200, **2012a**.

- Paixao J., Dinis T.C., and Almeida L.M. "**Protective role of malvidin-3 glucoside on peroxynitrite-induced damage in endothelial cells by counteracting reactive species formation and apoptotic mitochondrial pathway"**. *Oxid Med Cell Longev*, 428538, **2012b.**
- Pal Martin, Mark A. Febbraio, and Graeme I. Lancaster. "**The roles of c-Jun NH2-terminal kinases (JNKs) in obesity and insulin resistance**." *The Journal of physiology* 594.2: 267-279, **2016**.
- Pasparakis M. "**Regulation of tissue homeostasis by NF-κB signalling: implications for inflammatory diseases.**" *Nature Reviews Immunology* 9.11: 778, **2009**.
- Passamonti S., Vrhovsek U., Vanzo A., and Mattivi F. "**The stomach as a site for anthocyanins absorption from food"**. *FEBS Lett* 544, 210-213, **2003**.
- Pati S., Liberatore M.T., Gambacorta G., Antonacci D., La Notte E. "**Rapid screening for anthocyanins and anthocyanin dimers in crude grape extracts by high performance liquid chromatography coupled with diode array detection and tandem mass spectrometry"**. *J Chromatogr* A. 1;1216(18):3864-8, **2009**.
- Patras A., Brunton N.P., O'Donnell C. "**Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation"**. *Trends Food Sci Technol*;21(1):3–11, **2010**.
- Pedersen B.K., Febbraio M.A. "**Interleukin-6 does have a beneficial role in insulin sensitivity and glucose homeostasis**". *J Appl Physiol*; 102: 814–816, **2007**.
- Peng K., L. Liu, D. Wei, Y. Lv, G. Wang, W. Xiong, X. Wang, A. Altaf, L. Wang, D. He, "**P2X7R is involved in the progression of atherosclerosis by promoting NLRP3 inflammasome activation"**. *Int. J. Mol. Med*. 35: 1179–1188, **2015**.
- Perkins Neil D. "**Integrating cell-signalling pathways with NF-κB and IKK function**." *Nature reviews Molecular cell biology* 8.1: 49, **2007**.
- Perry R.J., V.T. Samuel, K.F. Petersen and G.I. Shulman. "**The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes".** *Nature*. 510: 84–91, **2014**.
- Pessin J.E., and Kwon H. "**Adipokines mediate inflammation and insulin resistance.**" *Frontiers in endocrinology,* **2013**.
- Peters W.H. and Roelofs H.M. "**Time-dependent activity and expression of glutathione S-transferases in the human colon adenocarcinoma cell line Caco-2**". *Biochem J*, 264 (2): 613-616, **1989.**
- Pinto M., Robine-Leon S. and Appay M.D. "**Enterocyte-like differentiation and polarization of the human cell line CaCo-2 in culture**". *Biol. Cell*., 47: 323-330, **1983**.
- Potenza M.A., Marasciulo F.L., Chieppa D.M., Brigiani G.S., Formoso G., Quon M. J., & Montagnani, M. "**Insulin resistance in spontaneously hypertensive rats is associated with endothelial dysfunction characterized by imbalance**

between NO and ET-1 production". *American Journal of Physiology-Heart and Circulatory Physiology*, 289(2), H813-H822, **2005**.

- Poulos S.P., Dodson M.V., Hausman G.J. "**Cell line models for differentiation: preadipocytes and adipocytes**" *Exp. Biol. Med;* 235, 1185–1193*,* **2010**.
- Poulos S.P., Dorothy B., Hausman and Gary J. Hausman. "**The development and endocrine functions of adipose tissue.**" *Molecular and cellular endocrinology 3*23.1: 20-3,4, **2010**.
- Poynten A. M., Gan S. K., Kriketos A. D., Campbell L.V., Chisholm D. J."**Circulating fatty acids, non-high density lipoprotein cholesterol, and insulin-infused fat oxidation acutely influence whole body insulin sensitivity in nondiabetic men**." *The Journal of Clinical Endocrinology & Metabolism* 90.2 : 1035-1040*,* **2005**.
- Prasad S., Phromnoi K., Yadav V.R., Chaturvedi M.M., & Aggarwal B.B. "**Targeting inflammatory pathways by flavonoids for prevention and treatment of cancer**." *Planta medica* 76.11: 1044, **2010.**
- Prior RL., and Wu X.. "**Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities**" *Free Radic Res;* 40,1014-1028*,* **2006**.
- Prior RL., Wu X., Gu L., Hager T., Hager A., Wilkes S., Howard L. "**Purified berry anthocyanins but not whole berries normalize lipid parameters in mice fed an obesogenic high fat diet**". *Mol Nutr Food Res. Nov;* 53(11):1406-18*,* **2009**.
- Prior RL., Wu X., Gu L., Hager T.J, Hager A., Howard LR. "**Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity**". *J Agric Food Chem.* Feb 13; 56(3):647-53, **2008.**
- Qi Y., Nie Z., Lee Y.S., Singhal N.S., Scherer P.E., Lazar M.A."**Loss of resistin improves glucose homeostasis in leptin deficiency"**. *Diabetes* 55, 3083–3090, **2006**.
- Qin B., Anderson R.A. "**An extract of chokeberry attenuates weight gain and modulates insulin, adipogenic and inflammatory signalling pathways in epididymal adipose tissue of rats fed a fructose-rich diet"**. *Br J Nutr*;108:581–587, **2012**.
- Qu Dan, Qu D., Liu J., Lau C. W., & Huang, Y."**IL-6 in diabetes and cardiovascular complications**." *British journal of pharmacology* 171.15: 3595-3603, **2014.**
- Quan W., Kim H.K., Moon E.Y., Kim S.S., Choi C.S., Komatsu M. "**Role of hypothalamic proopimelanocortin neuronautophagy in the control of appetite and leptin response".** *Endocrinology* 153, 1817–1826. doi:10.1210/en.2011-1882, **2012**.
- Ràfols Montserrat Esteve. "**Adipose tissue: cell heterogeneity and functional diversity.**" *Endocrinología y Nutrición (English Edition)* 61.2: 100-112, **2014**.
- Ramirez-Zacarias JL., Castro-Munozledo F., and W. Kuri-Harcuch. "**Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O.**" *Histochemistry* 97.6: 493-497, **1992**.

- Ranganathan G., R. Unal, I. Pokrovskaya, A. Yao-Borengasser, B. Phanavanh, B. Lecka-Czernik, N. Rasouli, and P. A. Kern. "**The lipogenic enzymes DGAT1, FAS, and LPL in adipose tissue:effects of obesity, insulin resistance, and TZD treatment"**. *J. Lipid Res*. 47: 2444–2450, **2006**.
- Rausch M.E., Weisberg S., Vardhana P.,"**Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration"**. *Int J Obes* (Lond);32:451-63, **2008.**
- Reaven G.M. "**Insulin resistance and its consequences :type 2 diabetes mellitus and coronary heart disease" 2000.**
- Roberts DJ., and Miyamoto S. "**Hexokinase II integrates energy metabolism and cellular protection: Akting on mitochondria and TORCing to autophagy**." *Cell death and differentiation* 22.2: 248*,* **2015**.
- Rosen Evan D., and Ormond A. MacDougald. "**Adipocyte differentiation from the inside out**." *Nature reviews Molecular cell biology* 7.12: 885, **2006**.
- Rosenow A., Arrey T.N., Bouwman F.G., Noben J.P., Wabitsch M., Mariman E.C. " **Identification of Novel Human Adipocyte Secreted Proteins by Using SGBS Cells**". *J Proteome Res.* 9: p. 5389-401*,* **2010**.
- Rossi A., Serraino I., Dugo P., Di Paola R., Mondello L., Genovese T. "**Protective effects of anthocyanins from blackberry in a rat model of acute lung inflammation**." *Free radical research* 37.8: 891-900, **2003**.
- Ruan H., Pownall H.J. "**Overexpression of 1-acyl-glycerol-3-phosphate acyltransferase-_ enhances lipid storage in cellular models of adipose tissue and skeletal muscle"**. *Diabetes*;50:233–40, **2001**.
- Ruan Hong and Lily Q. Dong. "**Adiponectin signaling and function in insulin target tissues.**" *Journal of molecular cell biology* 8.2 101-109, **2016**.
- Ruan Hong, and Harvey F. Lodish. "**Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-α**." *Cytokine & growth factor reviews* 14.5: 447-455, **2003**.
- Ruderman N.B., Carling D., Prentki M., Cacicedo J.M. "**AMPK, insulin resistance, and the metabolic syndrome"**. *J Clin Invest*;123(7): 2764–72, **2013**.
- Rui L., Aguirre V., Kim J.K, Shulman G.I, Lee A., Corbould A., Dunaif A., White M.F. "**Insulin/IGF-1 and TNF-α stimulate phosphorylation of IRS-1 at inhibitory Ser307 via distinct pathways"**. *J Clin Invest*; 107:181-189, **2001**.
- Rui Liangyou. "**Brown and Beige Adipose Tissues in Health and Disease**." *Comprehensive Physiology* **2017**.
- Russo A., La Fauci L., Acquaviva R., Campisi A., Raciti G., Scifo C.,Galvano F. "**Ochratoxin A-induced DNA damage in human fibroblast: protective effect of cyanidin 3-O-β-D-glucoside**". *The Journal of nutritional biochemistry*, 16(1), 31-37, **2005**.
- Sacheck J. "**Pediatric obesity: an inflammatory condition?**" *J Parenter Enteral Nutr*; 32: 633- 637, **2008**.
- Sachithanandan N., Graham K.L., Galic S.,"**Macrophage deletion of SOCS1 increases sensitivity to LPS and palmitic acid and results in systemic inflammation and hepatic insulin resistance".** *Diabetes.*; 60: 2023-2031, **2011**.

- Saltiel A.R., Kahn C.R. "**Insulin signaling and the regulation of glucose and lipid metabolism**" *Nature*.; 414: 799-806, **2001**.
- Samuel V.T., and Shulman G.I. "**Mechanisms for insulin resistance: common threads and missing links**". *Cell* 148, 852–871, **2012**.
- Sánchez F., García R., Alarcón F., Cruz M. "**Adipocinas, tejido adiposo y su relación con células del sistema inmune".** *Gac. Méd. Méx*, 141, 505–512, **2005**.
- Sarma A.D., Sreelakshimi Y., Sharma R. "**Antioxidant ability of anthocyanins against ascorbic acid oxydation"**. *Phytochem*. 45: 671–674, **1997.**
- Sárvári A. K., Doan-Xuan Q. M., Bacsó Z., Csomós I., Balajthy Z., & Fésüs L. **"Interaction of differentiated human adipocytes with macrophages leads to trogocytosis and selective IL-6 secretion"**. *Cell death & disease*, *6*(1), e1613, **2015**.
- Sasaki R., Nishimura N., Hoshino H., Isa Y., Kadowaki M., Ichi T. "**Cyanidin 3-glucoside ameliorates hyperglycemia and insulin sensitivity due to downregulation of retinol binding protein 4 expression in diabetic mice"**. *Biochem Pharmacol*;74(11):1619–27, **2007**.
- Scazzocchio B., Vari R., Filesi C., D'Archivio M., Santangelo C., Giovannini C., et al. "**Cyanidin-3-O-beta-glucoside and protocatechuic acid exert insulin-like effects by upregulating PPARgamma activity in human omental adipocytes"**. *Diabetes*;60(9):2234–44, **2011**.
- Schaffer Jean E. "**Lipotoxicity: many roads to cell dysfunction and cell death: introduction to a thematic review series**." *Journal of lipid research* 57.8 : 1327, **2016**.
- Schaffler A., Neumeier M., Herfarth H., Furst A., Scholmerich J., and Buchler C. "**Genomic structure of human omentin, a new adipocytokine expressed in omental adipose tissue".** *Biochim. Biophys.Acta* 1732, 96–102, **2005**.
- Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. "**A novel serum protein similar to C1q, produced exclusively in adipocytes"**. *The Journal of Biological Chemistry*, 270(45):26746-9, **1995**.
- Schipper H.S.,Rakhshandehroo M., Vande Graaf S.F.,Venken K.,Koppen A.,Stienstra R."**Natural killer T cells inadi pose tissue prevent insulin resistance"**. *J. Clin.Invest*. 122, 3343–3354, **2012**.
- Schulte D.M., Kragelund D., Muller N., Hagen I., Elke G., Titz A. "**The wingless-related integration site-5a/secreted frizzled-related protein-5 system is dysregulated in human sepsis"**. *Clin Exp Immunol* 180, 90-97, **2015**.
- Schulz T.J., Huang T.L., Tran T.T."**Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat"**[J]. *Proc Natl Acad Sci U S A*, 108(1): 143–148, **2011**.
- Sears Barry, and Mary Perry. "**The role of fatty acids in insulin resistance."** *Lipids in health and disease* 14.1: 121, **2015**.
- Seeram N.P., Momin R.A., Nair M.G., Bourquin L.D. "**Cyclooxygenase inhibitory and antioxidant cyaniding glycosides in cherries and berries"**. *Phytomedicine*. 8: 362-369, **2001**.
- Sell H., Eckel J. "**Adipose tissue inflammation: novel insight into the role of macrophages and lymphocytes".** *Curr Opin Clin Nutr Metab Care*;13:366-70, **2010**.

- Semple R.K., Chatterjee V.K., O'Rahilly S. "**PPAR gamma and human metabolic disease**". J Clin Invest;116:581–9, **2006**.
- Senol-Cosar O., Flach R.J.R., Di Stefano M., Chawla A., Nicoloro S., Straubhaar J. et al. "**Tenomodulin promotes human adipocyte differentiation and beneficial visceral adipose tissue expansion**." *Nature communications;* 7: 10686, **2006**.
- Sezer Havva. "**Insulin resistance, obesity and lipotoxicity**." *Obesity and Lipotoxicity*. Springer, Cham. 277-304, **2017**.
- Shepherd P.R. "**Mechanisms regulating phosphoinositide 3-kinase signalling in insulinsensitive tissues**". *Acta Physiol Scand*; 183: 3-12, **2005**.
- Sherwood "**Fondamentals of Human Physiology**", *Piccin Nuova libraria* s.p.a., Padova **2012.**
- Shi H., Kokoeva M.V., Inouye K., Tzameli I., Yin H., Flier J.S: "**TLR4 links innate immunity and fatty acid-induced insulin resistance**". *J Clin Invest*; 116: 3015–3025, **2004**.
- Shi H., Tzameli I., Bjorbaek C., Flier J.S: "**Suppressor of cytokine signaling 3 is a physiological regulator of adipocyte insulin signaling**". *J Biol Chem*; 279: 34733–34740, **2004**.
- Sikaris, K. "**The clinical biochemistry of obesity**". *Clin. Biochem. Rev*., 25,165–181, **2004**.
- Speciale A., Canali R., Chirafisi J., Saija A., Virgili F., Cimino F. "**Cyanidin-3-O-glucoside protection against TNF-alpha-induced endothelial dysfunction: Involvement of nuclear factor-kappaB signaling".** *J Agric Food Chem*;58(22):12048–54, **2010**.
- Speciale A., Chirafisi J., Saija A., Cimino F. "**Nutritional antioxidants and adaptive cell responses: an update".** *Curr Mol Med*. 11(9):770-89, **2011.**
- Speciale A., Cimino F., Saija A., Canali R., and Virgili F. "**Bioavailability and molecular activities of anthocyanins as modulators of endothelial function".** *Genes Nutr* 9, 404, **2014**.
- Spranger J., Kroke A., Mohlig M., Hoffmann K., Bergmann M.M.,Ristow M. "**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**.
- Standaert M.L., Bandyopadhyay G., Galloway L., Soto J., Ono Y., Kikkawa U., Farese R.V., Leitges M. "**Effects of knockout of the protein kinase C gene on glucose transport and glucose homeostasis**". *Endocrinology*; 140: 4470-4477, **1999**.
- Stanley T.L., Zanni M.V., Johnsen S., Rasheed S., Makimura H., Lee H."**TNF-alpha antagonism with etanercept decreases glucose and increases the proportion of high molecular weight adiponectin in obese subjects with features of the metabolic syndrome".** *J. Clin. Endocrinol.Metab*. 96, E146–E150, **2011**.
- Steffes M.; Gross M.; Lee D.; Schreiner P.; Jacobs D. "**Adiponectin, visceral fat, oxidative stress and early macrovascular disease: The coronary artery risk development in young adults study**". *Obesity*, 14, 319–326, **2006**.

- Steppan C.M.,Wang J., White- man E.L.,Birnbaum M.J., and Lazar M.A. "**Activation of SOCS-3 by resisti**n". *Mol.Cell. Biol*. 25, 1569 1575, **2005**.
- Stoner G. "**Modulation of genetic and epigenetic biomarkers of colorectal cancer in humans by black raspberries: a phase I pilot study"**. *Clin. Cancer Res*., 17, 598-610, **2011.**
- Stratford S., Hoehn K.L., Liu F., and Summers S.A. "**Regulation of insulin action by ceramide: dual mechanisms linking ceramide accumulation to the inhibition of Akt/protein kinase B"**. *J. Biol. Chem*. 279: 36608–36615, **2004**.
- Stucchi A., Reed K., O'brien M., Cerda S., Andrews C., Gower A., et al. "**A new transcription factor that regulates TNF-α gene expression, LITAF, is increased in intestinal tissues from patients with CD and UC**." *Inflammatory bowel diseases* 12.7: 581-587, **2006**.
- Suganami T., Ogawa Y. "**Adipose tissue macrophages: their role in adipose tissue remodeling". ***J Leukoc Biol;* 88: 33-39, **2010**.
- Sultana A., Cochran B.J., Tabet F., Patel M., Torres L.C., Barter P.J., & Rye K.A. "**Inhibition of inflammatory signaling pathways in 3T3-L1 adipocytes by apolipoprotein AI**." *The FASEB Journal* 30.6. 2324-2335, **2016**.
- Summers S.A. "**Ceramides in insulin resistance and lipotoxicity"**. *Prog. Lipid Res*. 45: 42–72, **2006**.
- Sun K., Kusminski C.M, Scherer P.E. "**Adipose tissue remodeling and obesity"**. *J Clin Invest* ;121:2094e101, **2011**.
- Sun X.J., Goldberg J.L., Qiao L.Y., Mitchell J.J. "**Insulin induced insulin receptor substrate-1 degradation is mediated by the proteasome degradation pathway"**. *Diabetes*; 48: 1359-1364, **1999**.
- Swaroop Jatla Jyothi, Duggirala Rajarajeswari, and J. N. Naidu. "**Association of TNF-α with insulin resistance in type 2 diabetes mellitus**." *The Indian journal of medical research* 135.1: 127, **2012**.
- Takada Y., H. Ichikawa A., Pataer S., Swisher and B.B. Aggarwal. **"Genetic deletion of PKR abrogates TNF-induced activation of Ikappa B alpha kinase, JNK, Akt and cell proliferation but potentiates p44/p42 MAPK and p38 MAPK activation"**. *Oncogene.* 26: 1201–1212, **2007**.
- Talukdar S.,Oh D.Y., Bandyopad- hyay G., Li D.,Xu J., McNelis J. "**Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase". ***Nat.Med*. 18, 1407–1412, **2012**.
- Tan Bee K., Raghu A., and Randeva H.S. "**Omentin: a novel link between inflammation, diabesity, and cardiovascular disease.**" *Trends in cardiovascular medicine* 20.5: 143-148, **2010**.
- Tanaka Y., Sasaki N., Ohmiya A., "**Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids"**. *Plant. J.,* 54(4), 733-749, **2008.**
- Tang Q.Q., Lane M.D. "**Adipogenesis: from stem cell to adipocyte"**. *Annual Review of Biochemistry*, 81:715-36, **2012.**
- Tang X., Guilherme A., Chakladar A., Powelka A.M., Konda S., Virbasius J.V. "**An RNA interference-based screen identifies MAP4K4/NIK as a negative regulator of PPARγ, adipogenesis, and insulin-responsive hexose transport"**. *Proc Natl Acad Sci USA*;103:2087–2092, **2006**.

- Taylor Roy. "**Insulin resistance and type 2 diabetes**." *Diabetes* 61.4: 778-779, **2012**.
- Thorens Bernard and Mike Mueckler. "**Glucose transporters in the 21st Century**." *American Journal of Physiology-Endocrinology and Metabolism* 298.2: E141-E145, **2009**.
- Tilg H., and Moschen A.R. "**Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis**." *Hepatology* 52.5: 1836-1846, **2010**.
- Titta L., Trinei M., Stendardo M., Berniakovich I., Petroni K., Tonelli C., Riso P.,Porrini M., Minucci S., Pelicci P.G. "**Blood orange juice inhibits fat accumulation in mice**". *Int J Obes* (Lond) 34, 578-588, **2010**.
- Todoric J., Löffler M., Huber J., Bilban M., Reimers M., Kadl A. "**Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n− 3 polyunsaturated fatty acids**." *Diabetologia* 49.9: 2109-2119, **2006.**
- Torskangerpoll K., Andersen Ø.M. "**Colour stability of anthocyanins in aqueous solutions at various pH values**". *Food Chem*;89(3):427–440, **2005**.
- Trayhurn P. "**Endocrine and signaling role of adipose tissue:New perspectives on fat**". Acta Physiol Scand. ;184:285 93.7, **2005.**
- Trayhurn P., Wood I.S. "**Adipokines: inflammation and thepleitropic role of white adipose tissue**". Br J Nutr;92,374-355.9, **2004**.
- Tsoyi K, Park HB, Kim YM, Chung J.I., Shin S.C., Lee W.S.,Kim, H.J. "**Anthocyanins from black soybean seed coats inhibit UVB-induced inflammatory cylooxygenase-2 gene expression and PGE2 production through regulation of the nuclear factor-jB and phosphatidylinositol 3-kinase/Akt pathway**". *J Agric Food Chem*;56:8969–8974, **2008**.
- Tsuda T., Ueno Y., Aoki H., Koda T., Horio F., Takahashi N."**Anthocyanin enhances adipocytokine secretion and adipocytespecific gene expression in isolated rat adipocytes**". *Biochem Biophys Res Commun.*;316(1):149–57, **2004**.
- Tsuda T., Ueno Y., Yoshikawa T., Kojo H., Osawa T. "**Microarray profiling of gene expression in human adipocytes in response to anthocyanins**". *Biochem Pharmacol*;71(8):1184–97, **2006**.
- Tzanavari T., Giannogonas P., and Karalis K.P. "**TNF-α and obesity**." *TNF Pathophysiology*. Vol. 11. Karger Publishers; 145-156, **2010**.
- Ueki K., Kondo T., Tseng Y.H."**Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse".** *Proc Natl Acad Sci USA*; 101: 10422-10427, **2004**.
- Unamuno X., Gómez-Ambrosi J., Rodríguez A., Becerril S., Frühbeck G., & Catalán V. "**Adipokine dysregulation and adipose tissue inflammation in human obesity**." *European journal of clinical investigation*: e12997, **2018**.
- Uysal K.T., Wiesbrock S.M., Marino M.W., Hotamisligil G.S: "**Protection from obesity induced insulin resistance in mice lacking TNF- a function".** *Nature*; 389: 610–614, **1997**.
- Vachharajani V., Granger DN. "**Adipose tissue: a motor for the inflammation associated with obesity**". *IUBMB Life* 61:424-30, **2009**.

- Van Camp J.K., Beckers S., Zegers D., Verrijken A., Van Gaal L.F., Van Hul W. "**Common genetic variation in sFRP5 is associated with fat distribution in men".** *Endocrine* 46, 477-484, **2014**.
- Van de Voorde J, Pauwels B, Boydens C. "**Adipocytokines in relation to cardiovascular disease".** *Metabolism*;62:1513–1521, **2013**.
- Vendrame S., Tsakiroglou P., Kristo A.S., Schuschke D.A., & Klimis-Zacas, D. "**Wild blueberry consumption attenuates local inflammation in the perivascular adipose tissue of obese Zucker rats**". *Applied Physiology, Nutrition, and Metabolism*, *41*(10), 1045-1051, **2016**.
- Vermeulen L., De W.G., Van D.P., Vanden Berghe W., Haegeman G. "**Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1)**". *EMBO J.* 22:1313-24, **2003**.
- Villarroya J., Cereijo R., and Villarroya F. "**An endocrine role for brown adipose tissue?.**" *American Journal of Physiology-Endocrinology and Metabolism* 305.5: E567-E572, **2013**.
- Wabitsch M., Brenner RE., Melzner I., Braun M., Möller P., Heinze E., & Hauner H. "**Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation**." *International journal of obesity* 25.1: 8, **2001.**
- Waki and P. Tontonoz, "**Endocrine functions of adipose tissue**," *Annual Review of Pathology*, vol. 2, pp. 31–56, **2007**.
- Wallace T.C. "**Anthocyanins in cardiovascular disease**". Adv Nutr. ; 2:1–7, **2011**.
- Wang Q., Umar I., Yida Z., & Wang F. "**Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) as a Target for Concurrent Management of Diabetes and Obesity-Related Cancer**." *Current pharmaceutical design* 23.25 : 3677-3688, **2017**.
- Wang X., and Seed B. "**A PCR primer bank for quantitative gene expression analysis.**" *Nucleic acids research* 31.24: e154-e154, **2013**.
- Wang Yong-Xu. "**PPARs: diverse regulators in energy metabolism and metabolic diseases**." *Cell research* 20.2: 124, **2010**.
- Wang Li-Shu, and Gary D. Stoner. "**Anthocyanins and their role in cancer prevention.**" Cancer letters 269.2 : 281-290, **2008**.
- Wang R., Hong J., Liu R., Chen M., Xu M., Gu W. "**SFRP5 acts as a mature adipocyte marker but not as a regulator in adipogenesis".** *J Mol Endocrinol* 53, 405-415, **2014**.
- Ward C.W., Lawrence M.C. "**Ligand-induced activation of the insulin receptor: a multistep process involving structural changes in both the ligand and the receptor"**. Bioessays; 31: 422-34, **2009**.
- Weigert C., Hennige A.M., Lehmann R., Brodbeck K, Baumgartner F., Schauble M., Haring H.U., Schleicher E.D: "**Direct cross-talk of interleukin- 6 and insulin signal transduction via insulin receptor substrate-1 in skeletal muscle cells"**. J Biol Chem; 281: 7060– 7067, **2006**.
- Weisberg S.P., Mc Cann D., Desai M., Rosenbaum M., Leibel R.L., and A.W. Ferrante Jr., "**Obesity is associated with macrophage accumulation in adipose**

tissue," *The Journal of Clinical Investigation*, vol. 112, no. 12, pp. 1796–1808, **2003**.

- Weisberg S.P., McCann D., Desai M., Rosenbaum M., Leibel R.L., Ferrante Jr. A.W. "**Obesity is associated with macrophage accumulation in adipose tissue"**. J Clin Invest;112: 1796-1808, **2003**.
- Weisberg, Stuart P., Hunter D., Huber R., Lemieux J. "**CCR2 modulates inflammatory and metabolic effects of high-fat feeding.**" The Journal of clinical investigation 116.1: 115-124, **2006**.
- Wellen K.E., Hotamisligil G.S. "**Obesity-induced inflammatory changes in adipose tissue"**. J Clin Invest ;112:1785-8, **2003**.
- Wilcox Gisela. "**Insulin and insulin resistance**." *Clinical Biochemist Reviews* 26.2: 19, **2005.**
- Wilk S., Jenke A., Stehr J.,Yang C.A.,Bauer S.,Goldner K."**Adiponectin modulates NK-cell function"**. *Eur. J. Immunol*. 43, 1024–1033, **2013**.
- Wilk S., Scheibenbogen C., Bauer S., Jenke A., Rother M., Guerreiro M. "**Adiponectin is a negative regulator of antigen activated T cells"**. *Eur.J.Immunol*. 41,2323–2332, **2011**.
- Wrolstad Ronald E., Robert W. Durst, and Jungmin Lee. "**Tracking color and pigment changes in anthocyanin products.**" *Trends in Food Science & Technology* 16.9: 423-428, **2005**.
- Wu D., Ren Z., Pae M., Guo W., Cui X., Merrill A.H., Meydani S.N.: "**Aging up-regulates expression of inflammatory mediators in mouse adipose tissue"**. *J Immunol*; 179:4829–4839, **2007**.
- Wu J., Boström P., Sparks L.M., Ye L., Choi J.H., Giang, A "**Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human**." *Cell* 150.2: 366-376, **2012**.
- Wu Q., Ortegon A.M., Tsang B., Doege H., Feingold K.R., Stahl A. "**FATP1 is an insulin-sensitive fatty acid transporter involved in diet-induced obesity**." *Molecular and cellular biology* 26.9: 3455-3467, **2006**.
- Wu D., Molofsky A.B., Liang H.E., Ricardo-Gonzalez R.R., Jouihan H.A., Bando J. K."**Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis"**. *Science* 332, 243–247, **2011**.
- Xie L., Su H., Sun C., Zheng X., & Chen W. "**Recent advances in understanding the anti-obesity activity of anthocyanins and their biosynthesis in microorganisms**." *Trends in Food Science & Technology*, **2017**.
- Xu Haiyan, Barnes G.T., Yang Q., Tan G., Yang D., Chou C. J., Chen H.. "**Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance**." *The Journal of clinical investigation* 112.12: 1821-1830, **2003**.
- Xu P.T., Song Z., Zhang W.C., Jiao B.,Yu Z.B. **Impaired translocation of GLUT4 results in insulin resistance of atrophic soleus muscle.** *BioMed research international*, **2015**.
- Yamauchi T., Kamon J., Minokoshi Y., Ito Y.,Waki H.,Uchida S.**Adiponectin stimulates glucose utilization and fatty- acid oxidation by activating AMP-activated protein kinase**. *Nat.Med*. 8, 1288–1295, **2002.**

- Yang Q., Graham T.E., Mody N., Preitner F., Peroni O.D., Zabolotny J. M "**Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes".** *Nature*; 436: 356-62, **2005**.
- Yang R.Z., Lee M.J., Hu H., Pray J.,Wu H.B., Hansen B.C. "**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**.
- Yang G., L. Badeanlou J. Bielawski, A. J. Roberts, Y. A. Hannun, and F. Samad. "**Central role of ceramide biosynthesis in body weight regulation, energy metabolism, and the metabolic syndrome"**. *Am. J. Physiol. Endocrinol. Metab*. 297: E211–E224, **2009**.
- Ye D., Ma Y. and Ma T.Y. "**Molecular mechanism of tumor necrosis factor-α modulation of intestinal epithelial tight junction barrier**". *Am J Physiol Gastrointest Liver Physiol*. 290: G496–G504, **2006**.
- Ye, Jianping. "**Regulation of PPARγ function by TNF-α."** *Biochemical and biophysical research communications* 374.3: 405-408, **2008**.
- Yeo C.R., Agrawal M., Hoon S., Shabbir A., Shrivastava M.K., Huang S.,Vidal-Puig A. "**SGBS cells as a model of human adipocyte browning: A comprehensive comparative study with primary human white subcutaneous adipocytes**". *Scientific reports*, *7*(1), 4031, **2017**.
- You J., Hodge C., Wen L., McAvoy J.W., Madigan M.C., Sutton G. "**Tear levels of SFRP1 are significantly reduced in keratoconus patients"**. *Mol Vis* 19, 509, **2013**.
- Youakin A., Hauri H.P. and Sterchi E. "**Decrease in polyactosaminoglycans associated with lysosomal membrane glycoproteins durino differentiation of CaCo-2 human colonic adenocarcinoma cells**". *Cancer Res*, 49: 6889-6895,**1989**.
- Yue P., Jin H., Aillaud M., Deng A.C., Azuma J., Asagami T., Tsao, P.S.. "**Apelin is necessary for the maintenance of insulin sensitivity**". A*merican Journal of Physiology-Endocrinology and Metabolism*, 298(1), E59-E67, **2010**.
- Yue P., Jin H., Xu S., Aillaud M., Deng A.C., Azuma J."**Apelin decreases lipolysis via G(q), G(i), and AMPK-dependent mechanisms".** *Endocrinology* 152, 59–68, **2011**.
- Zamora-Ros R., Knaze V., Luja´n-Barroso L. "**Estimation of the intake of anthocyanidins and their food sources in the European Prospective Investigation into Cancer and Nutrition (EPIC) study"**. *Br J Nutr* 106(7):1090– 1099, **2011**
- Zebisch K., Voigt V., Wabitsch M., & Brandsch M. "**Protocol for effective differentiation of 3T3-L1 cells to adipocytes.**" *Analytical biochemistry* 425.1: 88-90, **2012**.
- Zechner R., R. Zimmermann T.O. Eichmann S.D. Kohlwein, G. Haemmerle A., Lass and F. Madeo. "**FAT SIGNALS–lipases and lipolysis in lipid metabolism and signaling".** *Cell Metab*. 15: 279–291, **2012**
- Zeyda M, Farmer D, Todoric J, Aszmann O, Speiser M, Gyori G, Zlabinger GJ, Stulnig TM: "**Human adipose tissue macrophages are of an anti-**

inflammatory phenotype but capable of excessive pro-inflammatory mediator production". *Int J Obes (Lond*); 31: 1420–1428, **2007**.

- Zhang Y, Lian F, Zhu Y, Xia M,Wang Q, Ling W, et al. "**Cyanidin-3- O-beta-glucoside inhibits LPS-induced expression of inflammatory mediators through decreasing IkappaBalpha phosphorylation in THP-1 cells"**. Inflamm Res;59(9):723–30, **2010**.
- Zhang Y., Yu H., Gao P., Chen J., Yu C., Zong C., et al. "**The effect of growth hormone on lipid accumulation or maturation in adipocytes.**" *Cellular Physiology and Biochemistry* 39.6: 2135-2148, **2016**.
- Zhou Yingjiang, and Liangyou Rui. "**Leptin signaling and leptin resistance**." *Frontiers of medicine* 7.2: 207-222, **2013**.
- Zhu W., Jia Q.,Wang Y., Zhang Y.,Xia M. "**The anthocyanin cyanidin-3- O-beta-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: Involvement of a cAMP-PKA-dependent signaling pathway".** *Free Radic Biol Med*;52(2):314–27, **2012**.
- Zoico E., Rossi A., Zamboni M. "**Adipochine e complicanze metaboliche dell'obesitá**". *Biochim Clin;35:10-9,* **2011**.
- Zuo Y., Quiang L., Farmer S.R. "**Activation of CCSST/enhancer-binding protein (C/EBP) expression by C/EBP during adipogenesis requires a peroxisome proliferator-activatedreceptor-associated repression of HDAC1 and C/ebp gene promoter".** *J Biol Chem*;281:7960, **2006**.