
***In vitro* interactions of adipocytes and macrophages: potential roles of vegetal extracts in obesity prevention**

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Summary

Obesity and overweight are a plague that concerns more than 20 % of the world population. People suffering from these states present excessive fat accumulation that could impair health. The main causes are an increased intake of energy-dense food and an increased physical inactivity. The energy excess is stored in the adipose tissue whose functions are deregulated, which induces obesity linked metabolic complications. Indeed, most of the time, obesity is accompanied by metabolic disorders, *i.e.* type 2 diabetes, cardiovascular disease... Medical interventions are possible but they often appear ineffective, this is why preventive actions need to be set up.

This study aimed at investigating the potential beneficial effects of fruit derivatives (*i.e.*, pomegranate husk extract and three apple extracts) on the development of obesity and its metabolic disorders by focusing on the adipose tissue functions.

In case of weight gain, the adipose tissues, responsible for the energy storage, have to increase their storage capacity by increasing the size of the adipocytes as well as their number by inducing the differentiation of preadipocytes (adipogenesis). This leads to an increasing body fat mass associated with the obesity complications. In order to identify potential natural candidates that could reduce the adipose tissue expansion linked to obesity development, we have studied the effect of a pomegranate husk extract and three apple extracts on adipogenesis and fat accumulation *in vitro*. Thanks to fluorescence microscopy technics, we have shown that the pomegranate husk and apple extracts could partially inhibit the adipogenesis and the lipid accumulation. These first results make of these extracts interesting candidates for additional investigations.

The second part of this work was focused on the adipose tissue inflammation. Indeed, the adipose tissue of obese people is characterized by a chronic low grade inflammation that could spread throughout the body and induce metabolic disorders. This inflammation is mainly due to an interaction between hypertrophied adipocytes and inflamed macrophages found in the adipose tissues. This condition has been simulated *in vitro* in order to investigate the potential anti-inflammatory effect of the extracts of interest (*i.e.*, the pomegranate husk extract and three apple extracts). The expression and secretion of pro-inflammatory markers were followed, *i.e.* IL-6, TNF α and CCL-2 as well as the anti-inflammatory adipokine, *i.e.* adiponectin. Although the pomegranate husk extract was able to reduce the inflammatory secretions of adipocytes or macrophages cultivated independently, the extract was no more able to decrease the inflammation maintained by the both cell types cultivated together. Nevertheless, the three apple extracts have revealed interesting anti-inflammatory effects on the cells monocultivated and cocultivated by reducing the secretion, as well as the expression, of pro-inflammatory mediators.

Taken as a whole, the results reported in this work allow to highlight that natural derivatives such as the pomegranate husk extract or apple extracts could be interesting candidate to prevent obesity development and/or complications by acting on adipose tissue functions. However, additional research has to be done to investigate the bioavailability of these extracts as well as their residual effects *in vivo*.

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List of Abbreviations

ADD	Adipocyte determination and differentiation factor
ADRP	Adipocytes differentiation related protein
AE	Apple extract
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BCA	BiCinchoninic acid
BMI	Body mass index
BSA	Bovine serum albumine
C/EBP	CCAAT/enhancer-binding proteins
cAMP	Cyclic adenosine monophosphate
CCL	Chemokine (C-C motif) ligand
CRP	C-reactive protein
CS	Calf serum
CVD	Cardiovascular disease
DAMP	Damage-associated molecular pattern
DEX	Dexamethasone
DHAP	Dihydroxyacetone phosphate
DMEM	Dubelcco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dsDNA	Double strand DNA
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallo catechin gallate
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
F-FDG	F-fluorodeoxyglucose
FABP	Fatty acid binding protein
FAT	Fatty acid translocase
FBS	Fetal bovine serum
FFA	Free fatty acid
G3P	Glycerol-3-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Glucose transporter
GPDH	Glycerol-3-phosphate dehydrogenase
HBSS	Hank's balanced saline solution
HPLC	High performance liquid chromatography
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSL	Hormone sensitive lipase

IBMX	Isobutyl methyl xanthine
IC ₅₀	Half maximal inhibitory concentration
ICAM	Intercellular adhesion molecule
IGF	Insulin-like growth factor
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LD	Lipid droplet
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LPH	Lactase phlorizin hydrolase
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MSC	Mesenchymal stem cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF- κ B	Nuclear factor κ -light-chain-enhancer or activated B cells
NO	Nitric oxide
PAI-1	Plasminogen activator protein 1
PAMP	Pathogen-associated molecular pattern
PAT	Perilipin ADRP TIP47
PET-SC	Positron emission tomography scan
PGC	PPAR gamma coactivator
PGE	Prostaglandin
PHE	Pomegranate husk extract
PPAR	Peroxisome proliferator activated receptor
PT	Phloretin
PZ	Phlorizin
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RXR	Retinoid X receptor
SCAT	Subcutaneous adipose tissue
SFRP	Secreted frizzled-related proteins
siRNA	Silencing ribonucleic acid
SREBP	Sterol regulatory element-binding
SVF	Stroma vascular fraction
TG	Triglyceride
TGF	Transforming growth factor

TIP	Tail interacting protein
TLR	Toll like receptor
TNF	Tumor necrosis factor
TZD	Thiazolidinedione
UCP	Uncoupling protein
VAT	Visceral adipose tissue
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
WAT	White adipose tissue
WHO	World health organization
WHR	Waist to hip ratio
Wnt	Wingless-type MMTV integration site

Context

According to the World Health Organization, the number of obese people has tripled since the eighties and nowadays all the countries (developed and developing) are concerned. Moreover, the adolescents, as well as young children, count among the concerned.

It is clear that the main causes are, on the one hand, a lack of physical activities and on the other hand, its combination with an increased consumption of energy-dense foods. Sadly, obesity is associated with the augmentation of metabolic complications such as type 2 diabetes, cardiovascular disease, cancers ...

Obesity and its linked metabolic complications are responsible for an important part of the mortality rate in our industrialised countries and the economic crisis that we are currently experiencing does not help to improve these facts. Indeed, people consume increasingly less expensive foods that often present a poorer nutritional profile and a higher calorie content.

Today, the medical approaches that treat the obesity and its consequences are, most of the time, ineffective.

Given these facts, it appears important to develop preventive strategy aiming at

- improving the quality of foods consumed whatever the socio-economic environment,
- promoting physical activity,
- informing people in order to help them to pick the best health choices.

It is in this context that was launched by the project of the “Delhaize Group Chair” with the contribution of the Fondation Louvain in 2009. The aim was to develop a multidisciplinary approach to prevent obesity by a high quality diet combined with physical exercises.

Concerning the diet, the purpose was to investigate on potential “functional” natural compounds presenting beneficial health properties. Such “functional” compounds could, for instance, be used as food supplements or be added to the usually consumed diet to potentially improve health.

The phenolic compounds naturally appeared as good candidates to ensure the role of functional natural compounds. Indeed, these substances, largely found in fruits and vegetables, present, most of the time, beneficial effects on health. They have already shown effect on chronic inflammation, cancers, ... and obesity development.

For this project, the *in vivo* part was carried out by Prof. Marc Francaux (Faculté des sciences de la motricité; Institute of NeuroScience, UCL) and his group who studied the impact of physical exercise combined with polyphenol enriched diets on obesity development.

Our contribution to this project was to develop an *in vitro* model allowing to identify and understand the action of potentially interesting vegetal extracts that could reveal beneficial effects on the development of obesity and its complications. As the adipose tissue is the organ that undergoes the main negative changes in case of obesity, we chose to focus on it. Indeed, in case of obesity, the cells constituting the bulk of the adipose tissue, *i.e.* the adipocytes, induce and maintain a low grade inflammatory chronic state by interacting with macrophages. This inflammation may spread to the whole body and contribute to the development of type 2 diabetes, cardiovascular diseases ... So we developed an *in vitro* model simulating the inflammatory conditions characteristic of an obese adipose tissue and tested on it vegetal extracts rich in polyphenols.

The *in vitro* investigation has allowed us to distinguish natural candidates interacting with the adipose tissue functions and potentially improving metabolic conditions linked to obesity development.

1. Introduction

1.1. The adipose tissue

The adipose tissue is a connective tissue designed to store large quantities of triacylglycerols, as well as fat-soluble substances. It derives from embryonic mesenchyme and its development begins during the second trimester of life¹. This tissue, today considered as an organ, is composed of several cell types: the mature adipocytes and various other cells, forming what is called the stroma vascular fraction (SVF). This fraction represents a very small part of the adipose tissue and contained different cell types: (i) preadipocytes, (ii) fibroblasts, (iii) mesenchymal stem cells, (iv) endothelial cells, (v) T cells, and (vi) macrophages that all have a role in the structure and function of this connective tissue²... Two types of adipose tissue have to be distinguished: the white adipose tissue (WAT) and the brown adipose tissue (BAT) that is spread in different parts of the body and could coexist at the same place than the WAT (**Fig 1.1**)³.

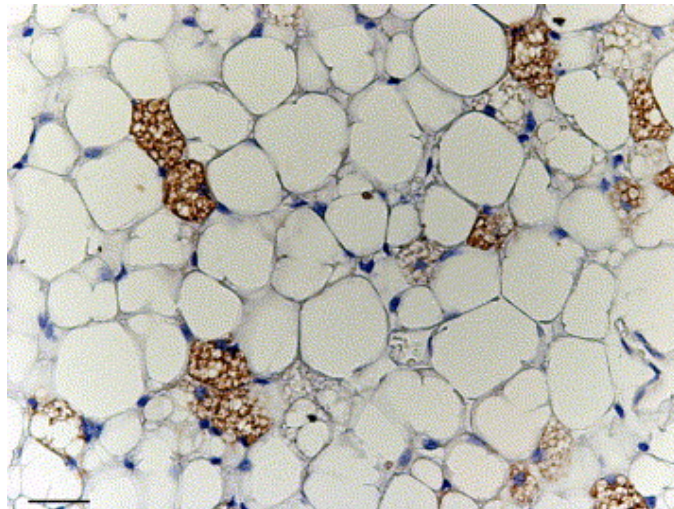


Fig 1.1. Light microscopy of mouse subcutaneous adipose tissue. BAT adipocytes (immunostained with UCP-1 specific antibodies, brown) show multiple lipid droplets. WAT adipocytes show a unique big lipid droplet (Cinti *et al.*, (2006))³

1.1.1. The brown adipose tissue

The BAT is a thermogenic organ highly present in small mammals and in newborns. This tissue produces heat by oxidizing lipids stored in the particular adipocytes constituting the tissue, the brown adipocytes. The

brown adipocytes contain numerous small lipid droplets, numerous mitochondria and are surrounded by a rich vasculature which confer a red-brown colour to the tissue. In the mitochondria membrane of the brown adipocytes, the presence of a unique protein, the uncoupling protein (UCP) 1, allows to dissipate the proton gradient required to produce ATP. Instead of ATP production, heat is generated⁴.

This tissue has long been considered as only occasionally present in human adult (2.5 to 45% according the sources). However, Van Marken Lichtenbelt *et al.* (2009) showed that this tissue is present in adult and that cold exposure increased the amount of active BAT suggesting the presence of non-active BAT in all adults (**Fig 1.2**)⁵. In children, the BAT is present in the interscapular region and disappeared often completely after the adolescence. However, other depots *e.g.* cervical, perirenal, periadrenal and perivascular, are maintained until the end of life. Furthermore, the BAT proportion is higher in women than in men and decrease with age and adiposity⁶.

As the activation of the BAT induces the oxidation of lipids stored in the brown adipocytes, this tissue is today considered as an interesting target to counter obesity.

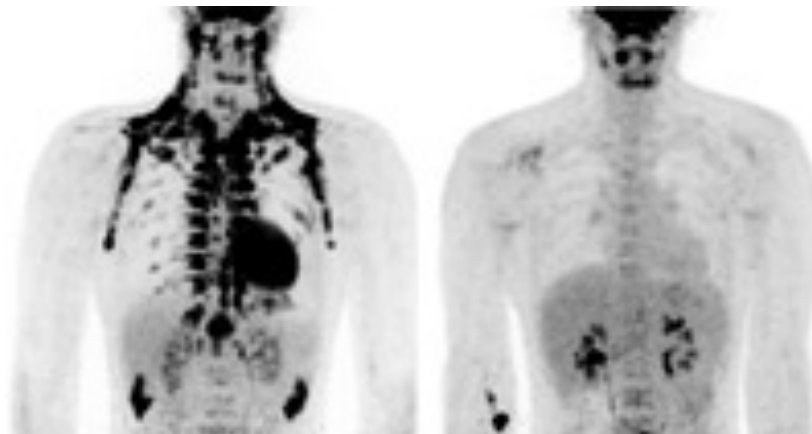


Fig 1.2. Physiologic uptake and distribution of ^{18}F -fluorodeoxyglucose (^{18}F -FDG) in adipose tissue. Results of PET-SC after cold exposure (left) or thermoneutral condition (right) for the same subject. The ^{18}F -FDG revealed the BAT localizations. (Van Marken Lichtenbelt *et al.* (2009))⁴

1.1.2. The white adipose tissue

The WAT is the main adipose tissue in human adults. It could occupy positions under the skin, *i.e.* the subcutaneous adipose tissue (SCAT), or around organs *i.e.*, the visceral adipose tissue (VAT).

The distribution and abundance of WAT depend on a variety of factors including age, sex, genetic and environment. One of the main functions of the WAT is to store the excess of energy intake. The adipocytes could use glucose to synthesize *de novo* free fatty acids (FFAs), which makes the adipose tissue as a major regulator of glucose and lipid homeostasis. This tissue has the ability to contain huge quantity of fat as triglycerides in its storage cells, the adipocytes, and to release energy in case of need. The excess of energy could be stored without limit in this tissue.

However, depending on their locations (SCAT or VAT), the adipose tissues respond more or less to the metabolic needs. **Table 1.1** resumes the main differences between both adipose tissues.

	SCAT	VAT
Proportion in lean state	$\pm 80\%$	$\pm 10\%$
Phenotype of adipocytes	Small adipocytes	Big adipocytes
Vascularisation	Poor	Rich
Insulin sensitivity	High	Low
Lipolysis rate	Low	High
Glucose uptake	Low	High
FFA uptake	High	Low

Table 1.1. Subcutaneous and visceral adipose tissues structural and functional differences. Resumed from Ibrahim (2010)⁷.

In addition to its homeostasis functions, the adipose tissue also regulates inflammation, immunity, cardiovascular function (...) by secreting biologically active factors. These two properties made of the adipose tissue both an endocrine and a storage organ that plays important roles in the body homeostasis. The main cells in charge of these functions are the adipocytes.

1.2. The adipocytes

The adipocytes derive from mesenchymal stem cells, as muscle or bone cells, which themselves derive from mesoderm. Pluripotent mesenchymal stem cells reside in the SVF and, under appropriate stimulation, they could be committed to the adipocyte lineage⁸. Some evidences indicate that brown adipocytes could also derivate from the same precursor cells than myocytes, *i.e*, the Myf-5 precursor⁹. Indeed, some characteristics of these cells are similar to those of muscle cells. The lipids stored in these adipocytes are mainly oxidized by mitochondria to produce heat. Their function is then drastically opposed to that of white adipocytes since brown adipocytes dissipate energy instead of storing it.

White adipocyte cells have to confront enormous challenges during their life cycle. On the one hand, they store huge quantities of lipids, facing severe architectural constraints and, on the other hand, they contribute to the regulation of physiological functions. Most of the time, mature adipocytes contain a large lipid droplet composed of triglycerides, cholesteryl esters, but also hydrophobic molecules including toxic pollutants. The unique droplet occupies most of the cell volume, the cytoplasm being almost absent and the nucleus rejected on the periphery. *In vivo*, the diameter of adipocytes is *ca.* 100 μm in diameter (**Fig 1.3**).

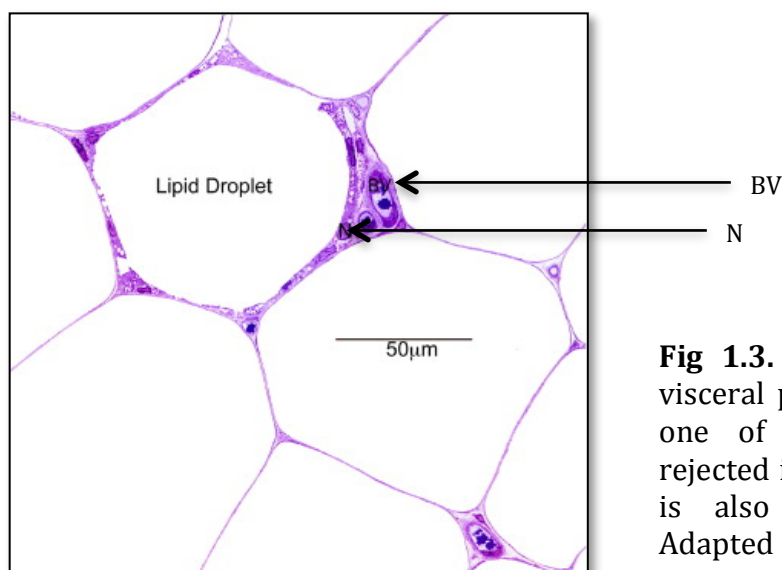


Fig 1.3. Adipocytes from a rat visceral pad. The nucleus (N) of one of them is visible and rejected in periphery. A capillary is also distinguishable (BV). Adapted from Henry *et al* (2000).

Playing a role in the lipid metabolism, the cells ensure their synthesis (lipogenesis), storage and release (lipolysis) according to the metabolic needs. During a period of food abundance, the energy excess is stored as triglycerides. In case of need, these triglycerides could be broken down to FFAs and glycerol. The FFAs are then, for example, catabolized by muscles and the liver to generate energy as ATP. Glycerol is used by liver and renal cells to synthesize glucose used by brain and other tissues¹⁰.

In humans, the number of adipocytes increases until the adolescence. An average adult has 30 billion fat cells (± 15 % of body weight in men and ± 25 % in women). Every year, ten percent of the adipocyte population are renewed¹¹. In case of obesity, the volume of fat content increased in pre-existing adipocytes (hypertrophy) and if the weight gain is more pronounced, as the capacity of storage is limited, new adipocytes could differentiate from preadipocyte cells (hyperplasia).

The contribution of hyperplasia and hypertrophy in the adipose tissue expand seems to be equal and gender or obesity state do not impact on these occurrences¹¹.

1.2.1. Adipocyte differentiation

The adipocyte differentiation or adipogenesis is a very complex phenomenon that begins with a mesenchymal stem cell (MSC) and that ends with the formation of fully functional adipocytes. These transformations include several stages of differentiation characterized by gene expression driven by specific molecular regulations.

1.2.1.1. Stage of differentiation

Unlike other organs, the adipose tissue is dispersed in many sites around the body¹². Before the apparition of the first preadipocyte biological markers, a capillary network appears in the future site of adipose tissue. The early event that promotes determination of precursor cells to the adipocyte lineage is not really established.

A variety of signals could induce the commitment, *e.g.* bone morphogenetic protein, transforming growth factor β , insulin-like growth factor 1, interleukin 17, fibroblast growth factor 1 and 2, and activin¹³. These signals initiate the commitment of the mesenchymal stem cell in the adipocyte lineage and, most of the time, this phenomenon occurs in clusters. *In vivo*, this first phase is commonly called the *determination phase*. At this stage, preadipocytes are morphologically similar to the stem cell but are blocked in the adipocyte lineage (**Fig 1.4**).

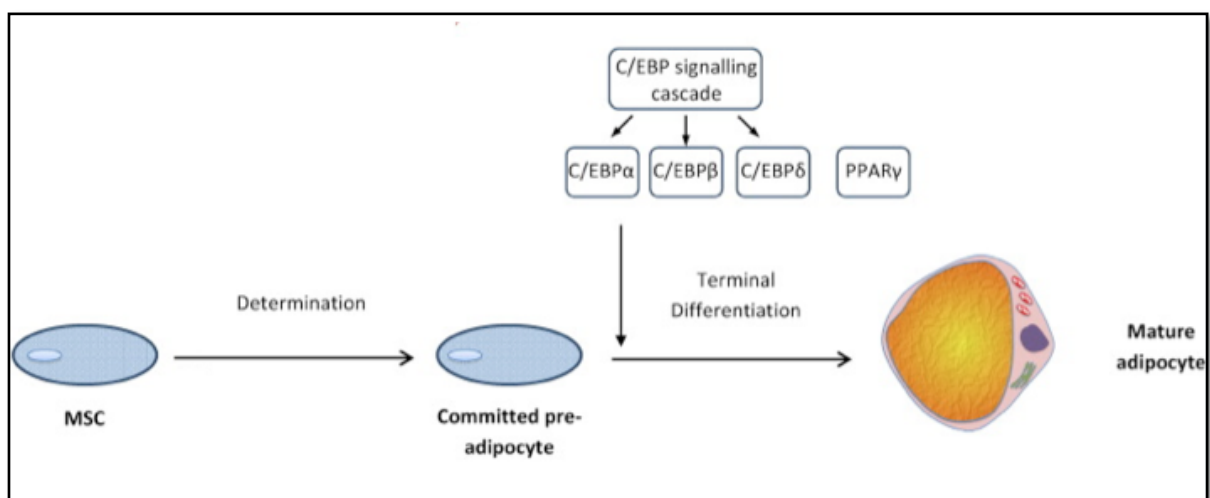


Fig 1.4. Overview of white adipocyte formation. A mesenchymal stem cell (MSC) is committed to the adipocyte lineage under the stimulation of specific markers. The preadipocyte after a clonal expansion, starts to express the main inducers of the adipocyte differentiation (C/EBP members and PPAR γ). Then, the cell undergoes the last part of the differentiation process to finally present the adipocyte phenotype. Adapted from Henry *et al.* (2012)¹

To initiate their differentiation, the preadipocytes need first to stop their growth. This arrest could be followed by a mitotic clonal expansion and a second growth arrest. These two steps are subjects of debates; even if these events clearly occur *in vitro*, no *in vivo* evidences exist¹². Anyway, after the growth arrest, the cells begin the *terminal differentiation phase*. During this phase, the preadipocytes undergo morphological changes. Their shape becomes rounded and they start to synthesize, transport and store triglycerides. They also acquire the ability to respond to hormones such as insulin. The morphological modifications are synchronized with changes in

the extracellular matrix components. This last one influences the expression of key adipogenic factors (see 1.2.2.3)¹⁴.

To clarify the molecular mechanisms of the differentiation process, we will focus on an *in vitro* cell line, *i.e.* the 3T3-L1 that has been the subject of numerous studies on the adipocytes differentiation. In this cell line, the different steps that lead from preadipocytes to mature adipocytes could be divided as: (1) preconfluent proliferation, (2) confluence/growth arrest, (3) hormonal induction/mitotic clonal expansion and (4) growth arrest/terminal differentiation. These four different steps are characterized by the expression pattern of specific genes¹⁵.

Preadipocytes have to reach confluence before they start to differentiate (step 1). Then the growth arrest (step 2) is achieved through contact inhibition and is required to move to the next step. At this stage, cells are blocked at the G₀-to-G₁ cell cycle phase. After this growth arrest, cells could be stimulated by a differentiation cocktail (see induction of the differentiation process) that will induce the beginning of the step 3. This stimulation re-starts the cell cycle and cells enter in the S-phase followed by a mitotic clonal expansion consisting of at least one round of DNA replication and cell doubling¹⁶. Nonetheless, some studies reveal that this duplication is not really essential for a good differentiation process (**Fig 1.5**)^{17,18}.

For a long time, it was thought that the number of adipocytes was determined during the childhood and that, in case of weight gain, only the size of the cells was affected. It is now known that, even in adults, the preadipocytes could differentiate into new adipocyte cells. This process would allow to increase the storage capacity in case of energy excess, but is also responsible for a high turnover of adipocytes¹⁹.

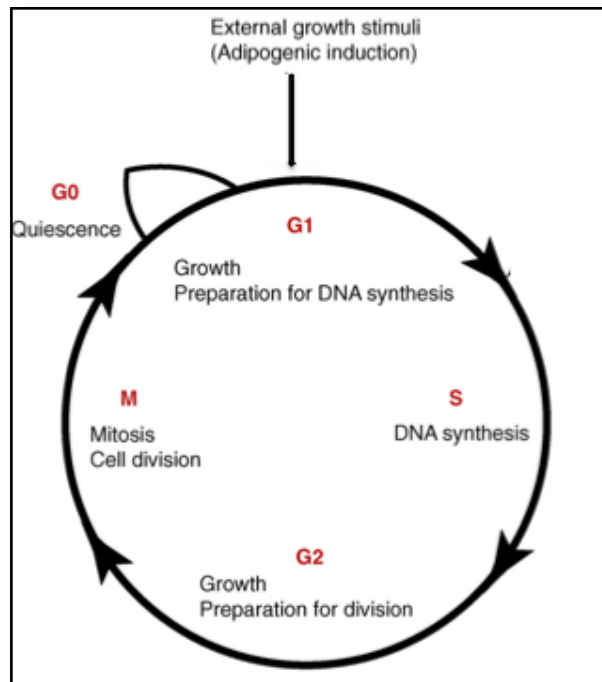


Fig 1.5. Cell cycle. Confluent adipocytes are blocked in G₀-to-G₁ phase. The adipogenic induction with an appropriate hormonal cocktail induces the DNA synthesis followed by the mitotic clonal expansion. After that, preadipocytes stop their growth and begin the differentiation process. The mature adipocytes cannot undergo any cell division. Adapted from Lefterova *et al.* (2009)¹⁷.

1.2.1.2. Induction of the adipogenesis

The *in vivo* induction of adipogenesis is not really known. Numerous *in vitro* studies have allowed to understand some of these aspects. To induce the differentiation in 3T3-L1 preadipocytes (step (3)), the cells need to be stimulated by hormones. The most effective cocktail is composed of insulin, a glucocorticoid, an agent that elevates intracellular cAMP level and fetal bovine serum. Traditionally, the cocktail contains a cAMP-phosphodiesterase inhibitor (most often the isobutyl methyl xanthine (IBMX)), insulin and dexamethasone (DEX) as glucocorticoid agonist and it is called the MDI cocktail.

Insulin increases the percentage of cells that differentiate and increases the amount of lipid stored in each cell. Surprisingly, preadipocytes do not express insulin receptors and insulin acts on the insulin-like growth factor 1 (IGF-1) receptor, which induces the PI₃K-Akt pathway and the Ras-MAP kinase pathway inducing the proliferation and the differentiation of

preadipocytes^{20,21}. DEX stimulates the glucocorticoid receptor pathway and induces, among others, the expression of C/EBP δ , a critical factor essential for the induction of adipogenesis (see point 1.2.2.3)²². DEX also reduces the expression of Pref-1, an inhibitor of adipogenesis. IBMX allows stimulating the cAMP-dependant protein kinase pathway that also plays a role in the early stage of the differentiation process. It is responsible for the increase of cAMP and has been shown to increase C/EBP β levels^{22,23}.

The MDI cocktail triggers the preadipocyte conversion by inducing the expression of regulatory molecules. All of these molecules interact with each other following a timed process.

Concerning the brown adipogenesis, it could be induced *in vivo* by cold exposure. The noradrenaline, via the sympathetic nervous system (hypothalamus) also regulates the BAT proliferation, differentiation and activity⁴.

1.2.1.3. Molecular regulation during the adipocyte differentiation

During the few days needed for the acquisition of adipocyte phenotype, the expression of 2,000 genes is modified^{24,25}. At the early beginning, two major inducers of the differentiation are activated, *i.e.* **C/EBP β and C/EBP δ** ¹⁹. They belong to the family of transcription factors named CCAAT/enhancer-binding proteins (C/EBPs). All the members of this family have a C-terminal leucine zipper domain to allow dimerization. The different C/EBPs members can homo- or heterodimerize and bind to the C/EBP consensus sequences via their basic domain. Both factors are required for WAT and BAT growth and adipogenesis. *In vitro*, ectopic expression of C/EBP β is sufficient to induce the differentiation of 3T3-L1 cells without any further hormonal stimulation²⁶. Embryonic fibroblasts lacking either C/EBP β or δ differentiate less easily than normal embryonic fibroblasts and if both are lacking, the differentiation does not occur²⁷. *In vivo* experiments on mice knock outs for one of these two transcription factors have reported no differences in the white adipose tissue compared with normal mice.

However, 85% of the C/EBP β (-/-) δ (-/-) mice died at the early neonatal stage²⁷.

The two inducers are known to act synergistically on the expression of C/EBP α and PPAR γ_2 via a C/EBP-binding sites present in their promoters.

The peroxisome proliferator activated receptor (PPAR) γ belongs to the nuclear receptor superfamily of ligand-activated transcription factors. Alternative promoter usage gives two PPAR γ isoforms. PPAR γ_1 is ubiquitous and expressed in WAT, BAT, macrophages, liver, skeletal muscle, kidney, colon... PPAR γ_2 has 28 (or 30 in rodents) more N-terminal residues than PPAR γ_1 . The isoform PPAR γ_2 is mainly expressed in adipose cells and is necessary and sufficient for the adipocyte differentiation process¹⁰. All the PPAR have to heterodimerize with the retinoid X receptor (RXR) in order to bind specific DNA sequence (peroxysome proliferator response element PPRE).

PPAR γ is called the **master regulator of adipogenesis**. Indeed, Rosen *et al.* (1999) showed that embryonic stem cells PPAR γ (-/-) are unable to differentiate into adipocytes¹². An other team working on 3T3-L1 cells showed that transduced 3T3-L1 preadipocytes expressing siRNA against PPAR γ_1 and PPAR γ_2 lose their ability to differentiate into adipocytes²⁸. Up to now, no factor able to rescue adipogenesis in the absence of PPAR γ has been identified. PPAR γ_2 is responsible for the expression of genes involved in lipid accumulation and many other adipocyte specific functions. Its role has also been demonstrated *in vivo*, although PPAR γ (-/-) mouse embryos are not viable, experiments using chimeric mice revealed that PPAR γ is required for adipogenesis¹².

This transcription factor is also essential for the brown adipogenesis⁴. In brown preadipocytes, the PPAR γ coactivator PGC-1 is expressed and interacts with PPAR γ to induce the expression of UCP-1. Forced PGC-1 expression in white adipocytes induced mitochondrial biogenesis and UCP-1 expression²⁹. In case of cold exposure or under β -adrenergic stimulation, the expression of PGC-1 increased in white adipocytes leading to a transdifferentiation and intermediate morphology features³⁰.

C/EBP α and PPAR γ_2 stimulate mutually their own expression and activate the downstream target genes essential for the development of adipocytes. C/EBP α , unlike PPAR γ_2 , is not sufficient to promote the adipogenesis and is even not really necessary. Indeed, an other study of Rosen *et al.* (2002) has shown that C/EBP α null fibroblasts undergo adipogenesis when PPAR γ is replenished³¹. They revealed that the main role of C/EBP α is to induce and maintain the expression of PPAR γ . Even whether C/EBP α does not play a crucial role in the adipogenesis, it is critical for the establishment of insulin sensitivity by inducing the expression of insulin receptor.

The sterol regulatory element-binding protein (SREBP)-1c is also a transcription factor acting on PPAR γ_2 production as C/EBP β and C/EBP δ ³². The adipocyte determination and differentiation factor (ADD) 1 is the murine protein and SREBP-1c is its human homolog. They are members of the basic helix-loop-helix-leucine zipper family and their expression is elevated in preadipocytes and increases during the differentiation under the action of insulin³³. It appears that ADD1/SREBP-1c is not essential for the differentiation process as revealed experiments on dominant-negative cells, however it could be responsible for the production of endogenous PPAR γ ligand that stimulates the adipogenesis³⁴. **Fig 1.6.** resumes the principal transcriptional controls that govern the adipogenesis.

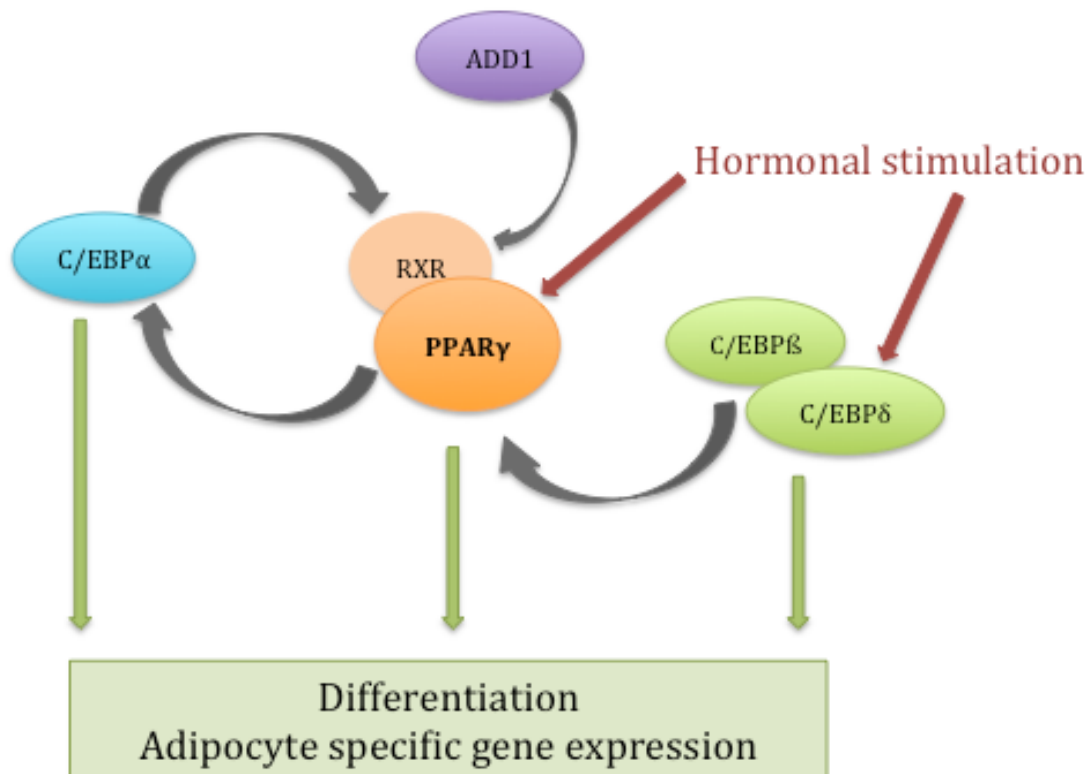


Fig 1.6. Transcriptional principal control of the adipogenesis in mouse. The first factors expressed (C/EBP β and C/EBP δ) act on PPAR γ expression. PPAR γ activates C/EBP α expression, which exerts positive feed back on PPAR γ . Efficiency of PPAR γ could be increase by linking to a ligand such as RXR. ADD1 also act on PPAR γ expression and stimulation. These factors act together on the expression of adipocyte specific genes.

Recently, some other signalling molecules have been identified as critical for the initiation of adipocytes differentiation. Among them, the Wingless-type MMTV integration site (Wnt) family members play crucial roles in preadipocytes determination through the inhibition of C/EBP α and PPAR γ_2 . The Wnt glycoproteins are secreted by preadipocytes and act via autocrine or paracrine pathways to regulate the proliferation, development and differentiation. By binding to cell surface receptors, Wnt10b activates β -catenin pathway that finally activates anti-adipogenic target genes. Another Wnt members, Wnt5a, acts also as inhibitor of adipogenesis. However, Wnt5b, unlike its congeners, promotes adipocyte differentiation by inhibiting β -catenin nuclear localization^{1,31}.

GATA-2 and GATA-3 are transcription factors that block the cells at the preadipocyte stage by acting on the PPAR γ_2 promoter. Pref-1 has also been

identified as an inhibitor of the differentiation process. Actually, DEX inhibits Pref-1 transcription and thereby allows for the first step of differentiation³⁵. Moreover, this transmembrane molecule probably interacts with extracellular matrix to control the adipogenesis.

So, adipogenesis can be divided in two phases: the early and the late phases. The proteins described above are part of the early phase, other typical proteins characterize the late phase. These proteins assume the glucose transport, insulin sensitivity, triglyceride storage, synthesis and degradation, as well as the endocrine role of adipocytes.

1.2.2. *In vitro* adipocyte models

Numerous cell lines are currently used to study and characterize the different steps in the life of an adipose cell. Even whether the *in vitro* environment will never be similar to that encountered *in vivo*, there exist several advantages to use immortalized cell lines.

The preadipocyte cell lines show a fibroblastic phenotype, as preadipocytes found in the SVF *in vivo*. The populations of clonal cell lines are homogeneous. Moreover, they allow a response to treatments resulting in a cell population that presents, in the same time, the same stage of differentiation. Finally, these cells could be cultured indefinitely.

The preadipocyte cell lines usually used are named 3T3-L1 and 3T3-F442A. They derived from disaggregated 17-19-day-old Swiss 3T3 mouse embryos³⁶. The 3T3-F442A cells are more advanced in the commitment of the adipocyte lineage than 3T3-L1 cells¹⁵. This latest cell line is one of the best characterized models for studying the conversion of preadipocytes into adipocytes. Green & Kehinde (1979) reported that this adipocyte lineage mimics live adipose tissue and could form fat pads *in vivo* undistinguishable from normal adipose tissue³⁷.

The C3H10T1/2 cells from 14-17-day-old C3H mouse embryos are **mesenchymal stem cells**. They can be differentiated into muscle, bone, cartilage or adipose cells. To differentiate them into adipocytes, a pre-treatment is needed before the induction by the adipogenic factors.

The ***adipose-derived stem cells*** are easy to obtain and purify although contamination by other types of cells is inevitable. These cells could be differentiated *in vitro*. A primary preadipocyte culture may be a good step to confirm data derived from preadipocyte lines because it reflects better the *in vivo* context.

These different cell lines allow to better understand the complex mechanisms of adipocyte differentiation.

The ***primary preadipocyte culture*** could be developed after the isolation of preadipocytes from the adipose tissue. The differentiation of these precursors could be then induced *in vitro*. This “fresh” culture also contains other cell types present in the SVF³⁸.

1.2.3. Lipogenesis and lipolysis

Among the proteins expressed during the adipocyte differentiation, some manage the triglyceride synthesis, called lipogenesis. This pathway occurs in the adipose cells when the energy balance is positive. It consists of transforming carbohydrates or lipids into triglycerides, which are stored in the lipid droplets. Insulin stimulates this metabolic pathway by: (1) activating glucose entry into the adipose cells; (2) activating FFAs entry into the adipose cells; and (3) stimulating the enzyme responsible for the synthesis of storage triglycerides, the triglyceride synthase.

The glucose transporter (GLUT)-4 is present in the adipocyte plasma membrane and allows the entry of glucose. Its expression starts four days after the induction of the differentiation process²⁹. Once the glucose is inside the adipocyte, it could undergo the glycolysis pathway, which transforms it into pyruvate, which after the action of pyruvate dehydrogenase may give acetyl-CoA. This molecule is the input to FFA synthesis. Acetyl-CoA is carboxylated to form malonyl-CoA. The fatty acid synthase synthesizes fatty acids using these two molecules. This is the *de novo* synthesis of fatty acid that occurs in the liver, lactating glands and adipose tissue. However, adipocytes can further take up and use circulating triglyceride to form new FFAs.

The lipoprotein lipase (LPL) is a membrane protein that hydrolyzes circulating triglycerides (TGs), transported by lipoproteins, into FFAs. This enzyme is expressed in the early phase of the differentiation process, well before the lipid accumulation³⁹. The FFAs are transferred into the cytoplasm via the fatty acid binding protein (FABP) and the fatty acid translocase (FAT). The acyl-CoA-synthase forms acyl-CoA used for triglyceride synthesis pathway. The cytosolic enzyme glycerol-3-phosphate dehydrogenase (GPDH) is expressed in the late phase and is also involved in the triglyceride synthesis and reduces dihydroxyacetone phosphate (DHAP), derived from glucose, into glycerol-3-phosphate (G3P). Three acyl-CoAs are esterified with one molecule of G3P to form a triglyceride (**Fig 1.7**)

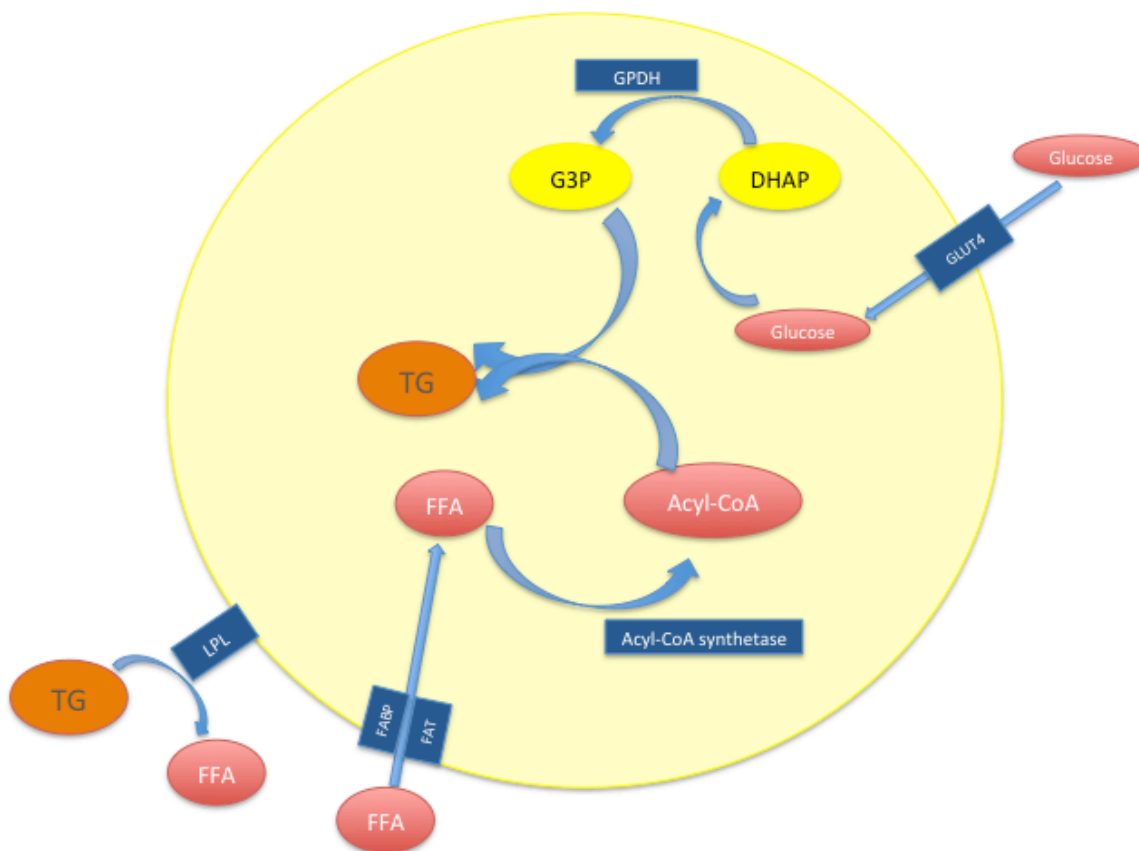


Fig 1.7. The lipogenesis. Triglycerides (TG) from chylomicrons or VLDL are hydrolyzed in free fatty acids (FFAs) by lipoprotein lipase (LPL). FFA and glucose enter in the adipocyte by their specific transporters. Inside the cell, glucose can be transformed in dihydroxyacetone phosphate (DHAP). Glycerol-3-phosphate dehydrogenase (GPDH) synthesizes glycerol-3-phosphate (G3P) from the DHAP. Three acyl-CoAs and one G3P allow the synthesis of one molecule of triglyceride. Glucose could also be used for *de novo* free fatty acid synthesis.

In case of need, the energy stored can be released thanks to the lipolysis. This process consists in hydrolyzing triglycerides into three FFAs and one glycerol molecule. Three consecutive steps realized by three different enzymes lead to the release of these molecules. The adipose triglyceride lipase (ATGL) catalyzes the initial step converting the triglyceride into a diacylglycerol. Then, the diacylglycerol is hydrolized by the hormone sensitive lipase (HSL) into a monoacylglycerol. These reactions occur at the surface of the lipid droplet. The FFAs released and the monoacylglycerol are transported into the cytoplasm where the monoacylglycerol lipase releases the last FFA and the glycerol molecule⁴⁰. The FFAs generated can move into the bloodstream, be bounded to albumin and be transported to the tissues that need energy such as liver, heart or muscles. Numerous fatty acid transporters present in the membrane of the target cell allow the FFA to cross the plasmatic membrane but simple diffusion mechanism could also be involved⁴¹.

1.2.4. The lipid droplet formation and growth

The lipid droplets allow the storage of triglycerides, sterol esters and lipophilic substances. Due to their hydrophobic character, a monolayer of phospholipids surrounds the droplet providing a unique separation between both phases, *i.e.* aqueous and organic⁴². Various proteins are also present in the monolayer such as the proteins of the PAT class. The proteins of the PAT class (for Perilipin, Adipocytes differentiation related protein (ADRP) and Tail interacting protein 47 (TIP47)) contain a central hydrophobic sequence for targeting the lipid droplet⁴³. Perilipin is adipocyte specific and regulates lipolysis. ADRP is ubiquitous and its expression is proportional to the amount of triglycerides stored in the cell. During the adipogenesis, the expression of ADRP happens early, but at later stages, ADRP is replaced by perilipin. TIP47 is a cytosolic enzyme shifted to lipid droplets when the level of FFA increases. Other proteins are also part of the lipid droplet membrane. Among them, the caveolins seem have an impact on the lipid droplet formation by favouring their growth⁴⁴. Their

expression increases during the differentiation process. These proteins are also present in high amount the plasma membrane of adipocytes and participate to the free fatty acid trafficking⁴¹.

The synthesis of TG explained above, seems to take place in the endoplasmic reticulum (ER)⁴⁵. The TGs accumulate in the ER and form the core of the lipid droplet. Three different models try to explain the mechanism by which the lipid droplet (LD) could be formed (**Fig 1.8**)⁴⁶.

- (i) Monolayer bulging and budding; the neutral lipids form a lens of oil in the ER bilayer that buds from the membrane. The cytosolic leaflet of the ER becomes then the phospholipid monolayer of the LD,
- (ii) Micelle formation; the lipid droplet forms between the two leaflets of the ER membrane that are excised. In this case, the LD is surrounded by a monolayer coming from the inner and the outer membranes of the ER.
- (iii) Vesicular budding; LDs are formed from a vesicle that buds from the lumen of the ER and stays connected to the ER that will fill the vesicle of lipid

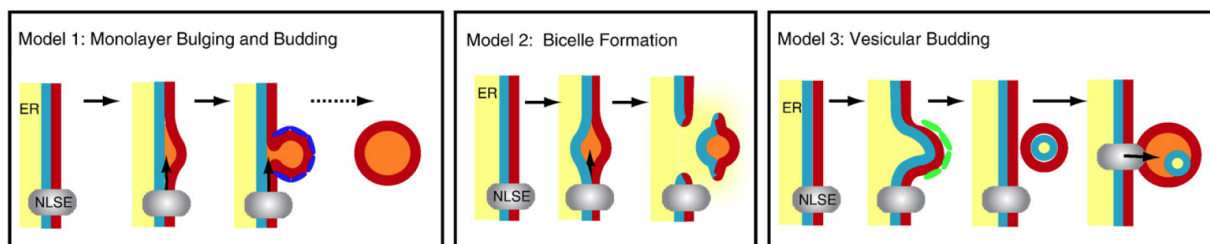


Fig 1.8. Proposed models for lipid droplet formation⁴⁶.

The way by which the LDs grow is relatively unknown. They could expand as a single organelle by the synthesis of TG inside the LDs or small droplets could fuse to form bigger LDs. Or, finally, if the LDs are connected to the ER, newly synthesized lipid could diffuse to the LD.

Anyway, LDs allow adipocytes to store huge amount of energy as triglycerides.

However, these cells have also the ability to secrete metabolically active molecules and give an endocrine role to the adipose tissue.

1.2.5. The adipokines

As already mentioned, the adipose tissue influences and regulates physiological functions. This tissue contains a lot of different cell types, likely to secrete regulatory molecules. The molecules secreted by adipocytes and having a metabolic role are called the **adipokines**. Their secretions vary in function of the body site, *i.e.* SCAT or VAT and in function of the body state, *e.g.* obesity⁴⁷. Before the discovery of leptin in 1994, some evidences underlined the existence of circulating factors derived from the adipose tissue. Coleman showed with the parabiosis experiments that circulating factors from congenitally obese mice had profound influence on the energy homeostasis in normal mice. Actually, this circulating factor was leptin⁴⁸.

Leptin is a hormone produced exclusively by adipocytes. This 16-kDa non-glycosylated protein acts on the hypothalamus to modulate body weight, food intake and fat stores. The hormone, by acting on the OB-receptors, informs the brain about the satiety state in order to regulate the different aspect of energy regulation. These OB-receptors are also present in peripheral tissues such as adipose tissue, skeletal muscles, pancreatic β cells and liver.

In humans, in function of the body state, leptin acts differently. Its circulating concentration is positively correlated with the total amount of body fat. If the weight is stable, leptin level reflects total body fat mass. However, if the energy balance is positive (weight gain) or negative (weight loss), leptin level changes in order to modulate the energy regulation pathways. High level of leptin informs the brain that there is an excess of energy. In response, the brain decreases the appetite sensation and increases the energy expenditure. Inversely, low level of leptin increases the appetite and decreases the energy expenditure. However, the receptors are less sensitive to high concentrations of leptin and obese people become quickly resistant to this hormone⁴⁹.

Adiponectin (also known as AdipQ, apMI and Acrp30) is the most abundant protein produced by the adipose tissue. Its plasmatic

concentration varies from 3 to 30 $\mu\text{g/ml}$, which is higher than the concentration of other known hormones⁵⁰. This globular hormone could form trimers that could also assemble by two to form hexamers or more.

Adiponectin acts on two receptors, ADPR1 and ADPR2, which are respectively primarily expressed in muscles and in liver. Disruption of these receptors abrogates adiponectin binding, causing insulin resistance and glucose intolerance⁵¹. Adiponectin is also an important factor that regulates several vascular functions as (i) vasodilation, (ii) reduction of inflammation caused by tumor necrosis factor (TNF) α , (iii) inhibition of the effect of oxidized low-density lipoproteins, (iv) increase of nitric oxide (NO) production, (v) stimulation of angiogenesis...

Numerous other proteins and non-proteins substances are secreted by adipocytes and could act on different targets. **Table 1.2** resumes the factors secreted by the adipocytes and their biological effects.

However, other cells, present in the adipose tissue, also secrete biological factors that act on the body functions. The other main sources of secretion are the macrophages. Depending on the “fat composition” of the individual, their secretions are more anti or pro-inflammatory.

Substances	Regulated by	Action by	Biological effects
Leptin ^{45,47,49}	Insulin (+) TNF α and IL1 (+)	OB receptor $\rightarrow$ JAK-STAT and PI3 kinase	- Repression of orexigenic pathways (nervous system) - Increase TNF α and IL-6 expression - Stimulation of angiogenesis
Adiponectin ^{44,50}	Insulin (+) TNF α (-)	ADPR1 and ADPR2 $\rightarrow$ AMPK and PPAR α T-cadherin	- Increase glucose absorption - Increase fat oxidation - Decrease hepatic gluconeogenesis - Increase insulin sensitivity and insulin secretion - Inhibition of VCAM-1 and ICAM-1 (endothelial cells) - Induction of NO production by endothelial cells - Decrease TNF α and IL-6 expression
SFRP5 ⁵¹		Binding on Wnt5	- Suppression of proinflammatory Wnt signalling, induction of adipogenesis
Chemerin ⁵⁰			- Anti-inflammatory effect by acting on the CMLK receptor 1 carried by macrophages - Regulation of adipocyte differentiation
Resistin ^{44,49} (produced by adipocytes in rodent and by monocytes in human)	Glucocorticoids and LPS (+)	Receptor unknown NF- κ B pathway	- Increase pro-inflammatory cytokine secretions by macrophages (IL-1, IL-6, TNF α) - Increase insulin resistance
Adipsin ⁴⁴			- Activation of the alternative complement pathway
Apelin ⁵⁰	Insulin (+)	Apelin receptor (APJ) $\rightarrow$ PI3K	- Inhibition of insulin secretion - Vasodilatation by stimulation eNOS expression - Stimulation of angiogenesis
Visfatin ⁵⁰			- Insulin mimetic effect (not clear)
TNFα ^{45,49,52}	Adiponectin (-)	TNF receptor NF- κ B pathway	- Increase insulin resistance - Decrease adiponectin expression - Induction of lipolysis - Increase TNF α , MCP-1 and IL-6 expression - Increase vascular inflammation

Substances	Regulated by	Action by	Biological effects
CCL-2 ^{51,53}		CCR-2	- Monocyte recruitment - Macrophages activation
C-Reactive Protein (CRP) ⁵²	IL6, TNF α (+)		- Increase vascular inflammation
Plasminogen activator protein (PAI-I) ^{45,52}	TNF α (+)		- Inhibition of plasminogen activation - Blocking fibrinolysis - Potential role in facilitating adipocytes enlargement
Angiotensinogen ^{45,51}	FFA, glucocorticoids, TNF α (+)		- Vasoconstrictive effect - Induction of VCAM-1 and ICAM-1 (endothelial cells) - Inhibition of insulin induced adipogenesis - Increase insulin resistance
Vascular Endothelial Growth Factor (VEGF) ^{53,54}	Insulin (+)		- Stimulation of angiogenesis

Table 1.2. Various molecules secreted by adipocytes and controlling body functions. (+) indicates that the molecule stimulates the expression and (-) that the molecule inhibits the expression

1.3. The macrophages

The macrophages derive from the differentiation of monocytes upon their migrations from blood into tissues. These phagocytic cells, present in the adipose tissue, allow the elimination of cellular debris that come from the apoptosis or tissue remodelling. This normal process of “cleaning” does not require a specific activation of the cells.

However, when macrophages are needed to eliminate debris from necrosis or when other endogenous dangers are detected, *i.e.* injury or infection, they completely change their physiology. In this case, the macrophages produce pro-inflammatory mediators and express specific surface proteins⁵⁷. According to their activation state, macrophages are designed as classically activated macrophages M1 or alternatively activated macrophages M2 (**Fig 1.9**).

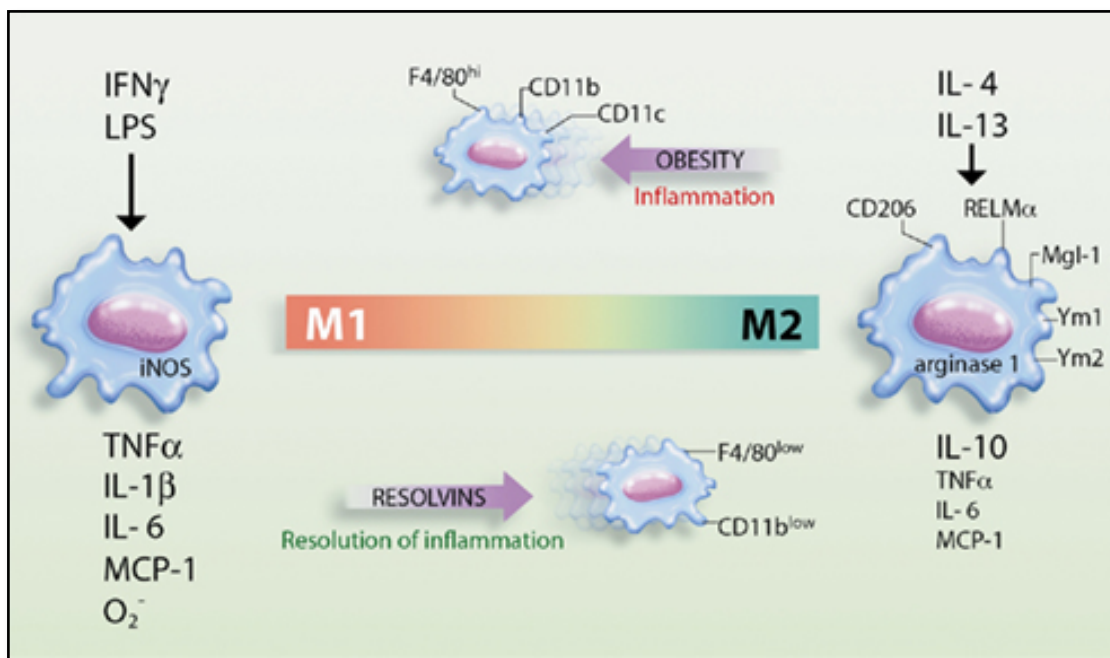


Fig 1.9. Macrophages polarization. Depending on the environment, macrophages differentiate into a pro-inflammatory profile (M1) or into an anti-inflammatory profile (M2). M1 macrophages express specific cell surface markers such as F4/80, CD11b and CD11⁵⁸. The M2 macrophages overexpress mannose (CD206) and galactose (Mgl-1) receptor, resistin-like molecule (RELM) α and chitinases Ym1 and Ym2. The activity of the arginase 1 is also higher in M2 macrophages.

The classically activated macrophages M1 are involved in host defence against pathogens. Interferon (IFN) γ and TNF α act together to activate the phagocytic cells, which will enhance their microbicidal and tumoricidal capacity and secrete high levels of pro-inflammatory cytokines (TNF α , interleukin (IL) 6, IL-1 β , chemokine (C-C motif) ligand (CCL) 2) and reactive oxygen/nitrogen species (ROS/RNS) via the activation of *e.g.* the inducible nitric oxide synthase (iNOS). Other pathogen associated molecules such as lipopolysaccharides (LPS), lipoprotein... could also promote the M1 phenotype. Many receptors allow macrophages to detect these signals⁵⁹. Among them, the Toll-like receptor (TLR) 4 recognizes LPS and activates via complex cascades the nuclear factor kappa-light-chain-enhancer or activated B cells (NF- κ B) transcription factor and three pathways of mitogen-activated protein kinase (MAPK) that lead to the expression of pro-inflammatory molecules⁶⁰. If M1 macrophages are thought to contribute to the tissue injury by stimulating the inflammation, M2 macrophages play a role in the resolution of inflammation through phagocytosis of apoptotic cells, reduction of pro-inflammatory cytokines secretions and assistance in tissue repair. Macrophages present the M2 phenotype when activated by IL-4, IL-13, IL-10. They produce soluble factors that will restore the non-inflamed state (IL-10, transforming growth factor (TGF) β ...) ⁵⁸. Some molecules derived from ω -3-polyunsaturated fatty acids, known as resolvins, could induce a switch to M2 phenotype in macrophages.

Depending on their microenvironment, macrophages could polarize into one phenotype or another (**Fig 1.9**). In adipose tissue from obese patients, an increased number of infiltrating macrophages is observed. These macrophages undergo a phenotype switch toward the classically activated M1 phenotype and are responsible for the inflammatory complication that occurs in obesity⁶¹.

1.4. Obesity

Today, the World Health Organization (WHO) considers obesity as a global epidemic. At least 300 millions of people are clinically obese and more than 1 billion of adults are in overweight. Sadly, this state also reaches children with, in 2010, 43 million of children younger than 5 years in overweight. Obesity is associated with a higher mortality rate than in non-obese people and with numerous health complications such as type 2 diabetes, cardiovascular diseases, dyslipidemia...

Obesity and overweight are classically defined via the body mass index (BMI) as the individual body mass divided by the square of their height. The BMI allows to categorize people according to their body state (**Table 1.3**). The obese category could still be divided in three groups according to the severity.

Category	BMI range (kg/m ²)
Underweight	< 18,5
Normal (Healthy weight)	18,5 - 25
Overweight	25 - 30
Obese	> 30

Table 1.3. BMI categories

These categories are applicable for most of the countries. However, for some ethnic groups, this calculation does not fit with the predicted body fat mass that normally correspond to the BMI. So, in some part of the world, like in Asian countries, a different BMI scale is used⁶². For Asian populations, BMI above 27.5 kg/m² revealed an obesity state and a BMI above 23 kg/m² is associated with overweight⁶³.

Nevertheless, even after a readjustment of the scale, the BMI does not always fit correctly with the body fat mass or distribution and it is not applicable to all ethnicity or age or even between gender. This is why other parameters are increasingly taken into account to measure the obesity state. The waist circumference, the waist to hip ratio (WHR), which evaluates more accurately the degree of visceral vs. peripheral

(subcutaneous) adiposity, or the skin fold thickness, are now measured to give a better idea on the “fatty state” of the patient.

Obesity and overweight are mainly caused by an excess of calories intake associated with a lack of physical activity. Obviously, genetic, environmental factors and gender may also influence the development of obesity.

The excess of energy accumulates in adipose tissue as triglycerides and leads to negative effects on health and reduction of the life expectancy. Nevertheless, recent report has highlighted that a moderate overweight could be associated with lower all-cause mortality⁶⁴. Numerous evidences reveal that the adipose tissue of obese individuals shows a chronic state of low grade inflammation having a role in the pathogenesis of obesity-related metabolic dysfunctions^{58,60,56}.

1.4.1. Obesity and adipose tissue remodelling

Under normal conditions, the SCAT is the main responsible for the storage of triglycerides, but once it reaches its maximum capacity, fat begins to be store in VAT or even in other tissues not suited for lipid storage (muscle, liver, pancreas)⁷. Fat accumulating in these tissues induces mitochondria dysfunctions leading to free radical release. To deal with the storage of the excess of energy, adipocytes have to increase their size (hypertrophy) and when they reach their critical size, preadipocytes need to differentiate into new adipocytes (hyperplasia). The adipose tissue expansion needs the formation of new blood vessels to allow oxygen and nutrients supplies along with extracellular matrix remodelling.

The big adipocytes release more pro-inflammatory cytokines that attract macrophages inside the tissue⁶⁵. The new arrivals perpetuate a vicious cycle of macrophages recruitment and exacerbated the production of pro-inflammatory mediators. Indeed, as already mentioned, the macrophages of obese adipose tissue show a M1 phenotype resulting in the production of pro-inflammatory molecules⁶⁶.

Weisberg *et al.* (2003) have clearly shown that the number of M1 macrophages in murine adipose tissue correlates with adipocyte size and that M1 macrophages are significantly more abundant (four times more) in adipose tissue of obese than in lean adipose tissue of lean people⁶⁷. They revealed the same correlation in human adipose tissue. The percentage of macrophages in adipose tissue ranges from less than 10% in lean individuals (mouse or human) to more than 50 % in extremely obese mice (*ca.* 40 % in obese human)⁵⁶. **Table 1.4** presents the main adipose tissue modifications observed in case of obesity when compared with lean state.

	Lean	Obese
Adipocyte hypertrophy/hyperplasia (%) ¹¹	53/47	50/50
Number of adipocytes ¹⁹	4 x 10 ¹⁰ adipocytes	8 x 10 ¹⁰ adipocytes
Renewing of adipocytes ¹⁹ (new cells/year)	3 ± 2 x 10 ⁹	8 ± 5 x 10 ⁹
Mean age of adipocytes ¹⁹	9.9 ± 3.5 years	9.7 ± 4 years
Proportion of macrophages in the AT ⁵⁶	10 %	50 %
Proportion of M1 activated macrophages among all the macrophages ⁶⁶	9 %	39 %

Table 1.4. Principal differences between the adipose tissue of lean and obese people. (AT: adipose tissue).

To resume, in case of energy excess, the adipocytes store more triglycerides and increase their size. This change induces modification in their secretions that attract macrophages inside the adipose tissue and activate them. These activated M1 macrophages contribute to the maintenance of the chronic low grade inflammatory state in the adipose tissue. Depending on the severity of the obesity state, crown-like structures are present in the adipose tissue. They are accumulations of macrophages around dead adipocytes in inflamed adipose tissue (**Fig 1.10**). It was observed that macrophages accumulate more in the VAT than in the SCAT. Actually, visceral adipocytes and macrophages are the principal producers of inflammatory cytokines that characterize the obesity state. This explains why the ratio of waist-to-hip circumferences is a good indicator of the risk of metabolic disorders^{66,68}.

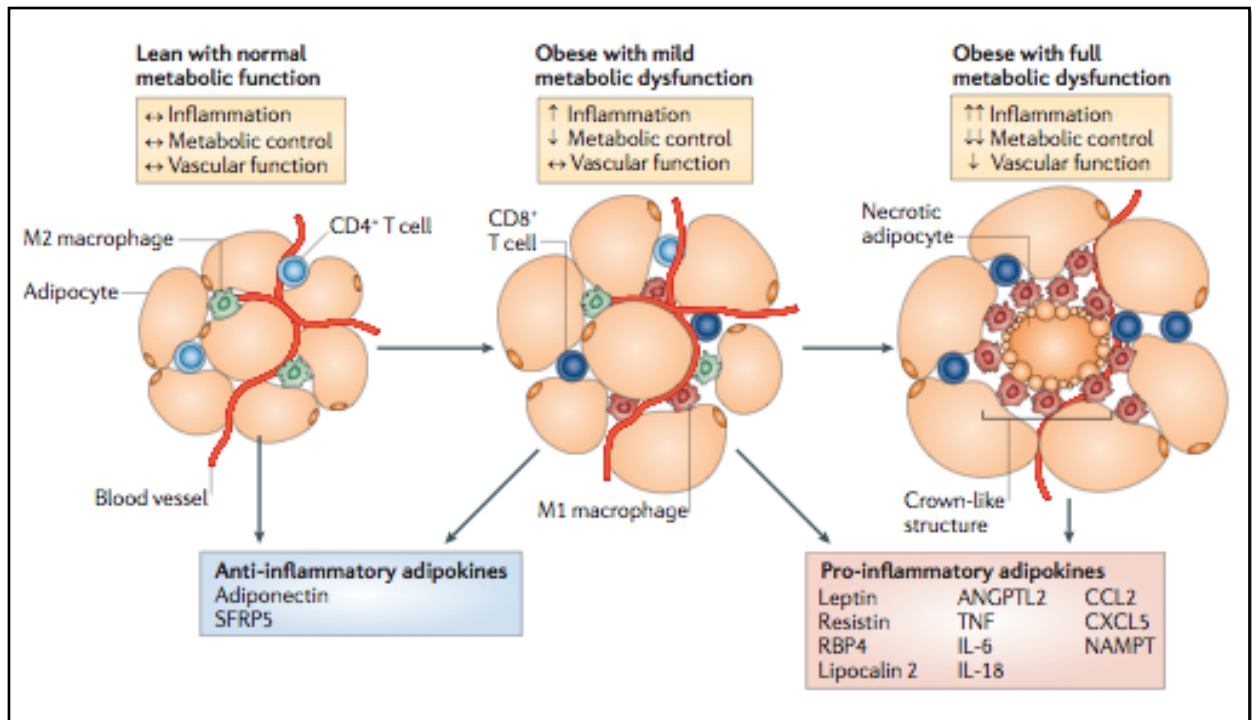


Fig 1.10. Modification of the adipose tissue in case of weight gain⁵⁴

1.4.2. Deregulation of adipose tissue secretions

In case of obesity, two major inflammatory pathways are activated in the adipose tissue: the NF- κ B pathway and c-Jun N-terminal kinase (JNK) cascade, which belongs to the MAPK pathways. These two pathways are linked to the induction of the expression of inflammatory genes such as IL-6, TNF α , CCL-2, IL-1 β in adipocytes and macrophages. In addition, they are linked to the development of insulin resistance by inhibiting the phosphorylation of insulin receptor substrates 1 and 2 (**Fig 1.11**)⁶⁹.

These pathways are activated by numerous factors such as cytokines, LPS, adipocytes death and lipids that interact with specific membrane receptors inducing the activation of the pathways⁷⁰. Inside the cells, the endoplasmic reticulum stress could induce the activation of the two pathways. The ROS present in the cell could also activate JNK.

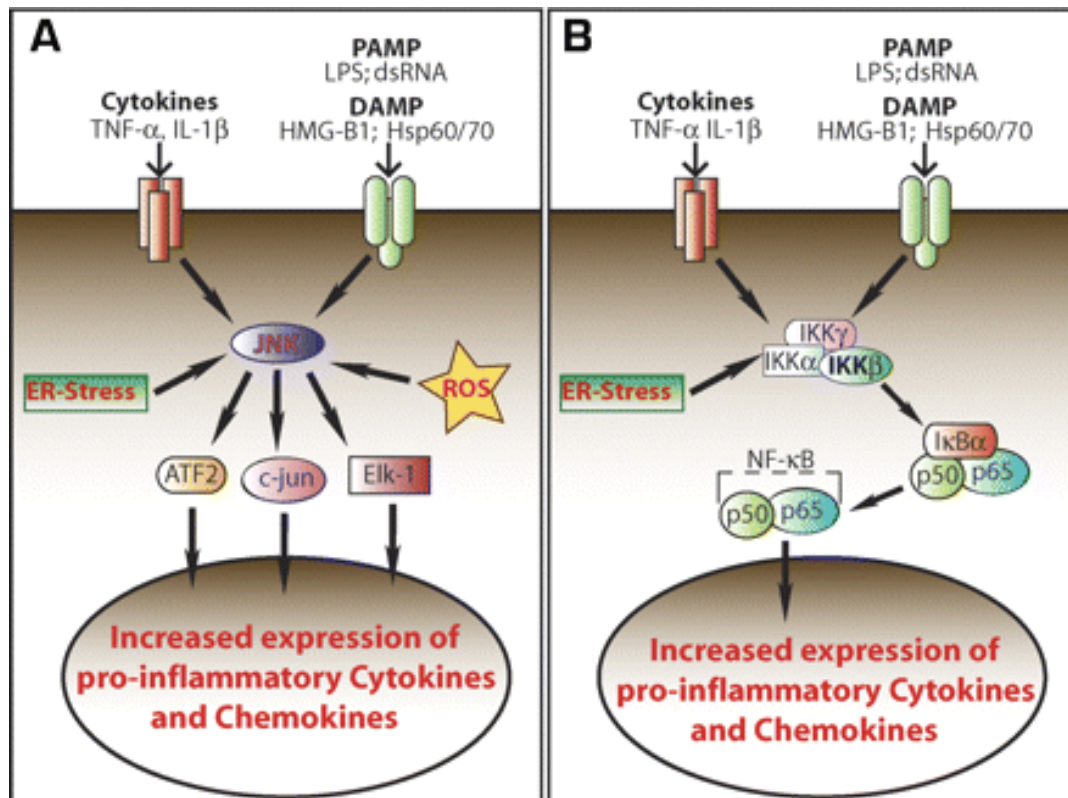


Fig 1.11. JNK (A) and NF- κ B (B) pathways. Numerous molecules could activate the two pathways by acting on specific receptors. Among them, the LPS and the double strand DNA (dsDNA) that are part of the pathogen-associated molecular pattern (PAMP) and the family of damage-associated molecular pattern (DAMP) could initiate the pro-inflammatory cascades. The two transcription factors induce the expression of pro-inflammatory cytokines and chemokines. Adapted from Giovanni Solinas *et al.* (2010).

Table 1.2 resumes all the molecules that could be secreted by adipocytes, the secretion of many of them being deregulated in case of obesity.

TNF α acts locally in adipose tissue through autocrine or paracrine mechanisms and induces IL-6 and leptin expression. This factor, by binding on its receptor, activates the three MAPK pathways that induce the lipolysis and increase FFA release from adipose tissue to the circulation. At the same time, TNF α inhibits LPL and thus blocks the lipid uptake by adipocytes. The final result is an increase of circulating FFA in the blood stream. It also inhibits the phosphorylation of the insulin receptor in muscles and adipose tissues, which promotes insulin resistance⁷¹.

CCL-2, also known as MCP-1, is a chemokine abundantly secreted in adipose tissue of obese people and responsible for the attraction of macrophages inside the tissue. Indeed, CCL-2 knockout mice accumulate fewer

macrophages in response to obesity induced by high fat diet. In the same study, transgenic mice expressing CCL-2 under the control of the aP2 gene promoter exhibited insulin resistance, macrophage infiltration into adipose tissue and increased hepatic triglyceride content⁷².

The C-Reactive Protein (CRP) produced by the liver, the leukocytes and the adipose tissue is commonly used as a systemic inflammation marker. It is produced in response to pro-inflammatory cytokines (IL-6, TNF α) so it is not surprising to measure a high level of CRP in the blood of obese patients⁶⁸.

Inversely, adiponectin is less produced by adipocytes in case of weight gain. Low level of adiponectin is associated with higher level of pro-inflammatory cytokines whereas infusions of adiponectin in animal models result in a decrease in systemic inflammation by unclear mechanisms⁶⁸. Interventions reducing the adipose mass or weight loss increase adiponectin level, which is concomitant with an improvement in insulin sensitivity⁷³. Actually, obese individuals who do not suffer from insulin resistance show significantly higher concentrations of circulating adiponectin than insulin resistant obesities, revealing a role of this adipokine in insulin sensitivity. Its expression is inhibited by TNF α and IL-6 and stimulated by PPAR γ agonists⁷⁴.

Obesity state is also linked to an oxidative stress, which corresponds to an overproduction of ROS and RNS not counterbalanced by antioxidant defences⁷⁵. ROS and RNS are metabolites normally formed during aerobic metabolism. These species, *i.e.* superoxide radical $\cdot\text{O}_2^-$, nitric oxide radical $\cdot\text{NO}$, hydroxyl radical $\cdot\text{OH}$, hydrogen peroxyde H_2O_2 and peroxynitrite ONOO^- , are essential for the body physiology and its protection. Nevertheless, a chronic exposure could lead to oxidative cell damages⁷⁵. Fat accumulation in adipocytes triggers an endoplasmic reticulum stress in adipocyte leading to an increase of the ROS/RNS production. Some pro-inflammatory transcription factors, *e.g.* NF- κ B, are activated by these

species. The resulting pro-inflammatory molecules, in turn, increase the ROS/RNS production and establish a vicious cycle⁶³.

Finally, the lipolysis rate is also increased in obesity leading to a high amount of FFA circulating in the blood stream. In normal conditions, the subcutaneous fat compartment acts as a lipid-buffering tissue and helps to maintain the lipid homeostasis⁷⁶. Normally, the SCAT contributes to more than 85% of systemic FFA release. In obese conditions, the VAT increases its FFA release that reaches 50 % of the total circulating FFA⁷⁷. Actually, the VAT tissue is less responsive to the storage/release of FFA reflecting the difference of insulin sensitivity between the two fat compartments. The VAT is also the source of the bulk of pro-inflammatory cytokines secreted in obesity. These facts made of the VAT the main responsible for the complications that are linked to obesity.

The deregulation of cytokines, chemokines and lipid secretions creates the chronic inflammation state that leads finally to metabolic disorders such as insulin resistance, cardiovascular diseases, ... all together named the metabolic syndrome.

1.5. Obesity and the metabolic syndrome

According to the World Health Organization, the metabolic syndrome is defined as the combination of type 2 diabetes, insulin resistance, impaired glucose tolerance or impaired fasting glucose with at least two of the following criteria, *i.e.* high blood pressure (> 140/90 mmHg), dyslipidemia, BMI > 30 kg/m², microalbuminuria. Very often, obesity coexists with one or more of these metabolic disorders.

1.5.1. Obesity, inflammation and insulin resistance/type 2 diabetes

The insulin resistance is the inability of the peripheral tissues to respond to a normal physiological level of insulin. Muscles, liver and adipose tissues do

not respond anymore to normal levels of insulin. The liver of patients suffering from insulin resistance releases continuously glucose in the blood resulting in hyperglycemia. This is a predisposition for the development of type 2 diabetes, which is a metabolic disorder characterized by high blood glucose in the context of insulin resistance. Obesity is thought to be the primary cause of type 2 diabetes. However, all obese people do not develop diabetes. Why do some obese people develop diabetes and not others is not really known. However it is observed that obese people presenting abdominal fat accumulation are more likely to have diabetic predispositions. Moreover, patients having enlarged adipocytes in their subcutaneous abdominal tissue are predisposed to develop diabetes⁷⁸.

As mentioned earlier, the VAT is less sensitive to insulin and it is thought to be a major actor in the development of insulin resistance, by releasing high amounts of FFAs and cytokines. In response to the high level of FFAs, the muscles and the liver oxidize more FFAs and the glucose normally used by muscle increases in the bloodstream. Moreover, liver in response to high FFA oxidation increases the gluconeogenesis, which also increases the blood glucose level. A hyperglycemia appears and contributes to the development of tissue insulin resistance^{70,76}.

The pro-inflammatory cytokines secreted by the adipose tissue also impact insulin resistance. Numerous studies have revealed that insulin resistance is correlated with systemic inflammation. Already in 1993, Hotamisligil and his colleagues have shown that the neutralization of TNF α in obese rats increased their response to insulin revealing an obvious role of TNF α on insulin resistance that accompanies obesity⁷⁹. However, the same approach in obese or insulin-resistant patients did not improve insulin sensitivity⁸⁰. In mouse, it appears that this cytokine stop the insulin signalling pathway by inducing the phosphorylation of the IRS subunits which blocks its interactions and the cascade⁸¹.

CCL-2 also impairs insulin sensitivity in fully differentiated adipocytes *in vitro*⁸². Concerning IL-6, its role in insulin resistance is still debated. What is

known is that circulating IL-6 level is increased in obese and insulin resistant subjects.

TNF α , CCL-2 and IL-6 act on adipocyte functions by decreasing their TG synthesis resulting in a reduced lipid accumulation in adipocytes and an increased of FFA released. In turns, the FFAs are able to activate the NF- κ B pathway that induces the synthesis of more pro-inflammatory molecules. TNF α also inhibits adiponectin secretion (Fig 1.12).

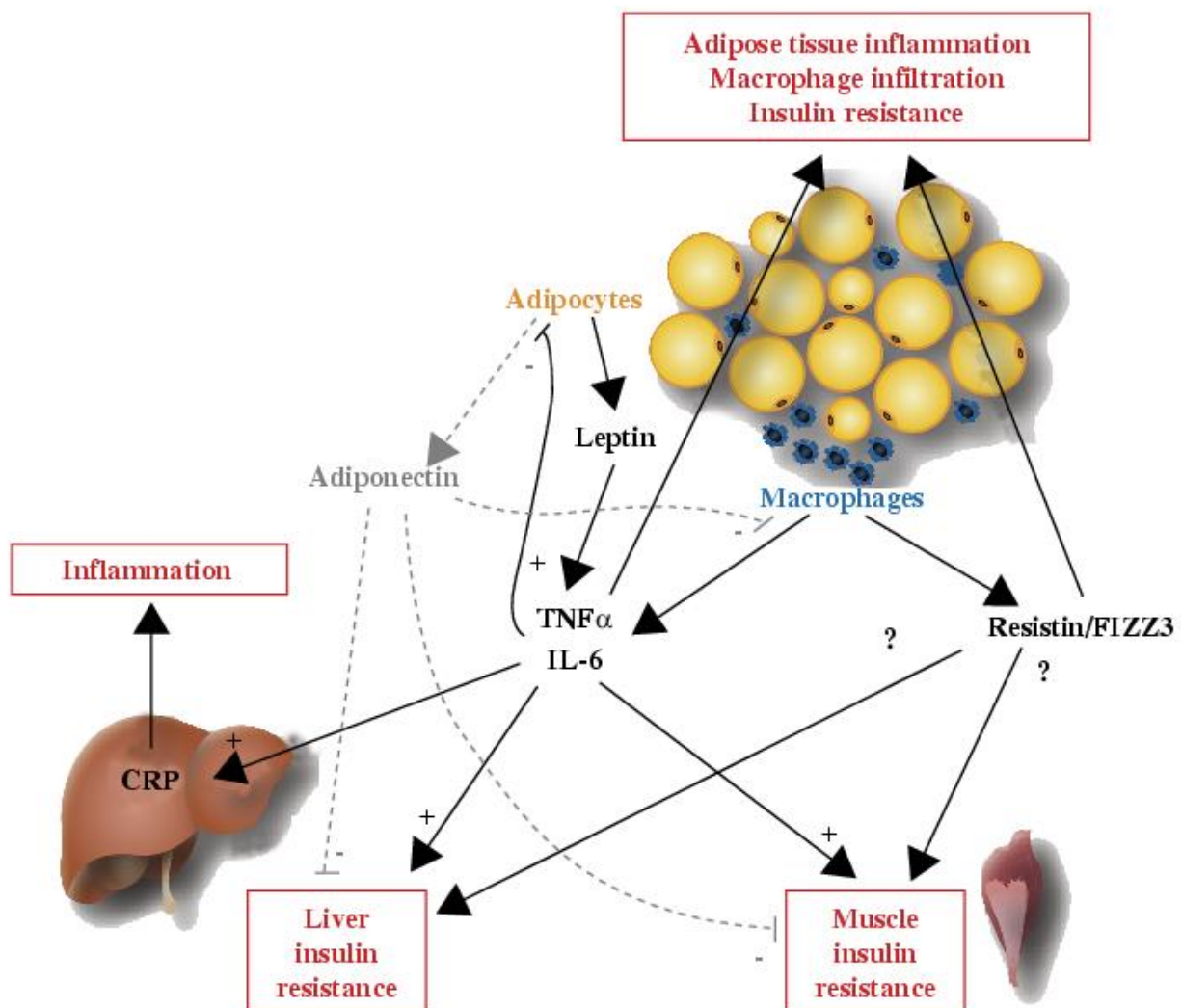


Fig 1.12. Deregulation of adipose tissue secretions in case of obesity. Infiltrated macrophages secrete high amount of TNF α , IL-6 and resistin that contribute to the development of insulin resistance of adipose tissue, liver and muscle. IL-6 induces the expression of the C-reactive protein (CRP) in the liver increasing the inflammatory state. TNF α decreases the adiponectin secretion by adipocytes strengthening the insulin resistance of the tissues. Figure adapted from Bastard *et al.* (2006)⁸¹.

1.5.2. Obesity, inflammation and cardiovascular disease

Cardiovascular diseases (CVD) describe all diseases that affect the cardiovascular system (heart, vessels and arteries). The main causes are atherosclerosis and/or hypertension. Obesity, among others such as age, gender, tobacco,..., is directly related to CVD. Manson *et al.* (2003) have shown in their study on 115,195 US women that BMI of non-smoking women was correlated with an increased risk of CVD death⁸³. The women with BMI > 32 kg/m² had a 5.8 times higher risk of dying of CVD than those with BMI values below 22 kg/m². The waist-to-hip ratio is also well correlated with CVD death revealing that abdominal fat plays an important role in the development of CVD⁷⁰.

Pro-inflammatory molecules are also involved in the CVD development, the majority of them coming from the adipose tissue. TNF α by inducing the development of diabetes could contribute to cardiovascular complications. Nonetheless, its direct implication on CVD is controversial as revealed by *in vivo* mice studies⁸⁴. However, this factor is known to induce the CRP production by the liver, which impacts on the vasculature.

IL-6 also induces CRP secretion and by inhibiting LPL activity, this favours the formation of foam cells, which are macrophages that have accumulated oxidized lipids. If foam cells accumulate in the bloodstream, they progressively form a necrotic centre of atherosclerosis, which could lead to a stroke or myocardial infarction.

The CRP is associated with an increased risk of CVD as well as of type 2 diabetes⁸⁵. CRP induces the production of endothelial adhesion proteins and secretion of inflammatory molecules by endothelial cells, leading to an increase of leukocytes adherence, cellular inflammation and foam cell accumulation in the vessels. It also inhibits endothelial and smooth muscle NO synthase contributing to hypertension.

Plasminogen Activator Inhibitor-1 (PAI-1), secreted by endothelium and adipose tissue, regulates the coagulation cascade. Its expression is

increased in obesity and contributes to a hypercoagulation state leading to higher risk to develop CVD.

Other factors such as angiotensinogen, vascular endothelial growth factor and leptin, are secreted by the adipose tissue and contribute to the apparition of cardiovascular complications⁵⁵.

Many others complications are associated with obesity like increased risk of cancers, respiratory disorders, Alzheimer's disease, ... According to public authorities and health care providers, there is a need to implement lifestyle change by providing an opportunity for preventive interventions against obesity linked complications. Drugs are obviously good candidates, but over the past two decades, phytochemicals gained the interest of public and scientists for their role in preventing diseases.

1.6. Prevention of obesity

The excessive fat mass, stored in adipocytes in case of obesity, is responsible for the development of systemic inflammation that is involved in major metabolic disorders leading to the metabolic syndrome⁸⁶. Global strategies focus on the restriction of calorie intake and increase of physical activity in order to reduce the amount of stored triglycerides and slow the obesity development.

In Europe, only two anti-obesogenic drugs are approved, *i.e.* Sibutramine and Orlistat. They allow to reduce the triglyceride absorption, but they present non-negligible side effects. Fortunately, research in nutrition has recently described some natural products that could counteract obesity development⁸⁷. One strategy is to act on adipocytes themselves by acting on preadipocyte proliferation, differentiation, lipid absorption, lipolysis, lipogenesis... A second strategy could be an action on the secretions of pro-inflammatory mediators by adipocytes or macrophages and thereby decrease the severity of complications linked to this state.

Among the phytochemical candidates, the family of polyphenols shows interesting health benefits. The phenolic compounds are plant secondary metabolites found in the human diet. Many polyphenols have already

shown anti-obesity properties. Some suppress growth of adipose tissue, inhibit the differentiation, stimulate lipolysis, induce apoptosis of adipocytes or decrease the pro-inflammatory cytokine production by the adipose tissue.

1.6.1. Prevention by acting on adipocyte life cycle

The first step where compounds could act to prevent obesity is the preadipocyte state. A slow down of their proliferation by inducing the arrest in the G₁ phase or an induction of their apoptosis by activating caspase 3 are two mechanisms that have already been observed after a treatment with resveratrol or quercetin, respectively.

Another way to interfere with the adipocyte development is to act on the maturation of the preadipocyte. Acting on the mitotic clonal expansion will reduce the number of differentiated adipocytes. Some phenolic compounds such as genistein or curcumin are known to inhibit this important step⁸⁸. As the number of adipocytes could increase but not decrease, reducing the number of newly differentiated adipocytes could be an interesting approach, since the capacity of storage would stay reduced. However, in case of weight gain, if the adipocytes begin too big, they start to produce more pro-inflammatory cytokines than smaller ones⁶⁵. Moreover, the excess of fat has to be stored and ectopic fat depots could appear if new adipocytes could not be formed in the adipose tissues. Finally, the differentiation of new adipocytes is a process that occurs in normal condition since the adipocyte population undergoes a turnover¹⁹. In conclusion, the blockage of the differentiation could be a possible way to prevent obesity development and complications if it is well controlled.

Obviously, the direct action on adipogenesis by reducing fat accumulation appears as an effective way to counter obesity. Numerous studies have highlighted the effect of vegetal compounds on PPAR γ expression and/or members of the C/EBP family. The down-regulation of these transcription factors inevitably leads to the adipogenesis inhibition. However, a well-known PPAR γ ligand family, the thiazolidinedione (TZD), activates the

transcription factor and improves the insulin sensitivity. Indeed, the activation of PPAR γ increases the fat storage capacity and leads to a decrease in circulating FFA in obese patients. In addition, the action of TZD on PPAR γ also leads to an increase of adiponectin secretion, which is known to improve insulin sensitivity. In conclusion, these observations show that the inhibition of adipogenesis could have some negative impacts on the development of obesity complications⁸⁹.

The stimulation of lipolysis in order to decrease the amount of fat stored in adipocytes is another strategy that has been proposed. However, it is known that in case of obesity, a high rate of lipolysis is observed and the released saturated fatty acids contribute to maintain the inflammatory state by acting on macrophages TLR4, which activates the NF- κ B pathway. Indeed, the saturated fatty acids are endogenous ligand for its receptor and may contribute to the polarization of macrophages toward the M1 profile⁹⁰. In conclusion, even if a physiological level of lipolysis is needed to ensure the energy supply, a higher FFA release could trigger insulin resistance by inducing pro-inflammatory secretions (**Fig 1.13**)⁷.

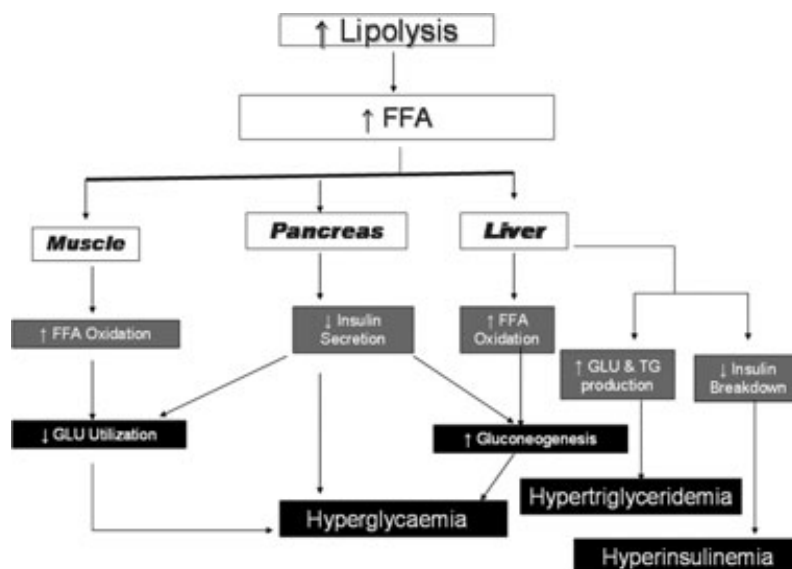


Fig 1.13. Negative effects of increased lipolysis. A high plasmatic concentration of FFA favours the FFA oxidation in muscles and liver leading to a reduction of the glucose utilization. The gluconeogenesis is stimulated in the liver, leading to an increase of the glucose plasmatic concentration. A chronic exposition to FFA could lead to a decrease of insulin production by the pancreas in patient predisposed to develop type 2 diabetes. Figure from Ibrahim (2010)^{7,91}.

Finally, the induction of the apoptosis of mature adipocytes is also a way to reduce the body fat content⁸⁸.

Table 1.5 sets out a non-exhaustive list of polyphenols that have already shown beneficial effects on adipocyte functions *in vitro*.

1.6.2. Prevention by acting on the inflammatory pathways

The low grade chronic inflammation of adipose tissue is one of the mechanisms underlying obesity complications. Several drugs, by acting on the inflammatory state, reduce their prevalence. It is known that the interaction of infiltrating macrophages with adipocytes is a crucial contributor to the development of this inflammation.

To decrease the adipose tissue inflammation, the blockade of the stimulation loop between the macrophages and the adipocytes appears to be a target. The candidate could act on the macrophage inflammation and/or on the adipocytes inflammation and by this way suppress the vicious cycle in the adipose tissue. Among other, the activation of PPAR γ appears to decrease the inflammation observed in adipose tissue; in addition to its role on metabolism, it also regulates inflammatory processes. This regulator represses the expression of inflammatory genes (*i.e.* iNOS), inhibits NF- κ B and modulates MAPK activity. TZD by linking PPAR γ increases fatty acid storage, but also reduces the adipocytes/macrophages inflammation.

In vitro models of coculture have allowed to study the impact of potential candidates on the inflammation state generated by the cohabitation of the two cell types. **Table 1.5** reports some candidates that come from studies on this field.

In vivo studies on rodents and humans have supported some of these *in vitro* observations. For example, genistein reduced food intake and body weight in ovariectomized female mice⁹². Grape-seed procyanidins decreased CRP, IL-6 and TNF α expression in mesenteric WAT of Zucker rats fed with high fat diets. Moreover, they also increased adiponectin mRNA in

the WAT⁹³. Resveratrol also reduces metabolic disturbance in obese Zucker rats. In this study, a positive effect of the polyphenols on the blood pressure is also described⁹⁴.

Numerous human intervention studies exist on the effect of polyphenols on metabolic dysfunction⁹⁵; for example catechins, including EGCG, may reduce the body weight and the waist circumference of obese patients after 90 days⁹⁶; resveratrol has shown a positive effect on insulin resistance in a study on 10 diabetic patients after four weeks of daily treatments; curcumin reveals positive effects on metabolic parameters of healthy or diabetic patients⁹⁵.

Targets for the obesity prevention		Potential Effects	Phytochemical
Adipocyte differentiation	Preadipocyte proliferation	Inhibition of proliferation (G ₁ phase arrest)	Chlorogenic acid ⁸⁸ , <i>o</i> -coumaric acid, <i>m</i> -coumaric acid, naringenin, rutin, naringin, genistein, resveratrol ⁹⁷
		Apoptosis (implication of Cdk2 and caspase 3)	Quercetin ⁹⁸ , EGCG, esculetin
	Induction of differentiation	Inhibition of the mitotic clonal expansion	Genistein, curcumin ⁹⁹
		Apoptosis	EGCG, esculetin, resveratrol
	Adipogenesis	Modification of PPAR γ and C/EBP expression	Capsaicin ⁹⁸ , curcumin ¹⁰⁰ , genistein, blue berry, EGCG, resveratrol, sakuranetin ¹⁰¹ , extract from kiwi ¹⁰²
	Mature adipocyte	Stimulation of lipolysis / Inhibition of lipid accumulation	Genistein ¹⁰³ , quercetin, luteolin, grape seed procyanidins, EGCG ⁸⁸ , resveratrol ¹⁰⁴
		Apoptosis	Green tea extracts, soy isoflavones ¹⁰⁵ , EGCG ⁸⁸
		Increases insulin sensitivity	Luteolin ¹⁰⁶ , Kaempferitrin ¹⁰⁷ , extract from kiwi ¹⁰²
Obesity-linked inflammation	Macrophages	Reduces the proinflammatory cytokines secretion and NO release	Luteolin ¹⁰⁸ , EGCG ¹⁰⁹ , resveratrol ¹¹⁰ , grape seed procyanidins ¹¹¹
	Adipocytes	Reduces the proinflammatory cytokine secretion	Grape seed procyanidins ¹¹² , oligomerized grape seed polyphenols ¹¹³
		Reduces the leptin secretion	Resveratrol ¹¹⁴
		Increases adiponectin secretion	Kaempferitrin ¹⁰⁷ , grape seed procyanidins ¹¹²
	Inflammatory vicious cycle between adipocytes and macrophages	Reduces the proinflammatory cytokine secretion and NO release	Naringenin chalcone ¹¹⁵ , luteolin ¹⁰⁸ , diosgenin ¹¹⁶ , paeoniflorin ¹¹⁷ , grape seed procyanidins ¹¹²

Table 1.5. List of polyphenols or vegetal extracts that have an effect on adipocyte functions or inflammatory state of adipose tissue. The elements in bold are compounds that stimulate the expression of the transcription factors.

1.7. Phenolic compounds bioavailability

Most of the studies have highlighted *in vitro* effects of phenolic compounds on adipocytes and/or macrophages as well as on their interactions. However, *in vivo*, if the compounds are orally ingested in a particular food matrix, they have to face digestive and metabolic steps before arriving in the bloodstream and then within the adipose tissue. The food matrix could modify the bioaccessibility of the compounds and the different digestion steps could result in modifications on their chemical structure.

Most of the time, the polyphenols found in the food are glycosylated. These glycosylated derivatives are hydrolysed by the lactase phlorizin hydrolase (LPH) or the cytosolic β -glucosidase within the enterocytes. However, if the glycosylated moiety is too big, the phenolic compound could not be absorbed at this level and therefore undergo modification by the colonic microflora. It is indeed known that polyphenols having a high molecular weight (glycosylated or not) are virtually not absorbed in the gut.

The phenolic compounds absorbed in the small intestine could be (re)conjugated with glucuronic acid, sulphate or methyl moieties. Then, the conjugates reach the liver or other organs through the portal vein or the bloodstream, respectively. Usually, all the circulating polyphenols are in a conjugated form, except when a high dose is administrated revealing that the conjugation pathway could be saturated. So, according to their size, the phenolic compounds are absorbed more or less quickly and their presence in the plasma could be observed between 0.5h to 9h after their ingestion. In the liver, as in other organs, these molecules could be further metabolised¹¹⁸.

Molecules presenting a big glycosyl moiety or (re)excreted in the bile are often hydrolysed by the microorganisms and absorbed through the colonic barrier. At this level, the aglycone form could also be degraded in smaller molecules. The phenolic compounds, their degraded forms and derivatives are finally eliminated by urine and bile (**Fig 1.14**).

The conjugated forms show differences in their biological activities compared to their free form. However, only a few investigations have been done on their effects, the majority of the studies focusing on the free forms. Nevertheless, it was already observed that conjugates could also undergo deconjugation at the cellular level¹¹⁹.

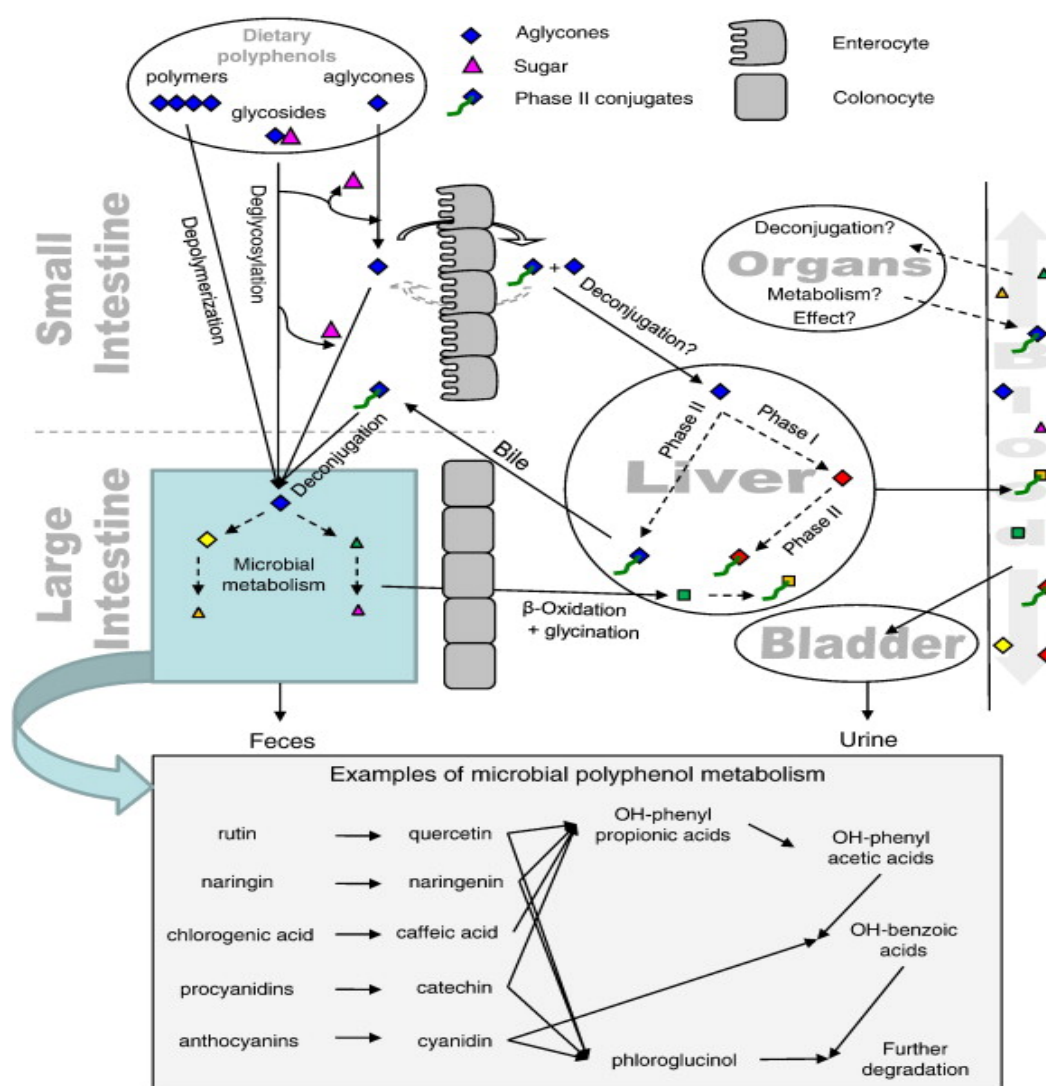


Fig 1.14. Metabolism of phenolic compounds. In small intestine, the polyphenols undergo depolymerization and/or deglycosylation. Once in their aglycone form, they could be absorbed by the enterocytes where they undergo conjugation or not. They are then sent to the liver where they could be again modified and sent to the bloodstream to gain access to tissues or the bladder to be excreted. Polyphenols could also be sent back from the liver to the intestinal tract through the bile. They will then reach the large intestine where they could be modified by the microflora (re)absorbed or excreted. Possemiers *et al.* (2011)¹²⁰

1.8. The chosen extracts

For this work, we have focused on the potential effect of two fruits and some of their main phenolic compounds. Apple is a truly common fruit, highly consumed in our continent. Numerous positive effects of apple consumption on different health parameters have already been observed. Indeed, epidemiological studies have highlighted that apple consumption is negatively correlated with the risks to develop chronic diseases such as coronary disease¹²¹, type 2 diabetes^{122,123}, asthma¹²² or lung and colon cancer^{124,125}. In this work, we have investigated the effect of three different apple extracts on biological functions and inflammation of adipose tissue *in vitro*. Another fruit, less common but very fashionable, has held our attention, the pomegranate. Its well-known antioxidant power and a few studies about its effects on body weight make it a good candidate to the investigation of its effects on adipogenesis and adipose tissue inflammation. We choose to work with an extract from the husk, richer in polyphenols than the flesh, since it has already been characterized and used in our group to investigate potential anti-inflammatory effects on the intestinal barrier, in an *in vitro* model consisting of Caco-2 cells.

1.8.1. Pomegranate: legendary power?

Persians believed Eve ate a pomegranate instead of an apple from the tree of knowledge. At the time of ancient Egypt, pomegranates were thought to offer eternal life and were buried with dead. In many other civilisations, this exotic fruit was believed to have positive vibes. Nowadays, scientific studies have revealed some real beneficial health effects of pomegranates. The pomegranate, scientifically named *Punica granatum*, originates from the region of Iraq and Iran. It is now cultivated in most Mediterranean countries, in Africa, in Latin America, in California and in some part of Asia. *Granatum* means abundant in grains, indeed, the pomegranate is composed of numerous seeds surrounded by a red colour pulp, which are the edible part. The seeds are embedded in a white membrane and a thick red husk covers the pomegranate (**Fig 1.15**)¹²⁵.



Fig 1.15. Pomegranate fruit

The fruit, but also other parts of the plant, has revealed positive effects on health. Currently, more than forty clinical studies are in progress in the United States to verify the impact of pomegranate consumption on various diseases such as prostate cancer, diabetes, oxidative stress, atherosclerosis...¹²⁶

The juice is the main consumed product from pomegranates. It contains vitamin C and B₅, potassium, natural phenols and unsaturated oils¹²⁷. A glass of pomegranate juice contains more antioxidants than the same amount of red wine, green tea or blueberries juice¹²⁸. However, extracts, for medical use or nutrient supplementations, are more and more used. These extracts from pulp, husk, leaves or flowers are enriched in active compounds such as phenolic compounds and other antioxidants. Phenolic acids (*e.g.* chlorogenic and ferulic acid) and flavonoids (*e.g.* quercetin, phloridzin, epigallocatechins...) have been identified in this fruit as bioactive compounds¹²⁸. Nonetheless, ellagitannins are the most prevalent compounds in pomegranates and the principal representatives are the punicalagins and the granatins. The majority of these hydrolysable tannins are contained in the husk of the fruit and could be more or less extracted during the process of juice preparation. If the fruit is well pressed, the polyphenol content of the juice can reach 2 g/L¹²⁹.

The bioavailability of these polyphenols remains controversial. Cerda *et al.* (2003) have reported that after 37 days of a diet supplemented with

pomegranate extract, the level of punicalagin in the plasma of rat reached 30 $\mu\text{g/ml}$. Its main metabolite, ellagic acid, reached 0.5 $\mu\text{g/ml}$ ¹²⁹. This is the only study that reported the presence of punicalagin in plasma after pomegranate product ingestion, whereas other studies in human and other mammals did not reveal the presence of this molecule in the plasma^{130,131,132}.

Punicalagin is a large molecule composed of ellagic acid and gallagic acid linked to a glucose molecule (**Fig 1.16**). It is found in two forms in pomegranate: isomers α and β . It is supposed to be responsible for the pomegranate health benefits¹³³.

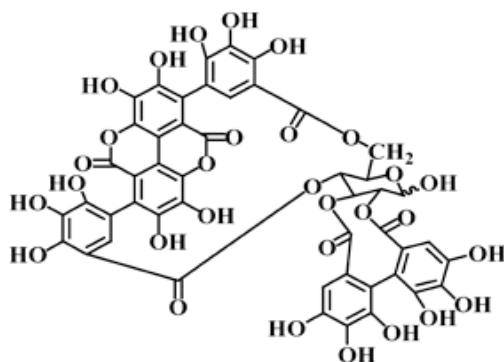


Fig 1.16. Punicalagin molecule

Upon pomegranate ingestion, ellagic acid is released from the hydrolysis of the ellagitannins such as punicalagin. The hydrolysis is due to the pH rather than to the presence of digestive enzymes. Ellagic acid is then absorbed, converted into other metabolites (urolithins D, C, A and B) and eliminated by the kidneys within 4 to 6h (**Fig 1.17**)^{125,134}.

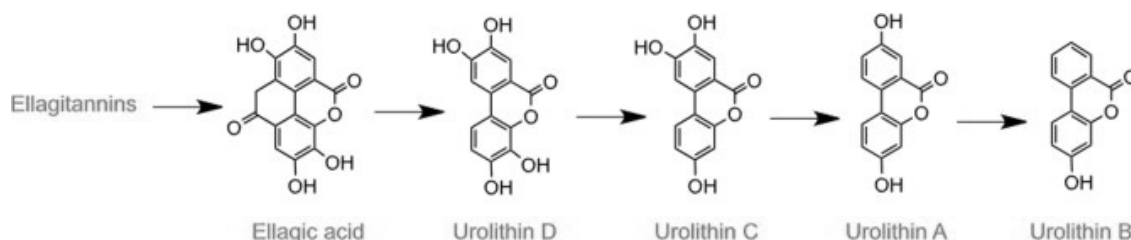


Fig 1.17 Proposed mechanism for ellagic acid metabolism¹³⁴

Despite the controversy about the bioavailability of the ellagitannins, the pomegranate has already proved its beneficial effect on health. We will review here the effect on obesity and related health complications. *In vitro* and *in vivo* evidences have revealed that pomegranates could improve the insulin sensitivity^{124,136}. This effect may be partially explained by an action on PPAR γ in adipocytes and macrophages^{135,137}. Possible effects on blood glucose level are controversy, some studies revealing that pomegranate is hypoglycemic^{138,139} whereas another does not observe any effect⁷. A recent study has revealed that pomegranate, punicalagin and ellagic acid inhibit adipogenesis and fatty acid synthase in 3T3-L1 cells¹²².

Medjakovic & Jungbauer (2012) have reviewed the numerous beneficial effects of pomegranate on obesity development and its complications¹²⁵. This fruit, as juice or extract, may reduce the body weight, total cholesterol, lipid profiles, presents anti-atherosclerotic effect by decreasing the LDL oxidative status and its negative impact ...

The anti-inflammatory effect of pomegranate in obese individuals has not really been investigated. However, pomegranate and its constituents have shown many anti-inflammatory actions in different cases such as:

- ✓ intestinal Caco-2 cells, as *in vitro* model of inflammation¹²⁴
- ✓ cultured RAW264.7 macrophages¹⁴¹
- ✓ cultured MC3T3-E1 osteoblasts¹⁴²
- ✓ serum TNF α level in dyslipidemic patients¹⁴³
- ✓ reduction of the vascular inflammation markers overexpressed in case of obesity in Zucker rats ¹⁴⁴
- ✓ reduction of the serum level of cholesterol and inflammation induced by high fat diet in the visceral adipose tissue and in the colon of obese mice¹⁴⁵

1.8.2. “An apple a day keeps the doctor away”

This well-known expression derived from the proverb “Eat an apple on going to bed, and you will keep the doctor from earning his bread” by J. P. Phillips in the English publication (1866) “Notes and Queries”¹⁴⁶. At this

time, the apple was already known for its beneficial effects on health. Today numerous researches have revealed the interesting potential of this fruit to counter various diseases. Apples are very popular in Europe and United States; in France, it is the best-selling fruit (20.3 % of the selling fruits each year) with 18 kg per household, per year¹⁴⁷.

This fruit exists in many varieties (Gala, Jonagold, Fuji ...) and is rich in carbohydrates (± 20 % of total weight) and fibres (± 3.5 %). These fibres are polysaccharides such as cellulose and hemicellulose, soluble fibres such as pectin, protopectin, which are present in the flesh and in the peel and the lignines found in the core of the fruit. Pectin is a soluble fibre present principally in the apple peel and is known to reduce the risk of cardiovascular diseases, as well as to decrease the cholesterol level. Apple also contains a high variety of vitamins; *i.e.* vitamin C, provitamin A and in a lesser extent some group B vitamins (B1, B2, PP, B5, B6, B9). The peel contains a higher amount of vitamin C and its concentration varies among cultivars. Potassium is the best represented mineral in apples although other numerous mineral compounds are present. The triterpenes are also found in high quantities in apples. Various health benefits are attributed to these molecules also found in plant and other fruits¹⁴⁸. Among these effects, powerful anti-inflammatory effects *in vitro* have already be reported by Andre *et al.*¹⁴⁸.

Finally, the phenolic compounds are important antioxidants contributing to the health benefit of apples. Depending on the variety, the antioxidant content significantly varies (**Fig 1.18**). Other parameters, such as maturity of the fruit or treatment (storage, heating, juice extraction...) modify the antioxidant content. For example, apple juice contains only 3 % of the antioxidants contained in the fresh fruit. Moreover, the phenolic compounds are non-uniformly distributed in the fruit and they are two to six times more abundant in the peel than in the flesh^{149,150,151}.

Different classes of phenolic compounds are present in apples: procyanidins, hydroxycinnamic acids, dihydrochalcones, flavonols, flavan-3-ols and anthocyanins. Some polyphenols, *i.e.* quercetin glycosides and

cyanidin-3-galactoside, are present exclusively in the peel. Others like flavan-3-ols are ubiquitous¹⁵¹. The antioxidant power of apple flesh is mainly attributed to the flavan-3-ols ($\pm 69\%$) followed by the chlorogenic acids ($\pm 31\%$). In the peel, the antioxidant effect is distributed among several groups, the flavan-3-ols ($\pm 71\%$), cyanidins ($\pm 10\%$), quercetin glycosides ($\pm 12\%$), chlorogenic acid ($\pm 7\%$) and dihydrochalcones ($\pm 0,1\%$)¹⁵².

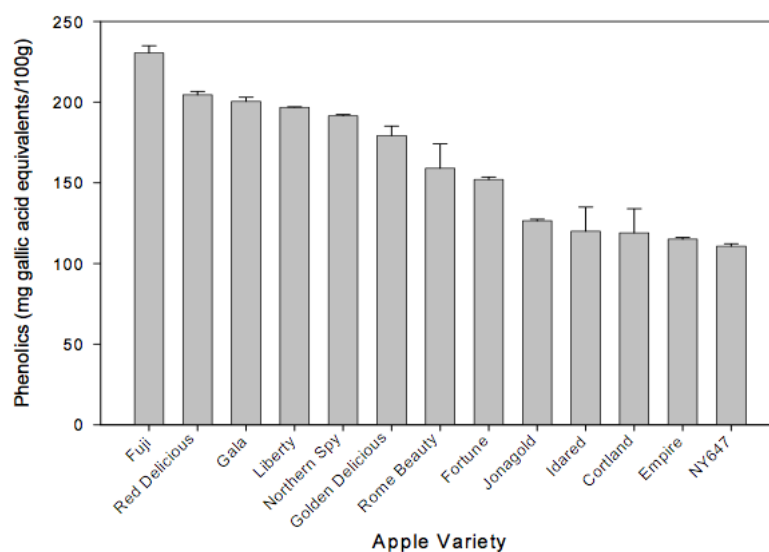


Fig 1.18. Phenolic composition of different apple varieties (Shoji *et al.* (2000))¹⁵³

These values are correlated with the concentrations of the polyphenols found in the fruit. The total phenolic content in apple as well as their antioxidant activity, is always very high compared with other “classical” fruits¹⁵⁴ (**Fig 1.19**).

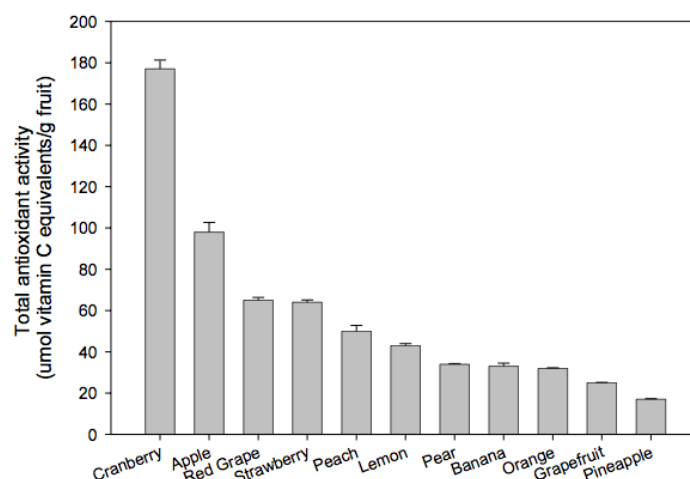


Fig 1.19. Total antioxidant activity of different common fruits (Tian *et al.* (2006))¹⁵⁴

Apples and their derivate products have been identified as potential candidates to prevent the development of obesity and related complications *in vitro*^{155,156,157} but also *in vivo*^{158,159}. Apple extracts could decrease the level of oxidized low-density lipoprotein (LDL) in human, and the effect is more pronounced after peel extract than after flesh extract ingestion¹⁵. A study in rat has demonstrated that apple is more effective than pear or peach to improve lipid metabolism and increase the plasma antioxidant potential. This difference is attributed to the polyphenols that are present in higher amounts in apple than in the two other fruits¹⁵⁹. An apple polyphenol extract administrated to rat during 3 weeks, significantly reduced the weight of visceral fat and the level of circulating leptin while increasing the size of adipocytes¹⁶⁰.

Among the polyphenols found in this fruit, quercetin, which is also present in onion, wine or tea, has revealed numerous effects on obesity development. This flavonol inhibits *in vitro* the pancreatic lipase activity (IC₅₀ of 21.5 µM)¹⁶¹ and decreases glucose intestinal absorption by acting on the GLUT2 in a non competitive way¹⁶³. It also enhances adiponectin secretion by adipocytes *in vitro*, inhibits adipogenesis in 3T3-L1 adipocytes¹⁶⁴ and decreases pro-inflammatory molecules secretion by macrophages *in vitro*¹⁶⁵. These observations clearly indicate that quercetin may be an interesting candidate to prevent obesity.

Others, but less studied, polyphenols, contain principally in the peel apple, may also contribute to the health benefits of the fruit. Phloretin and its glucoside conjugate phlorizin (phloretin 2-*O'*-glucoside), are dihydrochalcones from the family of flavonoids (**Fig 1.20**). Phlorizin has already shown an inhibitory effect on adipocyte differentiation *in vitro*¹⁶⁶, inhibition of the fatty acid synthase¹⁶⁷, reduction of the blood glucose levels and improvement of the metabolism in diabetic rats¹⁶⁸ and inhibition of intestinal and renal glucose absorption by acting on the sodium-linked glucose transporter¹⁶⁹. However, phlorizin is transformed in the intestinal tract by the lactase phlorizin hydrolase into phloretin^{170,171}, which could then be metabolized in phloroglucinol and phloretic acid by the



All of these results reveal that pomegranates and apples could be good candidates for the prevention of obesity development and complications. However, only a few studies report their potential effect on adipose tissue functions.

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2. Thesis aims

The chronic low grade inflammation state characteristic of the obese adipose tissues is strongly linked to the development of metabolic disorders. The weight gain forces the adipocytes to enlarge, which deregulates their secretions and leads to macrophages infiltration and activation.

According to this important fact, we firstly planned to study the impact of different vegetal extracts rich in polyphenols on lipid accumulation. Then we focused on the interaction between the adipocytes and macrophages that leads to this inflamed state. And finally, always in the context of obesity prevention, we evaluated the potential health benefits of different vegetal extracts on the inflammatory state characteristic of the obese adipose tissue .

In order to reach these objectives, we developed an *in vitro* model simulating this situation. By mimicking the inflammatory state of obese adipose tissue, this *in vitro* model has allowed us to investigate the potential positive effect of different extracts rich in polyphenols.

Our approach was based on acting on **two main aspects** contributing to the negative impact of obesity on health:

- the **adipocyte differentiation and lipid accumulation** occurring in the early stage of obesity development;
- the **chronic low grade inflammation in adipose tissue** linked to the obesity state and leading to metabolic complications;

As obesity is characterized by a hypertrophy of the adipocytes leading to a deregulation of their secretions, the identification of natural extracts that impact on the lipid accumulation appeared to be a good strategy to prevent obesity development and complications.

We have worked out the differentiation of 3T3-L1 preadipocytes in order to reach routinely a percentage of differentiated cells of more than 90 %. Once the differentiation process entirely controlled, we tested a pomegranate husk extract and its main constituents (*i.e.* punicalagin and ellagic acid) on the adipogenesis and the lipid accumulation profile. The results obtained

have been submitted to the PLOS ONE journal and are reported in the chapter 3. The effects of three apple extracts, phlorizin and phloretin on the adipogenesis and the lipid accumulation profile were also investigated. These results are presented in chapter 4.

Then, we have set up a coculture model of adipocytes and macrophages that allow to reproduce the inflammatory conditions characteristic of the inflamed adipose tissue. Based on previous studies, we chose to work with murine cell lines; the 3T3-L1 preadipocytes and RAW 264.7 macrophages. The chapter 5 carries forward the development of this model.

Once the coculture system clearly defined, we investigated the effect of the pomegranate husk and apple extracts on the inflammatory state simulated in the *in vitro* coculture model. The chapter 6 reports the effect of the pomegranate husk extract and its polyphenols on the inflammatory conditions of the coculture system. These results have been submitted to the Food and Function journal. The effects of the three apple extracts on inflammation are exposed in chapter 7.

Finally, results obtained in the first (adipogenesis) and second part (inflammation) have to be combined to discuss the potential preventive effect of pomegranate husk extract and apple extracts on the obesity linked metabolic disorders. This last chapter ends with final conclusion followed by the perspectives.

3. Pomegranate husk extract and its main phenolic compounds affected adipogenesis and lipid accumulation in 3T3-L1 adipocytes

The chapters 3 and 4 will report the effect of vegetal extracts on the differentiation of 3T3-L1 adipocytes *in vitro*. These murine cells present initially a fibroblast morphology. Under the action of a hormonal mixture, the differentiation process is induced and the preadipocytes begin their morphological change. The two first days, they round off and from day 4, they start to accumulate small lipid droplets. Eight days after the induction of the differentiation, the cells are considered fully differentiated although they could continue to store triglycerides by increasing their droplet size. This model will allow us to identify natural compounds that could interfere with the adipocyte differentiation and lipid accumulation with a view to identify candidate for obesity prevention by decreasing the fat storage.

In the chapter 3, the effect of a pomegranate husk extract on adipogenesis and lipid accumulation will be investigated. This extract has already been characterized by HPLC by Hollebeeck *et al.* (2012)¹ and contains various phenolic compounds, *i.e.* gallic acid, punicalin, pedunculagin I, punicalagin, strictinin, ellagic acid hexoside and ellagic acid. As punicalagin and ellagic acid are present in high concentration in this extract (60 μ M and 400 μ M per mg of extract/ml, respectively), their potential effect on adipogenesis will also be studied in order to verify whether they are the compounds responsible for the effect observed.

The differentiation of 3T3-L1 preadipocyte will be induced in the presence of the extract or one of the two polyphenols. Adipogenic markers will be followed as well as lipid accumulation until day 20.

The concentration of the pomegranate husk extract used (66 μ g/ml) corresponds to *ca.* 5 % of the daily amount that the provider suggests to take, *i.e.* 1 g. The concentrations of punicalagin and ellagic acid used in the experiments correspond to the amount found in 66 μ g/ml of the pomegranate husk extract.

The result will be presented in the form of the manuscript submitted for publication in “Plos One” and entitled “Pomegranate husk extract and its main phenolic compounds affected adipogenesis and lipid accumulation in 3T3-L1 adipocytes”. The authors are Julie Winand, Claude Remacle and Yves-Jacques Schneider

¹ Hollebeeck S, Winand J, Hérent M-F, During A, Leclercq J, Larondelle Y, et al. Anti-inflammatory effects of pomegranate (*Punica granatum* L.) husk ellagitannins in Caco-2 cells, an in vitro model of human intestine. Food and Function. 2012;3(8):875–85.

Abstract

Objective: This study aims to investigate the effect of a pomegranate husk extract (PHE) and its main phenolic constituents *i.e.* punicalagin and ellagic acid on the 3T3-L1 adipocyte differentiation and lipid accumulation. **Design and Methods:** 3T3-L1 cells were differentiated in the presence of the vegetal extracts. The effect on preadipocyte proliferation was measured by a MTT test. The impact on mitotic clonal expansion, percentage of differentiated cells and their lipid accumulation profile was followed by fluorescence microscopy analyzes and AdipoRed assay during 20 days. The expressions of adipogenic genes were followed as well as the secretion of adiponectin and the activity of glycerol phosphate dehydrogenase. **Results:** Punicalagin (4 μ M) and PHE (33 and 66 μ g/ml) interfered with the mitotic clonal expansion and reduced the number of differentiated adipocytes by 30 and 25 %, respectively. However no modification of the total triglyceride content was observed revealing a modification on lipid repartition in the adipocytes; cells treated with PHE contained 50 % more fat than control adipocytes, resulting of the presence of bigger fat droplets. **Conclusion:** PHE inhibited partially adipogenesis but promoted lipid accumulation in differentiating cells. Inversely, punicalagin slowed down the lipid accumulation. Ellagic acid did not affect adipogenesis.

3.1. Introduction

A high calorie intake forces the white adipose tissue to store more triglycerides by increasing the size (hypertrophy) and number (hyperplasia) of adipocytes. The expansion of adipose tissue deregulates adipocyte secretions, which leads to metabolic disorders. A recent study showed that even if the number of adipocytes remains relatively constant after adolescence, a remarkable turnover within adipocyte population occurs in adults¹. Accordingly, a possible way to prevent the development of obesity could consist in acting on the adipocyte hyperplasia, as well as its hypertrophy. Therefore, *in vitro* studies to identify compounds that will interfere with adipocyte differentiation or lipid accumulation are warranted. Many vegetal extracts and phenolic compounds have already shown effects on these two processes and could be useful to prevent the development of obesity and associated complications.

The *in vitro* mouse adipocyte cell line, 3T3-L1, has been widely used to investigate adipogenesis². Firstly, these fibroblast-like cells differentiate into adipocytes under the action of an empiric mixture³, which triggers two additional mitotic rounds of confluent preadipocytes. This process is known as mitotic clonal expansion⁴. At the same time, early adipogenic transcription factors are expressed and start to modify the cellular phenotype. PPAR γ is a key player in the initiation of the differentiation process and plays also a role in lipid storage⁵. This transcription factor, in parallel with C/EBP α , induces the expression of genes involved in lipogenesis, lipolysis and insulin sensitivity. The two regulators activate the expression of each other⁶. After four days, late adipogenic markers like adiponectin and glycerol phosphate dehydrogenase (GPDH) begin to be expressed and triglycerides accumulate in the form of small droplets.

Pomegranate (*Punica granatum* L.) and its constituents are the topic of studies related to obesity⁷. Positive effects on body weight, fat mass and insulin sensitivity have been observed in mice. Resveratrol is a polyphenol that has shown anti-obesity effect by acting on preadipocytes

differentiation, lipid accumulation, adipocyte apoptosis as reviewed by Baile *et al.* (2011)⁸. Most of these effects observed *in vitro* have been confirmed *in vivo* in rodents.

This study aims at investigating the potential effect of a pomegranate husk extract (PHE) and its main components, *i.e.* punicalagin and ellagic acid on the 3T3-L1 adipocytes differentiation. Resveratrol, known for its anti-adipogenic effect⁹, has been used as positive control.

3.2. Materials and methods

3.2.1. Materials and reagents

A hydroalcoholic extract of Pomegranate (*Punica granatum* L.) husk (PHE) was prepared from Moroccan cultivars and provided by Stiernon S.A. (Ghislenghien, BE) under the form of a dry powder. The phenolic compounds previously identified in the extract by HPLC are gallic acid, punicalin, pedunculagin I, punicalagin, strictinin, ellagic acid-hexoside and ellagic acid. Punicalagin (95.35 % purity) was purchased from Phytolab (Vestenbergsgreuth, DE); ellagic acid (> 90 % purity) from Extrasynthese (Lyon, FR); resveratrol, Triton X-100, dexamethasone (DEX), 3-isobutyl-1-methyl-xanthine (IBMX) and insulin from Sigma-Aldrich. (St Louis, MO); Dubelcco's Modified Eagle's Medium (DMEM), calf serum (CS), GlutaMax (100x), sodium pyruvate (100 mM), penicillin/streptomycin (100-100 µg/ml penicillin/streptomycin) and 0.05 % trypsin-0.02 % EDTA from Lonza (Verviers, BE) and fetal bovine serum (FBS) from PAA (Linz, AT).

3.2.2. Cell culture and treatment conditions

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10 % (v/v) CS, 1 mM Na pyruvate, 1 % (v/v) GlutaMax and 1 % (v/v) Pen-Strep in a 10 cm dish (Corning, Badhoevedorp, NL) at 37°C in a humidified 10 % (v/v in air) CO₂ atmosphere. For the differentiation process, cells were seeded in 12-well plates (Corning) and two-days postconfluent 3T3-L1 preadipocytes, designated as Day 0, were differentiated with DMEM supplemented with 10 % (v/v) FBS, 1 % (v/v) antibiotics, 1 % (v/v) Na pyruvate, 1 % (v/v) GlutaMax and an adipogenic mixture containing 170 nM insulin, 50 µM IBMX and 1 µM DEX for 2 days. Cells were then maintained in FBS-DMEM medium with insulin (170 nM) for another 2 days. Afterwards, the culture medium was replaced every 2 days by 10 % FBS-DMEM medium. Adipocyte differentiation was continued until days 8 or 20 according to the experiments.

PHE and the phenolic compounds were added in the culture medium during the 4 first days of the differentiation process. PHE was solubilized in Hank's balanced saline solution (HBSS) (Lonza) at the concentration of 2.5 mg/ml and added to the culture medium at the final concentration of 66 µg/ml corresponding to 4 µM of punicalagin and 24 µM of ellagic acid. Punicalagin was solubilized in 25 % (v/v) methanol at 50 mM and added to the culture medium at the final concentration of 4 µM. Ellagic acid and resveratrol were solubilized at 50 mM in DMSO and added to the culture medium at respectively 24 and 25 µM.

3.2.3. Cell viability, proliferation and cytotoxicity assays

Preadipocytes (5×10^3 cells) were seeded in 96-well tissue culture plate (Corning) in 200 µl of 10 % CS-DMEM containing various concentrations of PHE, punicalagin, ellagic acid or resveratrol and incubated at 37°C for 24 hours. Then, media were replaced with fresh 10 % CS-DMEM medium containing 0.2 mg/ml of MTT (3-(4,5-dimethyl -thiazol-yl-2)-2,5-diphenyl tetrazolium bromide) for 1h at 37°C. The medium was removed and the formazan violet precipitate solubilized in 50 µl of DMSO. Absorbance was read at 500 nm with a microplate reader (Spectramax PLUS, Molecular Devices, Berkshire, UK).

The effect on mitotic clonal expansion was assessed by counting the number of nuclei, at days 0, 2 and 4 of the differentiation process. Nuclei were stained with DAPI (Sigma). The detection is based on DAPI channel assay (SET 31000 DAPI (EX BP 360/40, BS LP 400, EM BP 460/50), which uses a 40x objective, a Nikon DS-Qi1Mc camera in combination with a 10x Nikon ECLIPSE TS100 microscope for image acquisition (Nikon, Brussels, BE). The parameters were optimized and fixed for all experiments (40x objective, gain of 1 x, 100 ms exposure). All images were analyzed with the NIS-Element Software (Nikon). For each well, 3 pictures were taken, with a random location of sampling. The area covered by the picture was always

0.25 mm² representing a number of cells varying between 45 and 125. The image analysis software automatically counted DAPI-stained nuclei.

The possible cytotoxicity of treatments was routinely checked through the determination of the LDH activity in the extracellular medium, with a cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, DE) used according to the manufacturer's instructions. The percentage of cytotoxicity was calculated using the maximum released LDH activity obtained upon complete lysis of 3T3-L1 cells in the same stage of culture with 1 % (v/v) Triton X-100.

3.2.4. AdipoRed assay

Preadipocytes (10³ cells) were seeded in 96-well tissue culture plate. Two days after confluence, preadipocytes differentiation was induced in the presence of various concentrations of PHE or the pure phenolic compounds. On day 8, cells were treated with AdipoRed (Lonza) and total lipid content was determined following the manufacturer's instructions. Briefly, 8 days old cultures were washed twice with PBS. Then, 200 µl of PBS were added to each well with 5 µl of AdipoRed reagent. The lipophilic assay reagent specifically distributed into the fat droplets of adipocytes. After 10 minutes, the fluorescence was read (485/535 nm).

3.2.5. Adiponectin assay

The adiponectin secretion in culture media was assessed by a commercially available sandwich ELISA kit (Millipore, USA), according to the protocol instructions. Culture media, at day 8, were harvested and centrifuged. The supernatant was diluted in order to reach measurable concentrations.

3.2.6. GPDH activity assay

Eight days after the induction of differentiation, cells were washed with PBS, and then harvested in ice-cold 20 mM Tris buffer (pH 7.3) containing 1 mM EDTA and 1 mM β-mercaptoethanol. Cell lysates were mixed and

maintained on ice until the GPDH activity assessed by measuring, at 340 nm, the oxidation of 125 μ M NADH (Sigma), in the presence of 200 μ M dihydroxyacetone phosphate (DHAP) (Sigma). Results were expressed as nmol/min x well.

3.2.7. Staining procedure

Adipocytes were stained with DAPI (Sigma) and/or AdipoRed. The cells were first washed twice with PBS, then incubated with 4 % (v/v) formaldehyde during 30 min at 37°C. Adipocytes were then permeabilized with a 1 % (v/v) triton-X-100 solution added 3 times during 5 min. The cells were then washed with PBS and DAPI (10 μ g/ml) and 0.2 % (v/v) Adipored was added to 500 μ l of PBS. After 10 min of incubation in the dark at 37°C, PBS was removed and replaced by fresh PBS.

3.2.8. Fluorescence image analysis

The method is based on a two channels assay, which uses a 40x objective, a Nikon DS-Qi1Mc camera in combination with a 10x Nikon ECLIPSE TS100 microscope for image acquisition. The parameters were optimized and fixed for all experiments (40x objective, gain of 1 x, 100 ms exposure). Channel 1 corresponds to the DAPI filter (SET 31000 DAPI (EX BP 360/40, BS LP 400, EM BP 460/50) and is the focus channel in which nuclei are identified. Channel 2 corresponds to the FITC filter (SET 31001 FITC (EX BP 480/30, BS LP 505, EM BP 535/40) and detected the fat droplets. All images were analyzed with the NIS-Element Software (Nikon). For each well, 3 pictures have been taken randomly. The area covered by the picture was always 0.25 mm² representing a number of cells varying between 45 and 125. The relative fluorescence of both DAPI and AdipoRed was measured by the NIS Software. The image analysis software automatically counted DAPI-stained nuclei. The lipid droplets were manually selected in the software, detected and then counted and classified according to their size by the software.

3.2.9. Gene expression study

Total RNA was extracted from 3T3-L1 using QIAzol reagent (Qiagen, Hilden, DE) according to the manufacturer's instructions. Quality and quantity of total RNA were assessed by Nanodrop spectrophotometry. Total RNA (1 µg) was reverse-transcribed using the Superscript III First-Strand Synthesis System (Invitrogen, Merelbeke, BE) following manufacturer's instructions. Then, qRT-PCR was performed in 96 well PCR plates (Bio-Rad, Nazareth Eke, BE), to estimate the mRNA abundance corresponding to either PPAR γ , C/EBP α , C/EBP β , SREBP, GATA2, GATA3, aP2, adiponectin, GLUT4 or LPL relatively to the housekeeping genes (β actin, GAPDH and HPRT), used for normalization. A total of 5 µl cDNA with the appropriate dilution, 0.2 µl primer mix and 4.8 µl SYBRGreen SuperMix (Bio-Rad) were added to each well. The PCR amplifications were performed in a MyIQ2 thermocycler (Bio-Rad) as follows: 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. Melting curves were systematically assessed for quality control of the primers. Gene expression values were normalized to the geometric means of the expression values of β -actin, GAPDH and HPRT and calculated on the basis of the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$). The primers used were reported in **Table 3.1**.

Gene name	Forward primer	Reverse primer
β actin	TAGACTTCGAGCAGGAGATGG	GGATTCCATACCCAAGAAGGA
GAPDH	GTGGAGATTGTTGCCATCAACG	CAGTGGATGCAGGGATGATGTTCTG
HPRT	ACATTGTGGCCCTCTGTGTG	TTATGTCCCCCGTTGACTGA
PPAR γ	GAAAGACAACGGACAAATCACC	CATACTCTGTGATCTCTTGCACG
C/EBP α	GCCAAACTGAGACTCTTC	GGAAGCCTAAGTCTTAGC
C/EBP β	GGGGTTGTTGATGTTTTTGG	CGAAACGGAAAAGGTTCTCA
SREBP	GCTTAGCCTCTACACCAACTGGC	ACAGACTGGTACGGGCCACAAG
GATA2	GGAGACGATTGTGCTGAGTCAA	TTAGGGTGCTCTGCAGCAACT
GATA3	CCGGCGGTACCTGTCTTTT	GCTGGTACGCTGTACCTTCATA
aP2	CCGCAGACGACAGGAAGGT	AGGGCCCCGCCATCT
Adiponectin	GGAGAGAAGGGAGAGAAAGGTC	AACAGTGACGCGGGTCTCC
Glut4	TGCTGGGCACAGCTACCC	CGGTCAGGCGCTTAGAC
LPL	TTCTCCTCCTACTCCTCCTC	TGTCCTCAGCTGTGTCTTCA

Table 3.1. Sequences of primers for different adipocyte genes followed by qRT-PCR

3.2.10. Statistical analysis

All the results were expressed as means $\pm$ SEM. Data were analyzed by one-way ANOVA followed by Dunnett's post-hoc test to identify mean differences between treatment and positive control. P-values less than 0.05 were considered significant. Statistical analyses were performed using the JMP® 10 software (SAS Institute, Cary, NC).

3.3. Results

3.3.1. Effect of treatments on cell viability and mitotic clonal expansion

The effect of increasing concentrations of PHE, punicalagin, ellagic acid or resveratrol on the proliferation of preadipocytes was evaluated by MTT test, after 24h incubation with 3T3-L1 cells. As shown in **Fig 3.1**, PHE, punicalagin and ellagic acid had no significant effect. Resveratrol, as already described¹⁰, strongly decreased the preadipocytes proliferation in a dose-dependent manner. This anti-proliferative effect was not due to cytotoxicity as LDH assays have confirmed (Data not shown).

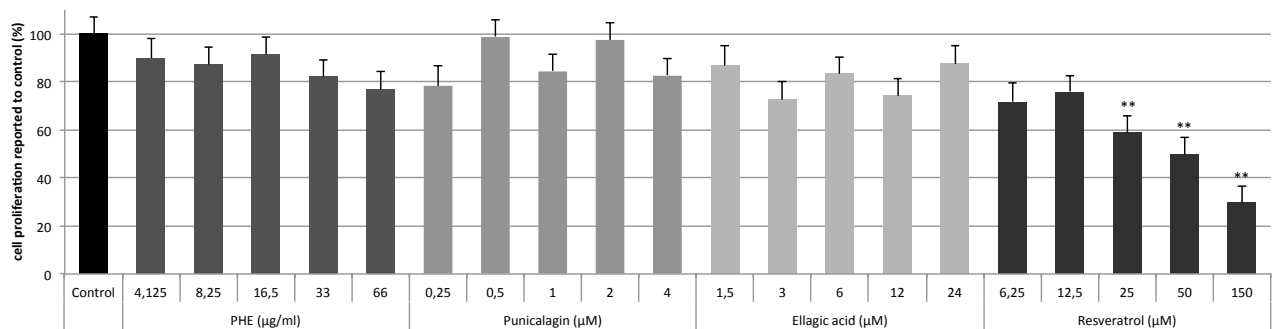


Fig 3.1. MTT colorimetric assay. Effect of PHE, punicalagin, ellagic acid and resveratrol on preadipocyte proliferation after 24h of treatment. Data are presented as means $\pm$ S.E.M, N=3 n=3, and compared to control value (** $p < 0.01$).

To determine whether PHE (33 $\mu\text{g/ml}$ or 66 $\mu\text{g/ml}$), punicalagin (4 μM), ellagic acid (24 μM) and resveratrol (25 μM) affected the mitotic clonal expansion that precedes the adipocyte differentiation, the total amount of nuclei was counted on days 0, 2 and 4. PHE, punicalagin and resveratrol prevented the mitotic clonal expansion (**Fig 3.2**). Ellagic acid caused a delay, which caught up on day 4 and maintained until day 20 (Data not shown).

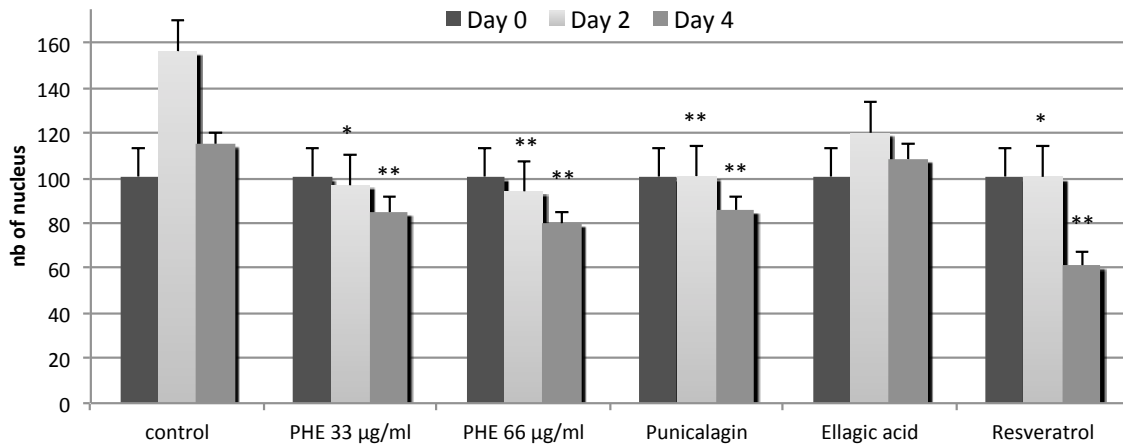


Fig 3.2. Nuclei number counting at day 0, day 2 and day 4 after induction of differentiation in the presence or absence of PHE (33 or 66 µg/ml), punicalagin (24 µM), ellagic acid (4 µM) or resveratrol (25 µM). Counting was assessed using DAPI staining followed by image analysis. Data are presented as means $\pm$ S.E.M, N=4 n=3, and compared to control values in the same condition (* p <0.05; ** p <0.01).

3.3.2. Effect of treatments on adipogenesis

The effects of PHE, its main constituents and resveratrol, were then tested on the differentiation of 3T3-L1 preadipocytes. After the 4 first days of differentiation in the presence or absence of these different compounds, cells were cultivated in a normal medium. At day 8, the number of differentiated cells was counted and reported to the total number of cells. 93 % of the cells were differentiated into adipocytes in the control condition. PHE (66 µg/ml), punicalagin (4 µM) and resveratrol (25 µM) reduced significantly the number of differentiated cells to, respectively, 68 %, 74 % and 61 % (**Fig 3.3**).

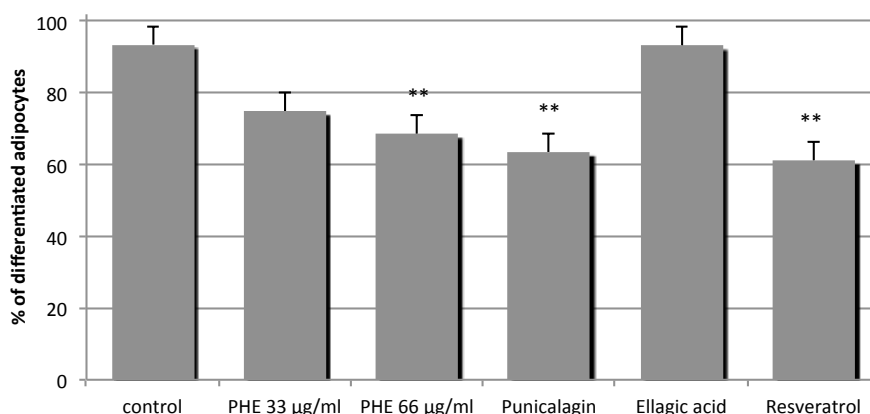


Fig 3.3. Percentage of differentiated adipocytes 8 days after the induction of differentiation in the presence or absence of PHE, punicalagin (4 µM), ellagic acid (24 µM) or resveratrol (25 µM). Differentiated cells were counted and reported to the total number of cells. Data are presented as means $\pm$ S.E.M, N=3 n=3, and compared to the control value (**p<0.01).

At day 8, GPDH activity was measured. Neither PHE, nor its phenolic compounds showed a significant effect on the GPDH activity excepted resveratrol that inhibited the enzyme in a dose-dependent manner. At 25 µM, it decreased the GPDH activity by about 30 % (Data not shown). The secretion of adiponectin was also unaffected by these extracts, as compared to control with the exception of cells treated with resveratrol. This stilbene, at 25 µM, reduced significantly the adiponectin secretion by more than 90 % (Data not shown).

3.3.3. Effect of treatment on lipid accumulation

During 20 days of culture, lipid accumulation has been followed after the treatment with PHE, punicalagin, ellagic acid or resveratrol. At day 8, the cells treated with PHE (66 µg/ml), punicalagin (4 µM) and resveratrol presented a lipid accumulation lower than in control cells (**Fig 3.4A**). However, only resveratrol was able to maintain this state until day 20. Nevertheless, as PHE reduced the mitotic clonal expansion and the proportion of differentiated cells, the number of lipid loaded cells was lower, as compared to control. When the total amount of accumulated triglycerides was reported to the number of cells (**Fig 3.4B**), it appeared

that adipocytes differentiated in the presence of PHE at 33 and 66 $\mu\text{g}/\text{ml}$ stored more triglycerides than control ones, 15 and 20 days after the differentiation. Interestingly, punicalagin and ellagic acid showed the same effect than PHE at day 20.

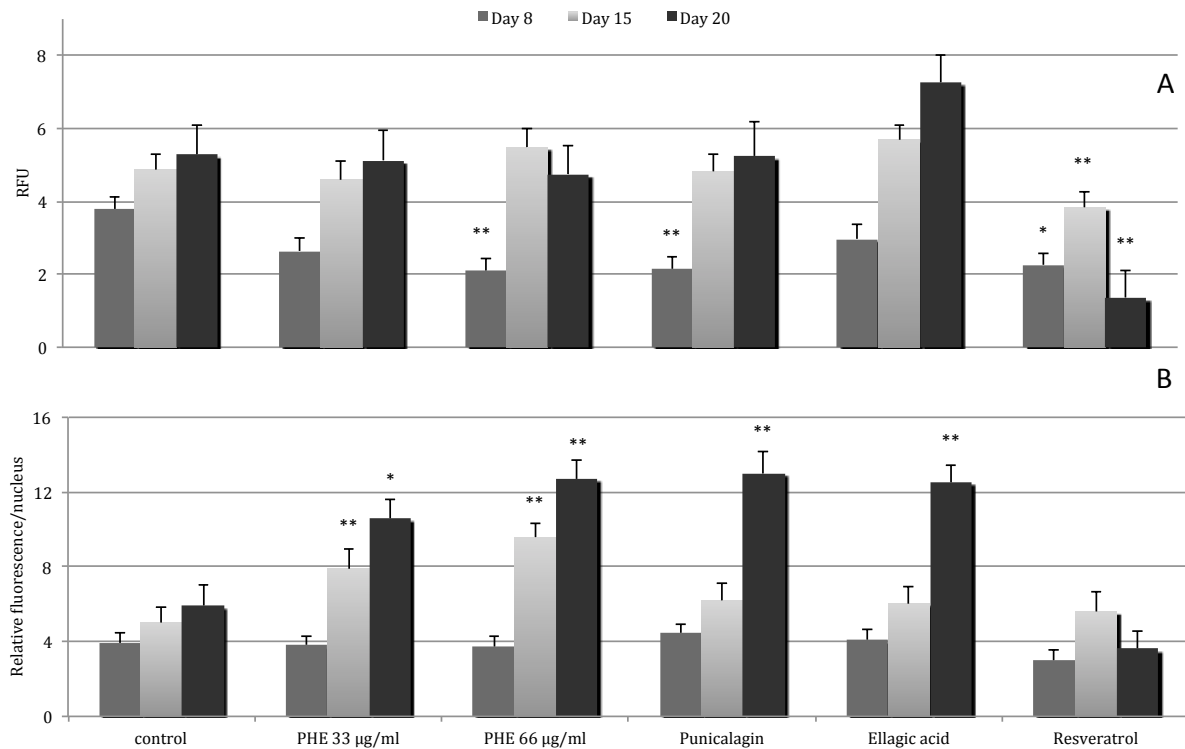


Fig 3.4. Triglyceride accumulation in 3T3-L1 during 20 days after induction of differentiation in the presence or absence of PHE, pinucalagin (4 μM), ellagic acid (24 μM) or resveratrol (25 μM). After 8,15 and 20 days, adipocytes were subjected to AdipoRed and DAPI staining, the relative fluorescence of AdipoRed was measured and the number of nuclei was counted. Figure A reports the relative fluorescence units (RFU) per conditions. Figure B is the RFU reported to the number of cells corresponding to each day. Data are presented as means $\pm$ S.E.M, N=3 n=3, and compared to the control value for the same day (* $p < 0.05$; ** $p < 0.01$).

To further support these observations, lipid droplets were counted and their size measured (**Fig 3.5 A**). At day 8 (**Fig 3.5 B**), cells treated with PHE at 66 $\mu\text{g}/\text{ml}$, punicalagin at 4 μM and ellagic acid presented bigger droplets (>10 μm of radius) than control adipocytes (0,24, 0,22 and 0,13 % respectively as compared to 0,12 % for the control adipocytes). However, only the cells treated with PHE maintained its differences until day 20 (**Fig 3.5 C and D**). As expected, resveratrol slowed down the process of triglycerides accumulation and maintained lipid droplet at smaller sizes.

Although, punicalagin also reduced the fat droplet growth, the total quantity of fat stored in adipocytes was not as low as after resveratrol treatment. Ellagic acid only had a small effect on triglyceride storage and repartition in adipocytes at the beginning of fat storage.

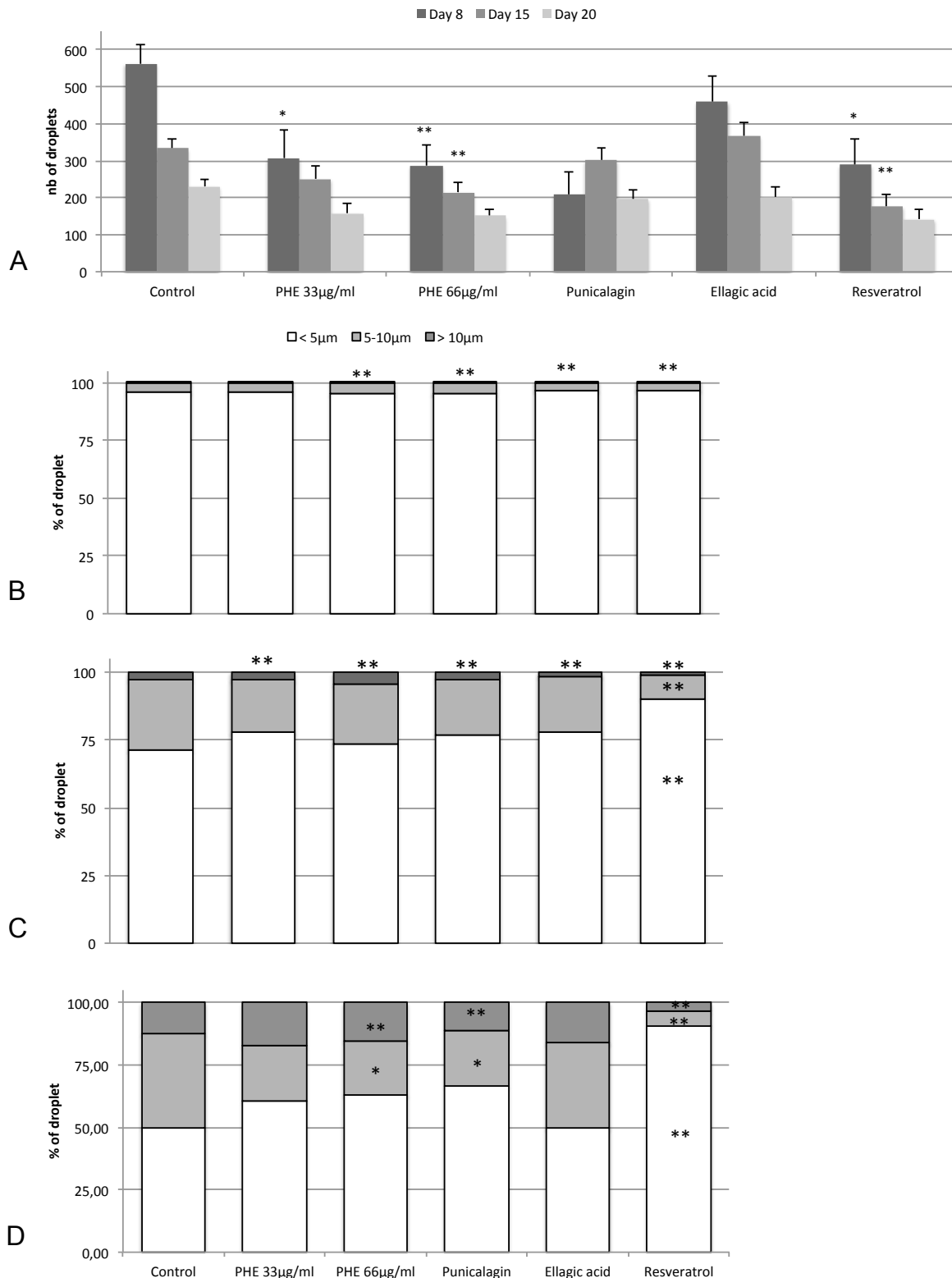


Fig 3.5. (A) Counting of lipid droplets in 0.25 mm² in 3T3-L1 after 8, 15 or 20 days of initiation of differentiation in the presence or absence of PHE, punicalagin (4 µM), ellagic acid (24 µM) or resveratrol (25 µM). Counting was assessed using AdipoRed staining followed by image analysis. Radius of each droplet were measured after 8 days **(B)**, 15 days **(C)** and 20 days **(D)** and classified by size (small: 0-5 µm, intermediate: 5-10 µm and big: >10 µm). Data are presented as means ± S.E.M, N=3 n=3, and compared to the control value (*p<0.05; **p<0.01).

Fig 3.6 shows pictures of the 3T3-L1 cells 15 days after the induction of their differentiation in the presence or absence of either PHE or phenolic compounds. These pictures clearly revealed: (i) that PHE induced the formation of bigger droplets, (ii) that punicalagin slightly increased the proportion of smaller droplets as resveratrol, and (iii) that ellagic acid treated cells presented the same phenotype than control cells

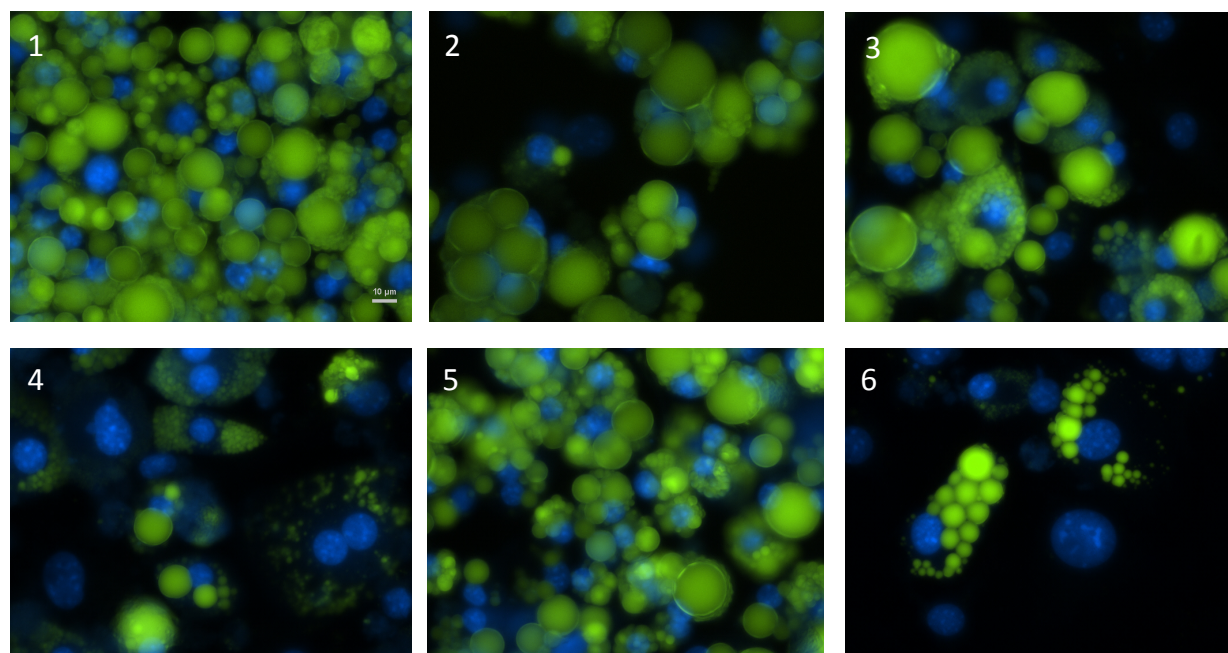


Fig 3.6. 3T3-L1 cells 15 days after the induction of differentiation in the presence or absence of the vegetal extracts. Cells were stained with DAPI and AdipoRed. All the pictures have been taken at the same magnification (400x) with scale representing 10 μm . Picture 1: control cells; 2: cells treated with PHE 33 $\mu\text{g/ml}$; 3: PHE 66 $\mu\text{g/ml}$; 4: punicalagin 4 μM ; 5: ellagic acid 24 μM and 6: resveratrol 25 μM .

3.3.4. Effect of PHE, punicalagin, ellagic acid and resveratrol on gene expression associated with adipogenesis

Figure 3.7 presents the relative gene expression in 3T3-L1 cells upon incubation with the tested compounds. Twenty-four hours after the induction of differentiation, the expression of adipogenic transcription factors (PPAR γ , C/EBP α , C/EBP β , SREBP, GATA2 and GATA3) was measured by qRT-PCR. PHE at 33 and 66 $\mu\text{g/ml}$, as well as resveratrol,

increased GATA2 expression. The expression of C/EBP β was significantly reduced by PHE at 66 μ g/ml. Ellagic acid reduced the GATA2 gene expression. No other significant effect was observed.

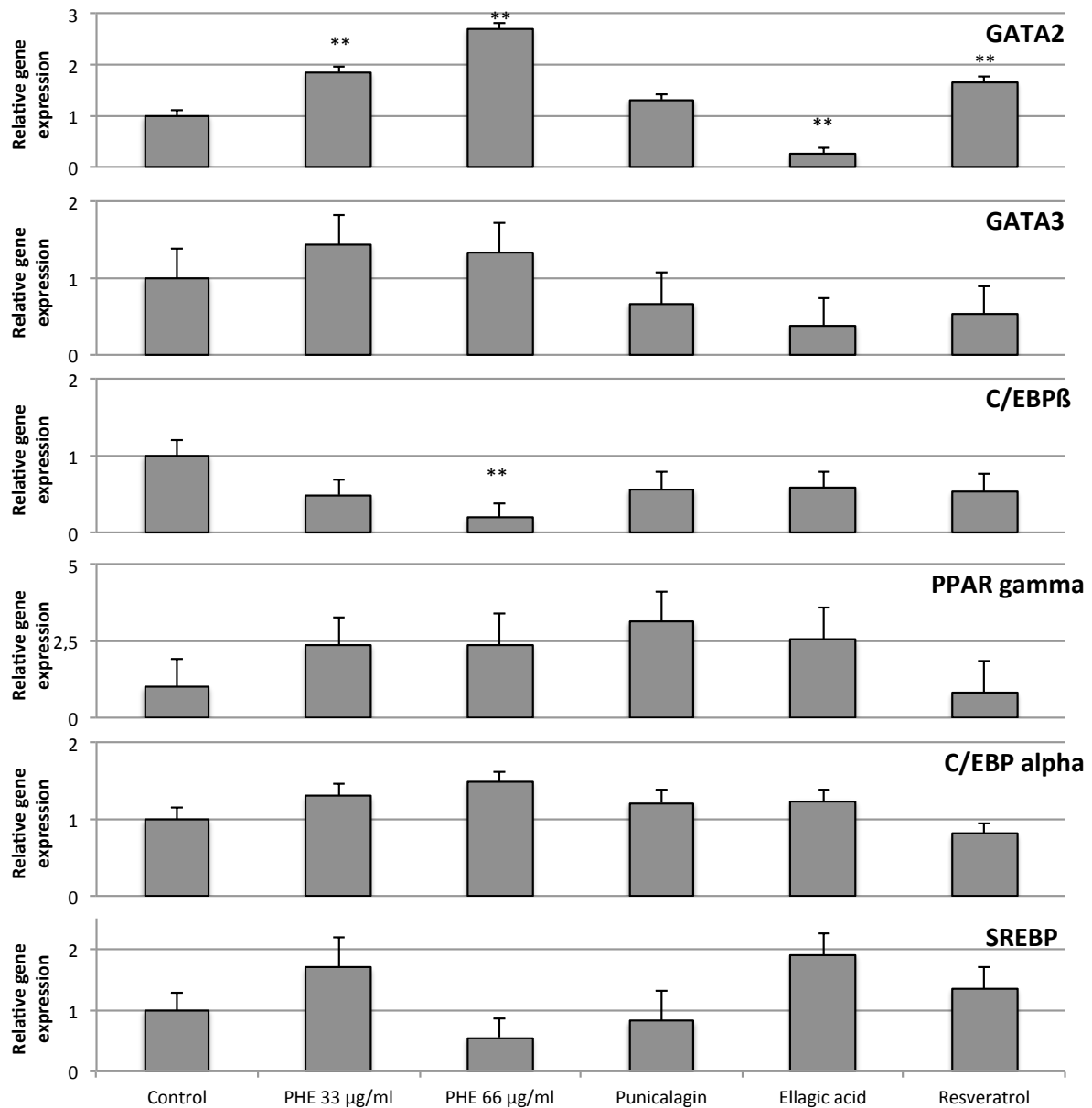


Figure 3.7 Relative gene expressions after 24h of treatment. Adipocytes markers were analysed by quantitative real-time PCR using mRNA extracted from 3T3-L1 after treatment with PHE, punicalagin (4 μ M), ellagic acid (24 μ M) or resveratrol (25 μ M). β -actin, GAPDH and HPRT were used as housekeeping genes and signals were normalized by the geometric mean of the three housekeeping genes and relative to control value. Data are presented as means $\pm$ S.E.M, N=3 n=1, (* $p < 0.05$; ** $p < 0.01$).

At day 8, the expression of differentiation markers (aP2, adiponectin), genes involved in glucose (Glut 4) or fat metabolism (LPL) were measured as well as PPAR γ gene expression. No significant effects were observed except an increased of adiponectin gene expression after the incubation with PHE at 66 μ g/ml (**Fig 3.8**).

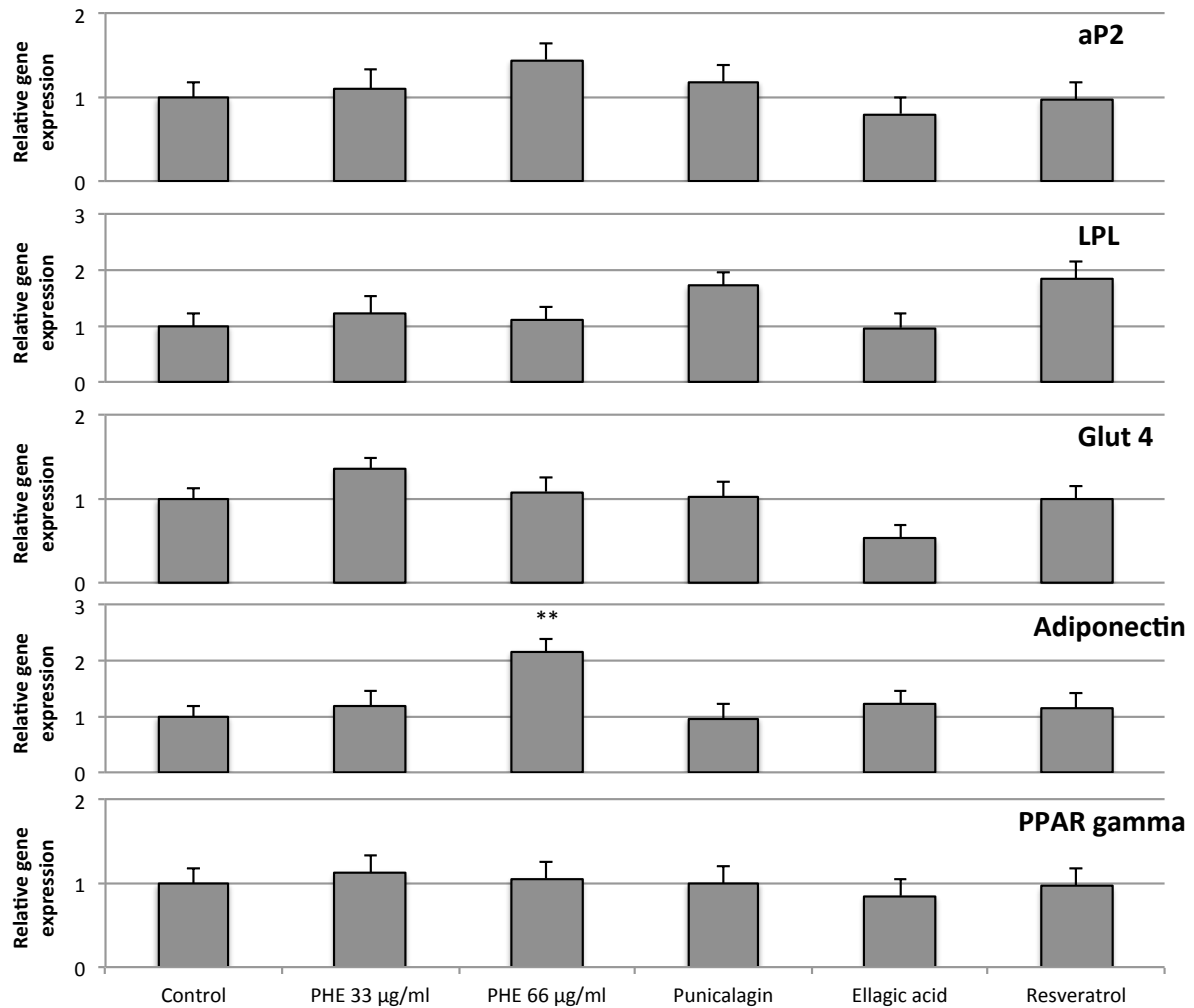


Figure 3.8 Relative gene expressions after 8 days of culture. Adipocytes markers were analysed by quantitative real-time PCR using mRNA extracted from 3T3-L1 after treatment with PHE, punicalagin (4 μ M), ellagic acid (24 μ M) or resveratrol (25 μ M). β -actin, GAPDH and HPRT were used as housekeeping genes and signals were normalized by the geometric mean of the three housekeeping genes and relative to control value. Data are presented as means $\pm$ S.E.M, N=3 n=1, (* $p < 0.05$; ** $p < 0.01$).

3.4. Discussion

Pomegranate is thought to be a good candidate to help to prevent the metabolic syndrome, as already suggested from *in vitro* and *in vivo* studies^{7,11}. The husk of this fruit is particularly rich in punicalagin, an ellagitannin having a high antioxidant¹² and a good anti-inflammatory effect¹³. We choose to work with PHE concentration that corresponds to *ca.* 5% of the daily amount recommended by the extract provider (*i.e.* 1 g)¹³. In this study, we used resveratrol as reference since this stilbene is known to inhibit adipocyte differentiation *in vitro*^{9,14,15} and since some studies have shown interesting effects on the metabolic syndrome *in vivo*^{16, 17,18}.

The results described in this work suggest that punicalagin and ellagic acid, the two main constituents of the pomegranate husk extract, should not be the only substances that do contribute to the effects of PHE on cell proliferation and fat accumulation. PHE and ellagic acid tended to reduce the proliferation of preadipocytes in a dose-dependent manner (**Fig 3.1**). However, no significant effect has been observed. The counting of nuclei revealed that, even though the anti-proliferative effect was not observed after 24h, PHE (33 and 66 µg/ml) and punicalagin (4 µM) reduced the number of cells after 48h and 96h (**Fig 3.2**) and this effect was still observed after 20 days of culture (Data not shown). At these concentrations, PHE and punicalagin would block the mitotic clonal expansion that occurs in the early stage of the differentiation process, resulting in a reduced number of cells that differentiate into adipocytes. The reference substance, *i.e.* resveratrol, as expected¹⁰, decreased the proliferation in a dose-dependent manner. Nuclei counting revealed that after 96h incubation, 25 µM resveratrol halved the nuclei number, as compared to control. Although this observation does not bring out the mechanism by which resveratrol acts, the results are similar to what was described by Chen *et al.* (2012) showing that resveratrol, already at 20 µM, has an apoptotic effect on 3T3-L1 preadipocytes by acting on the AMPK pathway¹⁰.

Ellagic acid alone showed no effect on preadipocyte proliferation, which is opposite to the results obtained by Zhi-Gang Yang *et al.* (2011) showing a decrease of 40 % in adipocytes viability upon cultivation during 8 days with 30 μ M of ellagic acid from *Euphorbia lunulata* ¹⁹.

Other studies have shown that some natural molecules modulate the mitotic clonal expansion^{20,21}. Curcumin or sulforaphane incubated during the first 2 days of preadipocyte culture inhibit their differentiation. These substances delayed the cell cycle progression that occurs during the mitotic clonal expansion, which is required to initiate the adipocyte differentiation. After 24h, preadipocytes treated with curcumin were mainly arrested in S/G₂ phase instead of G₀ phase. The expression of C/EBP β was not affected by curcumin suggesting an action either on C/EBP β localization or on other regulatory factors. Sulforaphane lowered C/EBP β expression in a dose-dependent manner resulting in a decrease of differentiated adipocytes. In our experimental model, PHE, punicalagin and resveratrol also decreased, or tended to decrease, C/EBP β gene expression (**Fig 3.7**).

PHE, punicalagin and resveratrol affected the differentiation process of adipocytes, by halving the number of differentiated cells, compared to control. Consequently, the amount of intracellular triglyceride was reduced in adipocytes upon treatment with resveratrol or punicalagin and adipocytes contained smaller lipid droplets than the control cells. Warnke *et al.* (2011) developed a method of detection of fat droplets in differentiated adipocytes. Microscopic analysis, similar to ours, allowed to quantify fat droplets in adipocytes treated with various dietary constituents²². In their work, 25 μ M resveratrol did not affect the lipid content, number and size of droplets after 7 days of treatment. Their gene expression analysis also revealed that resveratrol did not affect the expression of PPAR γ and C/EBP α , the master regulators of adipogenesis. These observations are, however, opposite of those of Rayalam *et al.* (2008) and ours^{8,9}.

Surprisingly, PHE reduced the number of differentiated cells but did not modify the total amount of triglycerides stored in all the adipocytes.

Therefore, as the total amount of stored neutral lipid remained high, the cells displayed larger droplets, with often a huge one rejecting the nucleus in periphery. This indicates that PHE clearly influenced the lipid droplet growth; however too little is known about the process of lipid droplet formation and further work should be done to investigate this effect. We could postulate that PHE will impede the lipolysis process by increasing the droplet size^{23,24}. Lipolytic assays should be done to investigate this hypothesis.

Punicalagin, by increasing the number of small droplets, also modified the adipocyte phenotype. This constituent of PHE has an effect similar to resveratrol although less strong. Punicalagin did not decrease the total fat content compared to control adipocytes but modified the triglyceride repartitions inside the cells.

Previous unpublished studies in our lab, using an *in vitro* digestion model of PHE indicated that punicalagin was degraded up to 88 % of the initial amount and that the amount of ellagic acid increased as a result of this process. The punicalagin concentration used for these experiments could therefore be probably higher than those reached *in vivo* in plasma since the phenolic compounds undergo digestion and metabolization. Nevertheless, a study on rats revealed that after 30 days of diet supplemented with pomegranate juice (6 % of punicalagin), punicalagin and its metabolites were present in plasma. Punicalagin plasma concentration was 28 μM and that of ellagic acid at 1.6 μM after 10 days of diet supplemented with pomegranate juice²⁵. The lower level of ellagic acid may be due to its high metabolization by the microflora.

Taken together, our results suggest that the effect of PHE on adipocytes differentiation could not be entirely explained by its main polyphenols, *i.e.* punicalagin and ellagic acid. However, PHE decreased the hyperplasia, but increased the lipid storage capacity of differentiated cells. This could be beneficial to prevent obesity complications. By decreasing the number of newly differentiated adipocytes, but in keeping the storage capacity of each cells, PHE may be a good candidate in limiting the hyperplasia but not the

hypertrophy that occurs in development of obesity. Punicalagin by decreasing the number of differentiated adipocytes and reducing the droplet sizes could also be a candidate for *in vivo* investigations.

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4. Apple extracts affected adipogenesis and lipid accumulation in 3T3-L1 adipocytes

The chapter 4, as the chapter 3, aims at investigating the potential effect of natural extracts on the adipocyte differentiation and the lipid accumulation. As the apples are fruits presenting interesting anti-obesity effects *in vivo*, investigations about their mechanisms of action are warranted¹.

Three extracts from different apple varieties will be tested for their potential effect on adipogenesis and lipid accumulation. These extracts are in form of dry powder and furnished by Stiernon S.A. The phenolic composition of these three extracts has been partially defined. The apple extract 1 (AE1), made from *Malus domestica Borkh*, contains more than 80 % of phenolic compounds of which ± 5 % is phlorizin, a typical polyphenol of apple. The extract 2 (AE2) is composed of 90 % of phenolic compounds

¹ Nakazato, K., Song, H. & Waga, T. Effects of dietary apple polyphenol on adipose tissues weights in Wistar rats. *Exp. Anim.* **55**, 383–389 (2006).

of which 15 % phlorizin. We do not have information about the variety of the apple used for the extract formulation. The third extract (AE3), made from *Malus pumila* Mill., contains more than 40 % of phenolic compounds whose 15 % of phlorizin.

In order to verify whether phlorizin and its unglycosylated form, phloretin, are the compounds conferring the effect of apple, they will be tested as pure compounds.

Abstract

Obesity is characterized by an expansion of the adipose tissues. Adipocytes that constitute these tissues have to increase their size to store the energy excess (hypertrophy). If these excesses of energy input continue, the preadipocytes present in the adipose tissue undergo their differentiation into adipocytes (adipogenesis) resulting in an increased number of storage cells (hyperplasia). Big adipocytes are known to secrete more pro-inflammatory molecules than small adipocytes that contribute to the development of obesity complications, *i.e.* type 2 diabetes, cardiovascular disease ... Apples are commonly consumed fruits in our European countries. It already appeared that apple consumption is correlated with various health benefits such as the improvement of obesity complications. In this work, we investigated the potential effects of three apples extracts (AE1, AE2 and AE3) on adipogenesis *in vitro*. We also investigated the effects of two dihydrochalcones found in apples, phloretin (PT) and phlorizin (PZ). 3T3-L1 cells were differentiated in the presence of the apple extracts or the polyphenols. Various markers of adipogenesis were followed as well as the lipid accumulation profile. We highlighted that according to the phenolic composition, the different extract did not reveal the same impact on adipogenesis. The AE1 and AE2 reduced partially the proportion of differentiated adipocytes by about 30 and 20 % respectively as compared to the control. Moreover, the resulting adipocytes presented 50% lesser lipid droplets than control cells. AE3, as PT and PZ, did not reduce the number of differentiated cells and even induced a slight increase of triglyceride accumulation as compared to the control adipocytes. Indeed, PT, and PZ to a lesser extent, stimulated adipogenesis resulting in an increased lipid content, glycerol-3-phosphate dehydrogenase activity and adiponectin secretion. These *in vitro* experiments have allowed to highlight that according to the apple varieties the effects on adipogenesis could differ. This observation could be related to the composition of phenolic compounds. However, by decreasing the size of droplets, AE1 and AE2 are

extracts presenting potential health benefits to counter obesity complications. We have also shown that PT, by increasing the number of differentiated cells, as the antidiabetic family drug thiazolidinedione, could be a good candidate to address the obesity associated metabolic disorders.

4.1. Introduction

When energy intake is higher than energy expenditure, the body stores the excess of energy as triglycerides in specialized cells of the adipose tissues, the adipocytes. These cells have the ability to increase their size (hypertrophy) to store more triglycerides in case of need. The number of adipocytes in the human body is fixed in adolescence although a turnover within adipocyte population occurs¹. Nevertheless, in case of obesity, as these cells have a limited storage capacity, they are able to increase their number and so respond to the storage need². The preadipocytes undergo their differentiation towards new adipocytes (adipogenesis), resulting in an increase of adipocyte population (hyperplasia). Furthermore, even if the weight loss empties partially the adipocytes, it does not decrease the number of fat cells facilitating a new storage of fat.

The size of adipocytes is linked to obesity complications: type 2 diabetic patients have adipocytes 19 % bigger than non diabetic subjects³. It also appears that adipocyte size is correlated with pro-inflammatory factor secretions. Big adipocytes secrete higher amounts of pro-inflammatory factors, *i.e.*, IL-6, TNF α , CCL-2, leptin ... than small ones^{4,5}.

Moreover, the thiazolidinediones (TZDs), a family of antidiabetic drugs, induce adipocytes differentiation and decrease the cells size resulting in a decrease of leptin and TNF α secretion and an improvement of insulin resistance⁶. The adipocytes treated with a TZD family member also present plurilocular droplets instead of unilocular one, providing more favourable conditions for fat exchange between cells and blood circulation⁷.

Nowadays, an increasing interest of natural compounds to counter obesity exists. Apples have the reputation to be good for health for several hundred years. Different *in vitro*^{8,9,10} and *in vivo*^{11,12} studies have confirmed their beneficial effects on health, *e.g.* decreasing cancer risks¹³, reducing low-density lipoprotein oxidation¹⁴..., and identified compounds in the fruit that have high beneficial potential¹⁵. The polyphenols that confer the majority of the antioxidant power of the apple¹⁶ are present mainly in the peel and the

total content is higher than in the other classically consumed fruits¹⁷. Among the phenolic compounds found in apple, the phloretin-2-*O'*-glucoside (phlorizin (PZ)), a dihydrochalcone, is present principally in the peel. According to the variety its concentration can reach 16 mg in an apple (0.05 % of total polyphenols content)¹⁸. Its unglycosylated form, phloretin (PT), is found in the apple juice and in the plasma after apple ingestion. A study on rat has highlighted the presence of PZ and PT in high concentration in the plasma 10h after the ingestion of a meal supplemented with PZ or PT¹⁹. The nature of the supplemented compound (PT or PZ) did not modify the proportion of PT and its glycosylated forms found in the plasma. The concentration of PT reached 10 μ M and its glycosylated forms reached 70 μ M. These two polyphenols have revealed opposite effects on adipocytes differentiation *in vitro*; PT enhanced the adipogenesis²⁰ while PZ inhibited it¹⁰. Najafian *et al.* (2012) have highlighted *in vivo* evidences of the PZ effect on negative parameters linked to obesity. The polyphenol was able to reduce the body weight of diabetic rats and improve their lipidic plasma profile¹².

To our knowledge, whole apple extract has never been tested *in vitro*. One *in vivo* study has demonstrated that Wistar rats fed during 3 weeks with a diet supplemented in apple extract showed reduced visceral fat weight and increased adipocyte sizes¹¹ but the mechanism of action of apple and its polyphenols has never been investigated. In this study, we have tested three apple extracts from different varieties on adipogenesis of 3T3-L1 cells. The three extracts differ in their polyphenol composition profile and have not revealed the same effect on adipogenesis. PT and PZ have also been tested as controls. PT, as expected, has increased adipogenesis whereas PZ has not interfered with the differentiation process.

4.2. Material and methods

4.2.1. Materials and reagents

Three extracts of whole apple fruits (AE1, AE2, AE3) were prepared from *Malus domestica Borkh* (AE1), an unknown variety (AE2) and *Malus pumila Mill.* (AE3) and provided by Stiernon S.A. (Ghislenghien, BE) under the form of a dry powder. **Table 4.1** presents the phenolic composition of these three extracts. Phlorizin and phloretin were purchased from Sigma-Aldrich (St Louis, MO). Triton X-100, dexamethasone (DEX), 3-isobutyl-1-methyl-xanthine (IBMX) and insulin from Sigma-Aldrich. (St Louis, MO); Dubelcco's Modified Eagle's Medium (DMEM), calf serum (CS), GlutaMax (100x), sodium pyruvate (100 mM), penicillin/streptomycin (100-100 µg/ml penicillin/streptomycin) and 0.05 % trypsin-0.02 % EDTA from Lonza (Verviers, BE) and fetal bovine serum (FBS) from PAA (Linz, AT).

	AE1	AE2	AE3
Total polyphenol content	> 80%	> 90%	> 40%
Catechin	0.4	24.2	2.9
Caffeic acid	1.3	1.1	0
Chlorogenic acid	27.9	107.8	10.9
Coumaric acid	0.7	1.9	0
Quercetin	0.3	2.8	0.5
Phlorizin	54.6	110.8	83
Epicatechin	2.6	41	4.2
Quercitrin	8.3	5.7	0.2
Isoquercitrin	5.5	0.7	0.1
Rutin hydrate	3.1	0.4	0.3
Phloretin	0.04	2.7	0.1
hyperoside	13.1	4.4	0.2
avicularin	12.3	1.5	0.18

Table 4.1. Concentration of polyphenols (µg/mg of extract) after filtration

4.2.2. Cell culture and treatment conditions

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10 % (v/v) CS, 1 % (v/v) antibiotics, 1 % (v/v) Na pyruvate and 1 % (v/v) GlutaMax in 10 cm dish (Corning, Badhoevedorp, NL) at 37°C in a humidified 10 % (v/v in air) CO₂ atmosphere. For the differentiation process, cells were seeded in 12-well plates (Corning) and two-days postconfluent 3T3-L1 preadipocytes, designated as Day 0, were differentiated with DMEM supplemented with 10 % (v/v) FBS, 1 % (v/v) antibiotics, 1 % (v/v) Na pyruvate, 1 % (v/v) GlutaMax and an adipogenic mixture containing 170 nM insulin, 50 µM IBMX and 1 µM DEX for 2 days. Cells were then maintained in 10% FBS-DMEM medium with insulin (170 nM) for another 2 days. Afterwards, the culture medium was replaced every 2 days by 10 % FBS-DMEM medium. Adipocyte differentiation was continued until days 8 or 20 according to the experiments.

Apple extracts or the phenolic compounds were added in the culture medium during the 4 first days of the differentiation process. Extracts were directly solubilized in the culture medium at appropriate concentrations. Phlorizin and phloretin were solubilized in DMSO at 50 mM and added to the culture medium to reach the corresponding concentration found in the extract. Control with vehicle was also realized (DMSO, 0.1%).

4.2.3. Proliferation and cytotoxicity assays

The effect on mitotic clonal expansion was assessed by counting the number of nuclei, at days 0, 2 and 4 of the differentiation process. Nuclei were stained with DAPI (Sigma). The detection is based on DAPI channel assay (SET 31000 DAPI (EX BP 360/40, BS LP 400, EM BP 460/50), which uses a 40x objective, a Nikon DS-Qi1Mc camera in combination with a 10x Nikon ECLIPSE TS100 microscope for image acquisition (Nikon, Brussels, BE). The parameters were optimized and fixed for all experiments (40x objective, gain of 1 x, 100 ms exposure). All images were analyzed with the NIS-Element Software (Nikon). For each well, 3 pictures were taken, with a

random location of sampling. The area covered by the picture was always 0.25 mm² representing a number of cells varying between 45 and 125. The image analysis software automatically counted DAPI-stained nuclei.

The possible cytotoxicity of treatments was routinely checked through the determination of the LDH activity released in the extracellular medium, with a cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, DE) used according to the manufacturer's instructions. The percentage of cytotoxicity was calculated using the maximum released LDH activity obtained upon complete lysis of 3T3-L1 cells in the same stage of culture with 1 % (v/v) Triton X-100.

4.2.4. AdipoRed assay

Preadipocytes (10³ cells) were seeded in 96-well plates. Two days after confluence, preadipocytes differentiation was induced in the presence of various concentrations of apple extracts or the pure phenolic compounds. On day 8, cells were treated with AdipoRed (Lonza) and total lipid content was determined following the manufacturer's instructions. Briefly, 8 days old cultures were washed twice with PBS. Then, 200 µl of PBS were added to each well with 5 µl of AdipoRed reagent. The lipophilic assay reagent specifically distributes into the fat droplets of adipocytes. After 10 minutes, the fluorescence was read (485/535 nm).

4.2.5. Adiponectin assay

The adiponectin secretion in culture medium was assessed by a commercially available sandwich ELISA kit (Millipore, Billerica, MA), according to the protocol instructions. Culture media, at day 8, were harvested and centrifuged. The supernatants were diluted in order to reach measurable concentrations.

4.2.6. GPDH activity assay

Eight days after the induction of differentiation, cells were washed with PBS, and then harvested in ice-cold 20 mM Tris buffer (pH 7.3) containing 1

mM EDTA and 1 mM β -mercaptoethanol. Cell lysates were mixed and maintained on ice until the GPDH activity assay, assessed by measuring, at 340 nm, the oxidation of 125 μ M NADH (Sigma), in the presence of 200 μ M dihydroxyacetone phosphate (DHAP) (Sigma). Results were expressed as nmol/min x well.

4.2.7. Staining procedure

Adipocytes were stained with DAPI (Sigma) and/or AdipoRed. The cells were first washed twice with PBS, then incubated with 4 % (v/v) formaldehyde during 30 min at 37°C. Adipocytes were then permeabilized with a 1 % (v/v) triton-X-100 solution added 3 times during 5 min. The cells were then washed with PBS and DAPI (10 μ g/ml) and 0.2 % (v/v) Adipored was added to 500 μ l of PBS. After 10 min of incubation in the dark at 37°C, PBS was removed and replaced by fresh PBS.

4.2.8. Fluorescence image analysis

The method is based on two channels assay, which uses a 40x objective, a Nikon DS-Qi1Mc camera in combination with a 10x Nikon ECLIPSE TS100 microscope for image acquisition. The parameters were optimized and fixed for all experiments (40x objective, gain of 1 x, 100 ms exposure). Channel 1 corresponds to the DAPI filter (SET 31000 DAPI (EX BP 360/40, BS LP 400, EM BP 460/50) and is the focus channel in which nuclei are identified. Channel 2 corresponds to the FITC filter (SET 31001 FITC (EX BP 480/30, BS LP 505, EM BP 535/40) and detected the fat droplets. All images were analyzed with the NIS-Element Software (Nikon). For each well, 3 pictures have been taken randomly. The area covered by the picture was always of 0.25 mm² representing a number of cells varying between 45 and 125. The relative fluorescence of both DAPI and AdipoRed was measured by the NIS Software. The image analysis software automatically counted DAPI-stained nuclei. The lipid droplets were manually selected in the software, detected and then counted and classified according to their size by the software.

4.2.9. Statistical analysis

All the results were expressed as means $\pm$ SEM. Data were analyzed by one-way ANOVA followed by a Dunnett post-hoc test to identify mean differences between treatment and positive control. P-values less than 0.05 were considered significant. Statistical analyses were performed using the JMP® 10 software (SAS Institute, Cary, NC).

4.3. Results

4.3.1. Effect of apple extracts, PT and PZ on cell viability and mitotic clonal expansion

Cytotoxicity was evaluated by measuring the proportion of the total lactate dehydrogenase (LDH) activity that was released in the culture media in comparison to a positive control (cells treated with 1 % (v/v) Triton X-100) and a negative one (cells cultured in the absence of any particular treatment). No cytotoxic effects of the different treatments were observed after 48 and 96h of exposure (Data not shown). The limit of cytotoxicity has been fixed at 10 % above the control value.

The AE1 concentration used in the following experiments is three fold higher than those of AE2 and AE3 in order to reach the PZ concentration normally found in apple (10 mg/apple). Indeed, AE1 contained 5 % of PZ whereas AE2 and AE3 contained 15 %. The chosen concentrations of PT and PZ are in the range of the concentrations found in the extracts.

In order to determine whether the apple extracts, PT or PZ affected the mitotic clonal expansion that occurs *in vitro* after the induction of the preadipocyte differentiation, the number of nuclei was counted at days 0, 2 and 4. The number of nuclei in the control condition doubled two days after the induction of differentiation. Treatments with PT and PZ did not modify this expansion. However, AE1, AE2 and AE3 prevented partially the cell division (**Fig 4.1**). The number of nuclei after AE1 and AE2 treatments stayed at values significantly lower than the control until day 20. However, since day 6, the treatment with AE3 did not reduced the number of nuclei as compared to the control condition (Data not shown). The loss of nuclei between days 2 and day 4 was associated with a slight increase of the cytotoxicity observed in all the conditions (Data not shown).

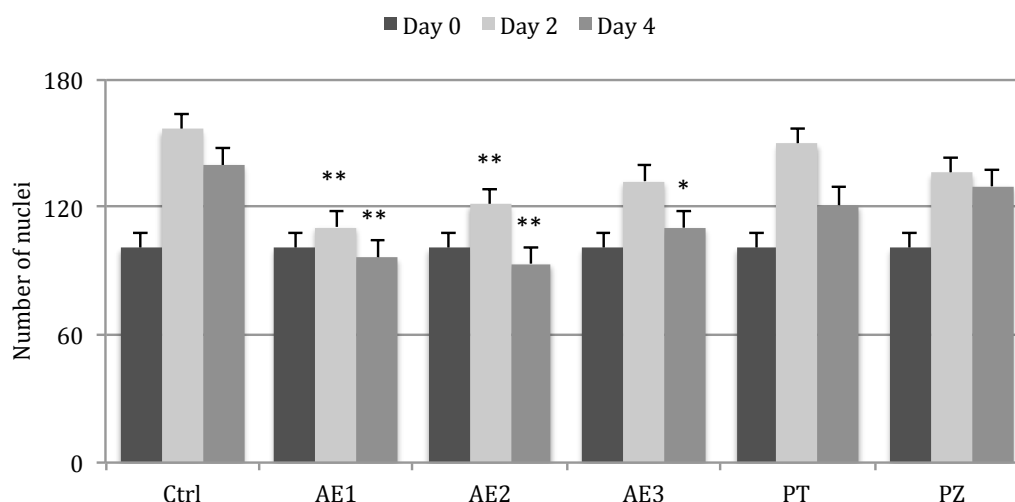


Fig 4.1. Nuclei number counting at days 0, 2 and 4 in 3T3-L1 adipocytes, after induction of differentiation in the presence or absence of AE1 (0.3 mg/ml), AE2 (0.1 mg/ml), AE3 (0.1 mg/ml), PT (5 μ M) or PZ (50 μ M). Counting was performed after DAPI staining followed by image analysis. Means $\pm$ S.E.M, N=4 n=3, are given and compared to the control value in the same conditions (* p <0.05; ** p <0.01).

4.3.2. Effect of apples extracts, PT and PZ on adipogenesis

Eight days after the induction of differentiation in the presence or absence of the extracts or phenolic compounds, the number of differentiated adipocytes were counted and reported to the total cell number (**Fig 4.2**). The percentage of differentiated cells in the control condition reached 95 % whereas AE1 and AE2 reduced this proportion to 68.5 and 80.0 %, respectively. AE3, PT and PZ did not affect the proportion of differentiated adipocytes.

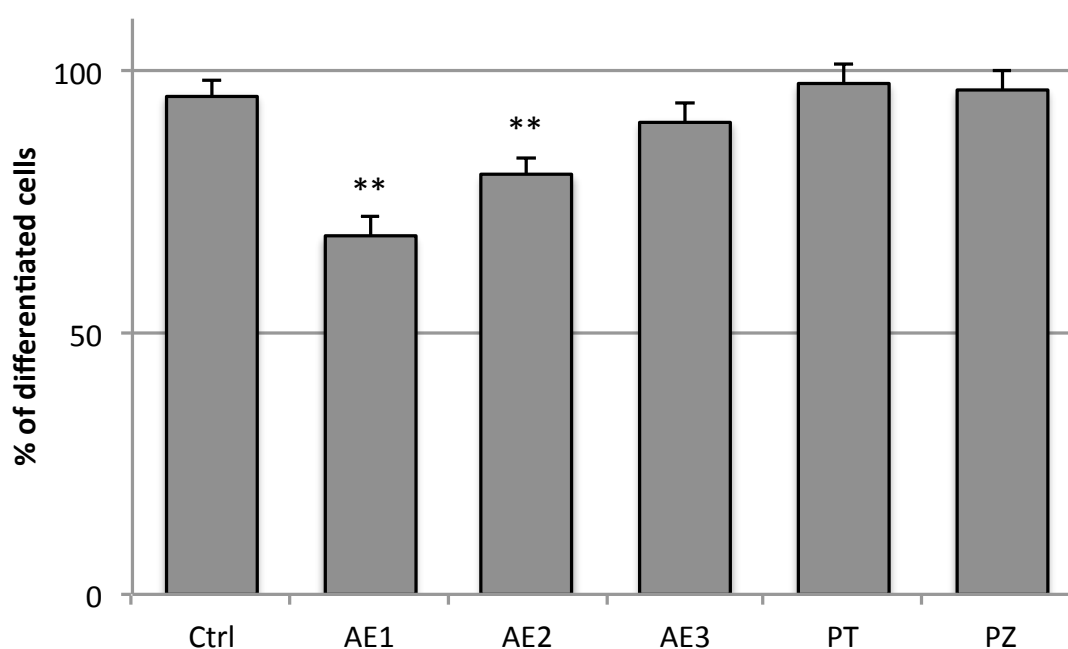


Fig 4.2. Percentage of differentiated adipocytes 8 days after the induction of differentiation of 3T3-L1 cells in the presence or absence of AE1 (0.3 mg/ml), AE2 (0.1 mg/ml), AE3 (0.1 mg/ml), PT (5 μ M) or PZ (50 μ M). Differentiated cells were counted and reported to the total number of cells. Means $\pm$ S.E.M, N=3 n=3, are given and compared to the control value (* p <0.05; ** p <0.01).

The effect of various concentrations of the extracts or the phenolic compounds on the lipid accumulation has been investigated at day 8 (**Fig 4.3**). The AdipoRed assay revealed that, at high concentrations, the three extracts inhibited almost totally the triglyceride accumulation; lower concentrations showed a smaller effect but still significant. PT (5 μ M) increased significantly the lipid content in adipocytes while PZ did not show any effect even at a high concentration.

The GPDH activity showed relatively the same profile that lipid accumulation for AE1 and AE2 but not for AE3 that remained without significant effect on the enzyme activity (**Fig 4.4**). PT and PZ tended to increase the enzyme activity at high concentrations.

Upon treatment with low concentrations of AE2 and AE3, adiponectin secretions were 50 % higher than in control conditions. The same effect was obtained with PT and PZ whatever the concentration. However at high concentrations, AE1, AE2 and AE3 decreased the adiponectin secretion (**Fig 4.5**).

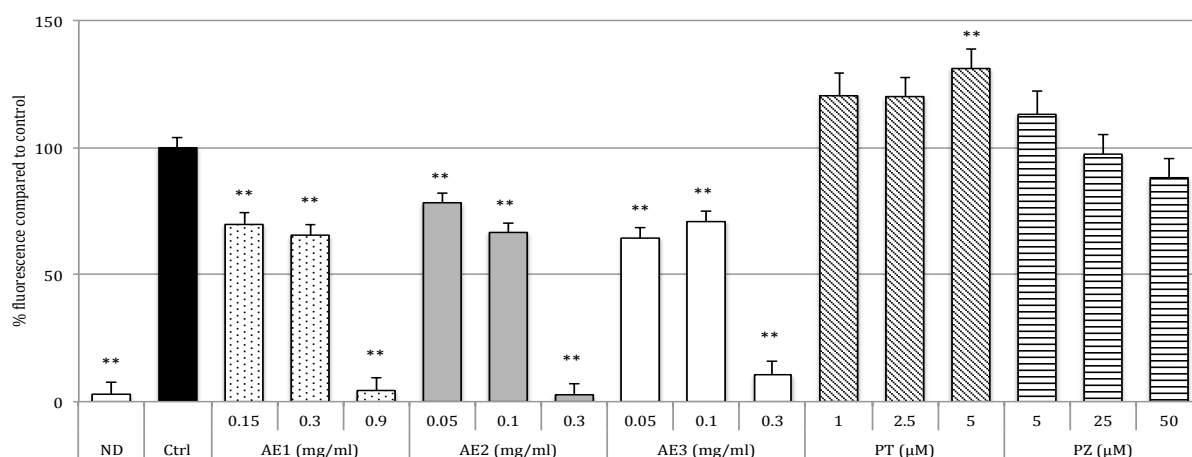


Fig 4.3. Determination of intracellular triglyceride accumulation in 3T3-L1 adipocytes differentiated for 8 days. Cells were treated during the first 4 days of differentiation with varying concentrations of AE1, AE2, AE3, PT or PZ. ND corresponds to the non differentiated cells. AdipoRed quantification was performed as described in Materials and Methods. Means $\pm$ S.E.M, $N=2/3$ $n=4$, are given and compared to the control value (* $p<0.05$; ** $p<0.01$).

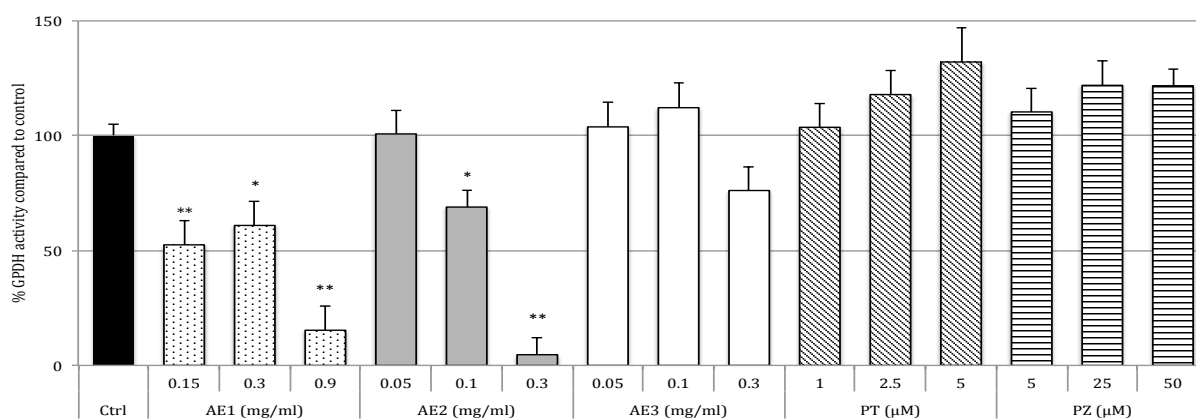


Fig 4.4. Assay of GPDH enzymatic activity in 3T3-L1 cells 8 days after the induction of differentiation in the presence or absence of the different extracts at various concentrations. Means $\pm$ S.E.M, $N=3$ $n=1/2$, are given and compared to the control value (* $p<0.05$; ** $p<0.01$).

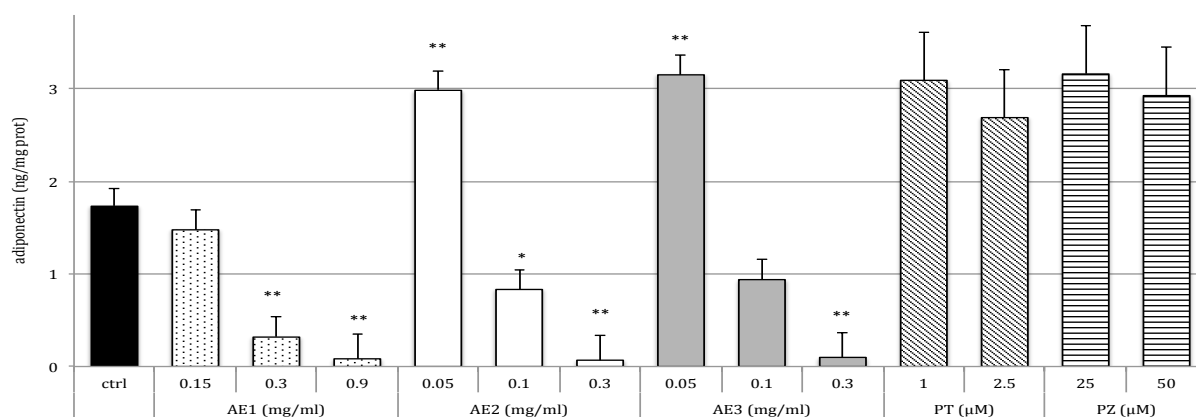


Fig 4.5. Adiponectin secretion by 3T3-L1 cells 8 days after the induction of differentiation in the presence or absence of AE1, AE2, AE3, PT or PZ at various concentrations. Means $\pm$ SEM, N=2/3 n=3, are given and compared to the control value (* p <0.05; ** p <0.01).

4.3.3. Effect of apple extracts, PT and PZ on the lipid accumulation profile

The differentiation of preadipocytes was induced in the presence or absence of AE1 (0.3 mg/ml), AE2 (0.1 mg/ml), AE3 (0.1 mg/ml), PT (5 μ M) and PZ (50 μ M). At day 8, 15 and 20, the lipid accumulation and repartition in adipocytes were followed by fluorescence microscopy analysis, after AdipoRed (lipids) and DAPI (nuclei) staining. The control cells accumulated steadily triglyceride over time (**Fig 4.6**). Adipocytes treated with AE1 and AE2 stored less triglycerides at day 8 as already measured in the AdipoRed assay (**Fig 4.3**), but at day 15 and 20, no significant reduction as compared to control was observed. In contrast, AE3, which induced a slight decrease of lipids accumulation at day 8, increased the lipid content at days 15 and 20. The same increase was observed at day 15 after PT treatment. PZ did not affect the lipid accumulation. If the lipid content is reported to the number of cell, it appeared that AE1, AE2 and AE3 induced a higher lipid content in adipocytes than control one, whereas, PT and PZ treated cells did not present a difference with control cells.

As indicated in **Fig 4.7**, the number of lipid droplets decreased with duration of culture, as the small droplets fused to form bigger ones. The number of droplets did not differ between the control, PT, PZ and AE3, excepted at day 8 for AE3, where the number of droplets was slightly lower. However, AE1 and AE2 decreased the number of droplet as compared to the control at the corresponding day.

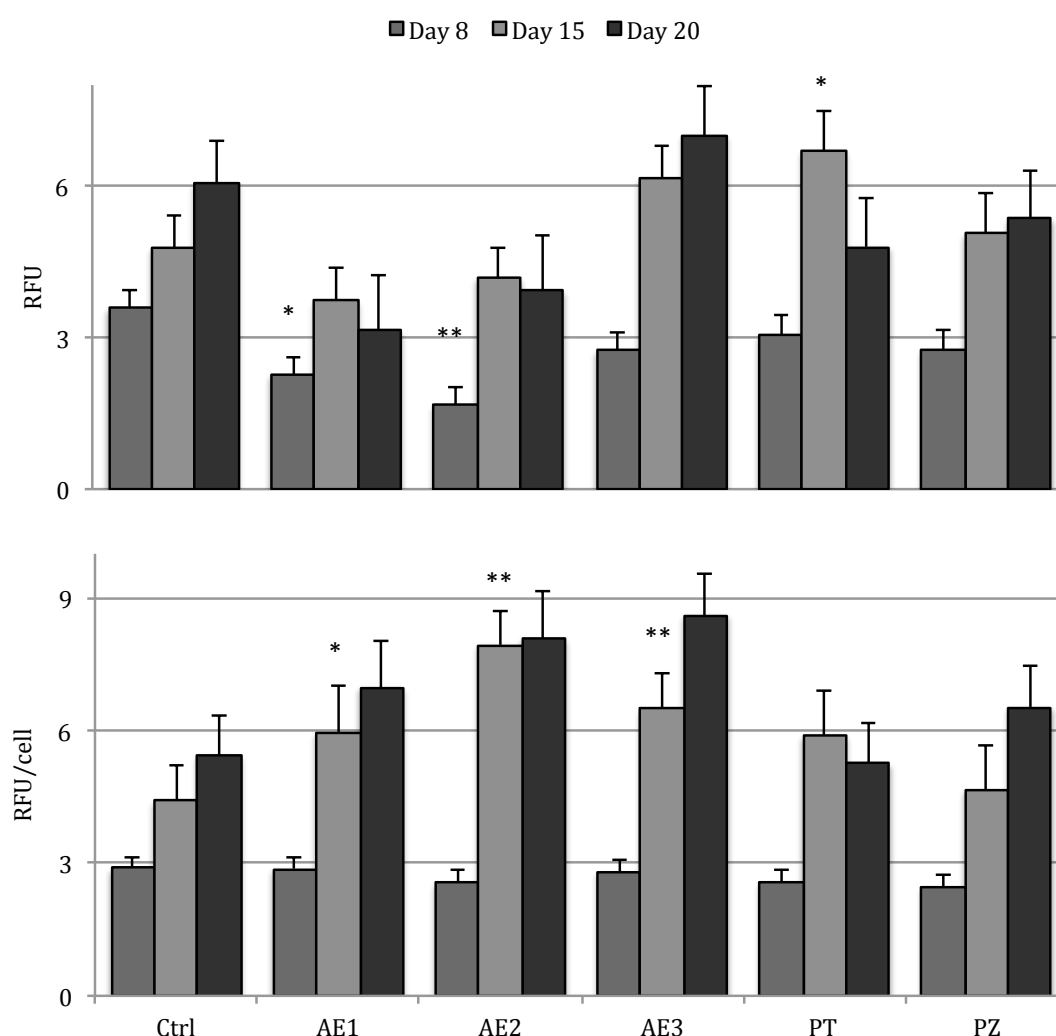


Fig 4.6. Triglyceride accumulation in 3T3-L1 during 20 days after induction of differentiation in the presence or absence of AE1 (0.3 mg/ml), AE2 (0.1 mg/ml), AE3 (0.1 mg/ml), PT (5 μ M) or PZ (50 μ M). After 8, 15 and 20 days, adipocytes were subjected to AdipoRed and DAPI staining, the relative fluorescence of AdipoRed was measured and the number of nuclei counted. A: relative fluorescence units (RFU) per conditions. B: RFU reported to the number of cells corresponding to each day. Means $\pm$ S.E.M, N=3 n=3, are given and compared to the control value for the same day (*p<0.05; **p<0.01).

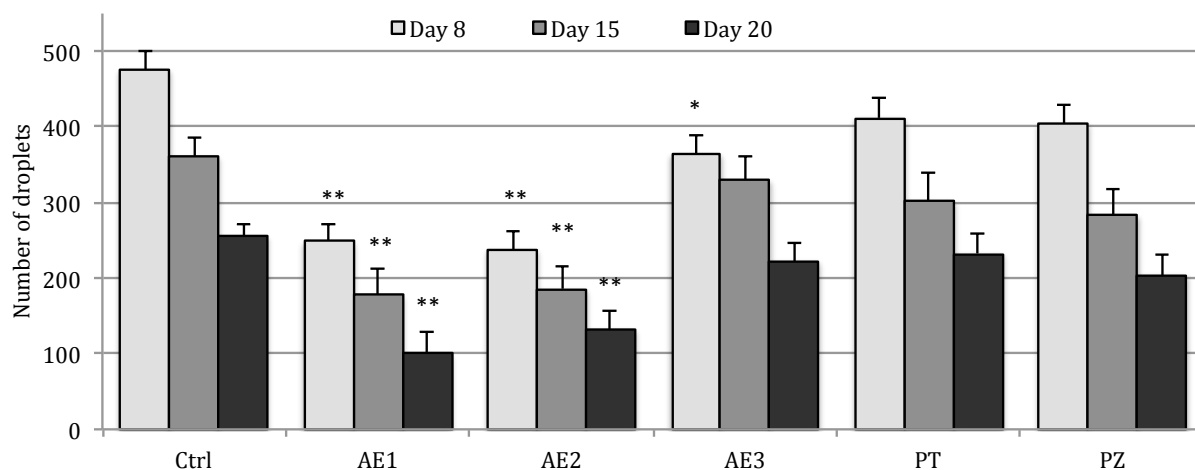


Fig 4.7. Counting of lipid droplets in 0.25 mm² culture area in 3T3-L1 cells after 8, 15 or 20 days of initiation of differentiation in the presence or absence of AE1 (0.3 mg/ml), AE2 (0.1 mg/ml), AE3 (0.1 mg/ml), PT (5 μM) or PZ (50 μM). Counting was performed after AdipoRed staining followed by image analysis. Means ± S.E.M, N=3 n=3, and compared to the control value (**p<0.01).

Regarding to the droplet size repartition of control adipocytes, smaller droplets (< 5 μm) were present in a higher proportion at day 8 (96 %), which decreased over time to reach 52 % at day 20 (**Fig 4.8**). This decrease was accompanied by the appearance of bigger droplets. AE1 and AE2 treatment reduced the droplet growth and the cells presented a higher amount of small droplets than control cells at day 20. AE3, PT and PZ did not affect the droplet size repartition. **Fig 4.9** represents pictures taken at day 20. Control cell, PT and PZ presented relatively the same phenotype whereas the treatments with AE1 and AE2 reduced the number of differentiated cells as well as the number and the size of droplets.

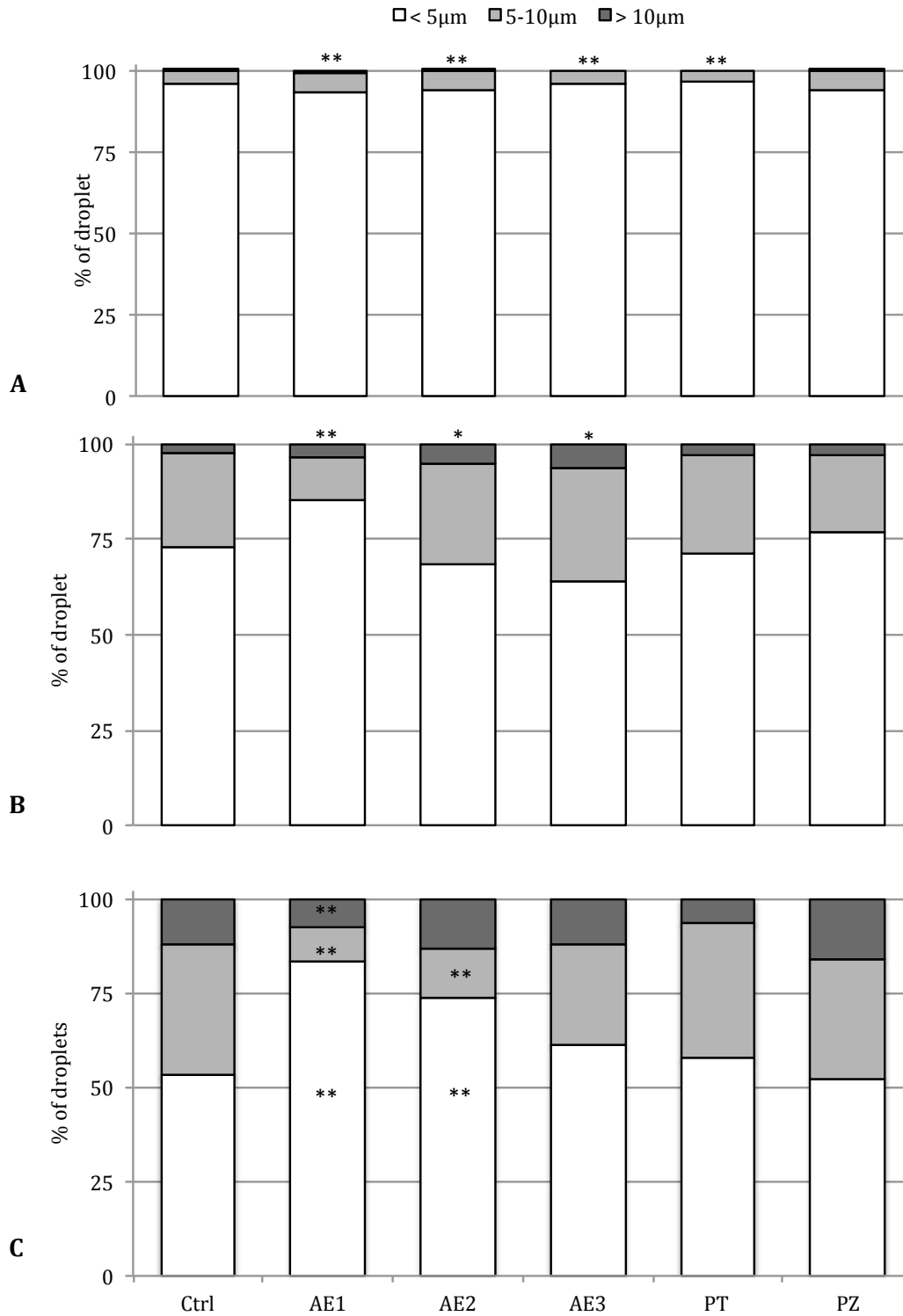


Fig 4.8. Radius of each droplet was measured in 0.25 mm² culture area and classified by size (small: <5 μm, intermediate: 5-10 μm and big: >10 μm) in 3T3-L1 cells after 8 (A), 15 (B) or 20 (C) days of initiation of differentiation in the presence or absence of AE1 (0.3 mg/ml), AE2 (0.1 mg/ml), AE3 (0.1 mg/ml), PT (5 μM) or PZ (50 μM). Counting was performed after AdipoRed staining followed by image analysis. Means ± S.E.M, N=3 n=3, are given and compared to the control value (*p<0.05; **p<0.01).

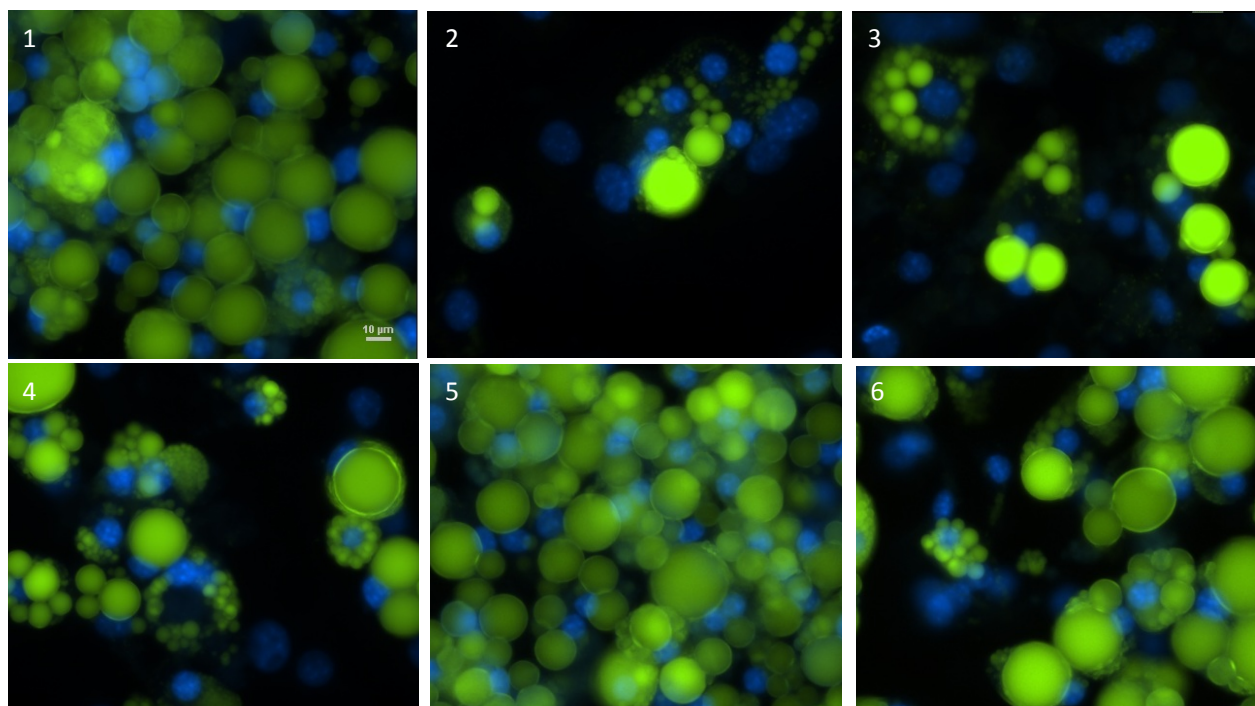


Fig 4.9. 3T3-L1 cells 20 days after the induction of differentiation in the presence or absence of the vegetal extracts. Cells were stained with DAPI and AdipoRed. All the pictures have been taken at the same magnification (400x) with scale representing 10 µm. Picture 1: control cells; 2: cells treated with AE1 (0.3 mg/ml); 3: AE2 (0.1 mg/ml); 4: AE3 (0.1 mg/ml) 5: PT (5 µM) and 6: PZ (50 µM).

4.4. Discussion

Apples are very common fruits, for example, in France, they represent each year 20 % of the total fruit sale. Among the numerous health benefits of apples, the prevention of obesity, through a regular consumption, begins to be investigated. A recent study has revealed that Wistar rats fed with a “high fat-sucrose” (HFS) diet supplemented with an apple extract present, after 56 days treatment, a lower weight gain, lower total white adipose tissue weight and lower insulin and glucose blood level, as compared to the control group fed only with the HFS diet²¹. Previously, other impacts of apple consumption on body weight and obesity complications have been studied but few *in vitro* studies have highlighted the effect of apple extracts on adipocytes differentiation.

In this work, we showed that apple extracts could impact adipogenesis and lipid accumulation, which gave a first clue to explain the beneficial effect of apple on obesity development.

In our experimental model, at day 8, cells treated with AE1 and AE2 showed a reduced activity of the GPDH activity (**Fig 4.3**). As the GPDH is an enzyme that allows adipocyte to synthesize triglycerides, it is not surprising to observe that the amount of fat stored in the cell was reduced (**Fig 4.4**). Shoji *et al.* (2000) have already shown that crude apple polyphenols decreased the GPDH activity and the lipid content in 3T3-L1 cells¹⁰.

However, as expected, PT, even at a low concentration (1 μM), increased the amount of fat stored. This is correlated with a slight increase of the GPDH activity. This is not the only report of the proadipogenic effect of this polyphenols. Hassan *et al.* (2010) have revealed that PT (50 μM) almost doubled the fat content and the GPDH activity of 3T3-L1 adipocytes. As in our work, they showed that PT increased the secretion of adiponectin, which is correlated with an increase of the gene expression. They proved that PT up-regulated the expression of PPAR γ and C/EBP α , the two master adipogenic transcription factors²⁰. Low level of adiponectin induces insulin resistance, and therefore increasing its secretion should improve the insulin sensitivity and other metabolic complications²².

Interestingly, AE2 and AE3, at low concentrations (0.05 mg/ml), decreased slightly the lipid accumulation but increased the adiponectin secretion whereas at higher concentrations they reduced the secretion of adiponectin (**Fig 4.5**). These surprising results may be explained by the complexity of these extracts. They could contain compounds that at high concentrations would inhibit adipogenesis and thus adiponectin secretion, whereas at lower concentrations other potent compounds would stimulate the adipogenesis resulting in high adiponectin secretion. For instance, such compounds could be PT, which at a low concentration of 1.25 μM already increased adiponectin secretion. Indeed in 0.05 mg/ml of AE2, PT was found at 0.5 μM . AE2 also contained catechin and epicatechin that had already shown an increase effect of adiponectin secretion and expression in 3T3-L1 adipocytes. At 5 μM , catechin increased the secretion by 20 %²³. In 0.5 mg/ml of AE2, catechin concentration reached 4 μM (1 μM in AE3 and AE1). The action of this polyphenol, in addition to the plausible effect of PT, could explain the effect of AE2 on adipogenesis and adiponectin secretion. The AE3 was the extract presenting the lowest amount of polyphenols. PZ was the most represented phenolic compound in AE3, with 81 % of the phenolic content. At 0.1 mg/ml of AE3, the concentration of PZ was 20 μM whereas other phenolic compounds reached a maximum of 0.14 μM . So we can postulate that the majority of the effect of AE3 could be attributed to PZ. Indeed, the effect of AE3 was close to the effect of PZ. However, the inhibition of the secretion of adiponectin by AE3 remains to be investigated. The reason why AE1 inhibited adipogenesis more efficiently than AE2 and AE3 could be the presence of high amount (30 %) of quercetin and derivative glycosylated forms in this extract (compared to 3 % and 0.9 % in AE2 and AE3, respectively). At 0.15 mg/ml of AE1, the concentration of quercetin and its glycosylated forms reached 16 μM (0.2 μM and 0.02 μM for AE2 and AE3, respectively, at 0.05mg/ml). It was already shown that quercetin (10 μM) reduced lipid accumulation and adipogenesis in 3T3-L1

and that at the same concentration, one of its glycosylated form is more efficient²⁴.

At 0.1 mg/ml AE2, as at 0.3 mg/ml for AE1, the mitotic clonal expansion was partially blocked resulting in a decrease of cell count as compared to the control. In addition to this reduction of the generation of potentially new adipocytes, AE1 and AE2 also decreased the proportion of differentiated cells to 68.5 and 80 % of the control condition (**Fig 4.2**). At day 20, in 0,25 mm², the number of differentiated adipocytes in the control condition was of 72 ± 6 cells, to be compared to 16 ± 2 and 38 ± 4 for AE1 and AE2, respectively (Data not shown). Nevertheless, it seems that reducing the number of adipocytes does not appear as a good strategy to improve insulin sensitivity⁶. Indeed, the amount of triglycerides stored in the cells treated with AE2, but surprisingly not with AE1, is higher than that found in control adipocytes. It has been proved that adipocytes containing high amounts of fat secreted less adiponectin but high levels of pro-inflammatory molecules contributing to the metabolic disorders linked to obesity⁵.

However, at day 20, adipocytes treated with AE2 had more small lipid droplets than control adipocytes (**Fig 4.8**). This phenotype promotes lipid exchanges; lipolysis as well as lipogenesis⁷. This difference was accentuated after the treatment with AE1. This extract decreased the total fat content in adipocytes but the differentiated cells presented the same amount of triglyceride stored in small droplets instead of big ones.

Antidiabetic drugs from the TZD family increase the adipogenesis²⁵ in addition to increasing the number of small droplets within adipocytes. At day 20, the treatment with PT (5 μ M) induced an increase of the number of differentiated adipocytes of 25 % as compared to the control (in 0,25 mm²; data not shown). This observation added to the effect on adiponectin secretion made of PT a potential candidate to prevent obesity complications since it seemed to act like the TZD drugs. However, more investigations about its effects on gene expressions have to be done.

However, less is known about the bioavailability of PT. This polyphenol is found almost exclusively in the peel of apples in various glycosylated forms. Compared to others apple phenolic compounds (quercetin, anthocyanin...), PT glycosides are present in very low quantities²⁶. Kahle *et al.* (2011) have followed the fate of apple polyphenols in a model of *in vitro* digestion and their systemic metabolism of apple polyphenols in human after 1L apple juice consumption²⁷. PT glycosides (among them PZ) were partially hydrolyzed by saliva, not affected by the gastric conditions, but hydrolyzed by duodenal digestion while the aglycone form *i.e.* PT remained unaffected. Hepatocytes were also able to conjugate PT to two glucuronides via phase II metabolism. Two hours after apple juice consumption, the concentration of PT and derivatives in human serum reached 0.17 μM whose the majority was phloretin-2'-*O*-glucoside (PZ) and $\pm 10\%$ was in the free form. After 24h, only 0.6 % of the ingested dose was measurable in urine. The apple juice used for this study did not contain detectable amount of free PT, the dihydrochalcone being present in glucoside forms, which were hydrolyzed during the digestion. With PT in its free form, higher plasmatic concentrations could be reached. Indeed, Crespy *et al.* (2001) have shown that 10h after native PT ingestion (22 mg), the free form of PT is found in the plasma of rats at concentration reaching 10 μM ¹⁹. However in this study, animals were fed with native PT in high amount, which is far from a real consumption.

According to these studies, the concentrations of PZ and PT that could be found in the plasma seems to depend on the form of ingestion (juice, extract, pure polyphenols). Moreover, as the PT plasmatic concentration stays very low, its detection is compromised. The low concentrations of PT, *i.e.* 1, 2.5 and 5 μM , used in this work, were in a plausible range regarding to the literature state. The PZ concentrations have already been detected in the plasma and could be reached after the ingestion of apple derivatives¹⁹.

This study has shown that depending of the cultivar, different effects were observed on 3T3-L1 adipocytes functionalities. AE1 and AE2 by reducing drastically the number of differentiated adipocytes could worsen the metabolic complications linked to obesity. However, the AE3 by increasing the adiponectin secretion at low concentration and in increasing the fat content in adipocytes may contribute to the improvement of insulin sensitivity. PT, as already shown, increased adipogenesis. Moreover, in this study, we showed that this effect was already observed at very low concentrations, more close to the plausible plasmatic concentrations that could be reached after a “normal” apple consumption.

4.5. References

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5. A coculture of adipocytes and macrophages as an *in vitro* model to simulate the inflamed adipose tissue

The two previous chapters have focused on the potential effect of vegetal extracts on the differentiation and the filling of adipocytes. An other important fact impacting on the obesity linked metabolic complications is the low grade inflammation state characteristic of the obese adipose tissue. This inflammatory state is mainly induced and maintained by adipocytes and macrophages that constitute the adipose tissue. The inflammation could spread in all the body and induce metabolic disorder such as type 2 diabetes, cardiovascular complications...

This chapter will present the adipose tissue model that we have developed in order to simulate the inflamed obese adipose tissue. This *in vitro* model was composed of two murine cell lines; the 3T3-L1 adipocytes and the RAW 264.7 macrophages.

The coculture model established has allowed us to study the potential anti-inflammatory effects of the vegetal extracts always in the aim to identify potential natural candidates that could prevent obesity development and complications

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5.1. Introduction

Adipose tissue of obese people is known to secrete pro-inflammatory molecules, such as TNF α and IL-6 that contribute to the development of obesity linked metabolic complications¹. The main cells responsible for these secretions are adipocytes and macrophages. In lean individuals, macrophages are present in the adipose tissue, but do not secrete inflammatory molecules. The macrophages assume their role in the repair and the remodelling of tissues². In case of weight gain, the secretions of adipocytes are upset and these storage cells start to secrete CCL-2, a chemokine, which attracts macrophages inside the adipose tissue. Hypoxia is also involved in macrophages recruitment³. Once in the adipose tissue, macrophages are activated by free fatty acids (FFAs) and CCL-2, released by adipocytes, and acquire a pro-inflammatory profile. The two cell types maintain a vicious cycle and stimulate each other to secrete pro-inflammatory signals. Adipocytes secrete more leptin and FFAs, which promote TNF α secretion by macrophages. TNF α , in turns, acts on adipocytes by stimulating the lipolysis, decreasing their adiponectin secretion and increasing their CCL-2 and IL-6 secretion, contributing to the inflammatory state aggravation.

In order to reproduce the inflammatory conditions observed in the adipose tissue of obese people, we developed an *in vitro* system that involved differentiated adipocytes and activated macrophages. We selected two cell lines already used in coculture for numerous studies, *i.e.* 3T3-L1 preadipocytes and RAW264.7 macrophages^{4,5,6}. The murine RAW264.7 line was established from a tumor induced by Abelson murine leukemia virus. It is known that the macrophages could be activated by a bacterial lipopolysaccharide (LPS)⁷. This stimulation induces the expression of inflammatory genes and the activated macrophages start to produce NO and TNF α ⁸, which corresponds to the activated M1 profile mainly represented in the obese adipose tissue.

Based on the model of Takayoshi Suganami *et al.* (2005), we have chosen to work with a bicameral coculture system. In this system, the two cell lines do not establish close contact (**Fig 5.1**). The interactions that occur are only due to secreted factors from both cell types. This allowed us to study only the effect of soluble factors and to harvest the cell separately in order to identify the cell type responsible for cytokines/chemokines secretions.

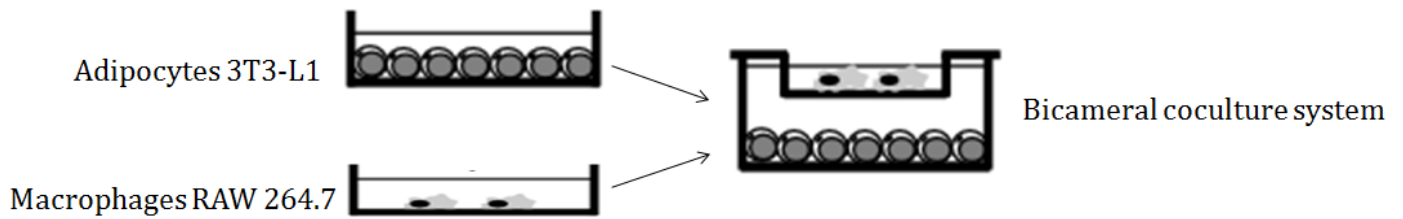


Fig 5.1. Bicameral coculture system. Differentiated adipocytes (day 8) are cocultivated with macrophages seeded on a porous membrane placed above them. Adapted from Suganami *et al* (2005).

5.2. Materials and methods

5.2.1. Materials and reagents

Triton X-100, LPS, dexamethasone (DEX), 3-isobutyl-1-methyl-xanthine (IBMX) and insulin were purchased from Sigma-Aldrich. (St Louis, MO); Dubelcco's Modified Eagle's Medium (DMEM), calf serum (CS), GlutaMax (100x), sodium pyruvate (100 mM), penicillin/streptomycin (100-100 µg/ml penicillin/streptomycin) and 0.05 % trypsin-0.02 % EDTA from Lonza (Verviers, BE) and fetal bovine serum(FBS) from PAA (Linz, AT).

5.2.2. Cell culture

RAW 267.4 murine macrophages (American Type Culture Collection, Manassas, VA) were subcultured in 25 cm² flasks (Corning, Badhoevedorp, NL) in DMEM containing 10 % FBS, 1 % (v/v) antibiotics, 1 % (v/v) Na pyruvate and 1 % (v/v) GlutaMax.

The activation tests were performed in 96 wells plate (Corning). Macrophages were seeded at appropriate concentrations and 24h later the medium was replaced by 10 % FBS-DMEM medium containing or not LPS. After 24h of incubation, media were harvested and stored at -20°C until ELISA and NO assays.

The day before the coculture, macrophages were activated by 10 % FBS-DMEM medium containing 1 µg/ml LPS. As control, the medium was replaced by fresh 10 % FBS-DMEM medium. After the 24h of incubation, macrophages were harvested and resuspended in 10 % of FBS medium for the coculture.

3T3-L1 preadipocytes (passage 14 to 18) (American Type Culture Collection, Manassas, VA) were subcultured in 10 cm Petri dish (Corning) in DMEM containing 10 % (v/v) CS, 1 % (v/v) antibiotics, 1 % (v/v) Na pyruvate and 1 % (v/v) GlutaMax. 70 % confluent cells were trypsinized and cultured in 12-wells culture plates for coculture assay (Corning). Two-days postconfluent 3T3-L1 preadipocytes, designated as "Day 0", were differentiated with DMEM supplemented with 10 % (v/v) FBS, 1 % (v/v)

antibiotics, 1 % (v/v) Na pyruvate and 1 % (v/v) GlutaMax, and an adipogenic mixture containing 170 nM insulin, 50 μ M IBMX and 1 μ M DEX for 2 days. Cells were then maintained in the same 10 % FBS-DMEM medium with insulin (170 nM) for another 2 days. Afterwards, the culture medium was replaced every 2 days by 10 % FBS-DMEM medium for four more days. Eight days after differentiation induction, fully differentiated 3T3-L1 adipocytes having accumulated large lipid droplets, were used for coculture experiments (day 8). The both cell types were cultured at 37°C under a humidified 10 % CO₂ atmosphere.

The cocultures were performed by plating RAW 264.7 macrophages (activated or not) in appropriate concentrations onto 0.4 μ m pore size bicameral inserts (Corning) and placing them in 12 wells plate containing the 8 days differentiated 3T3-L1 adipocytes for 24h.

As control, macrophages (activated or not) were also seeded in 12 wells plate in 10 % FBS-DMEM medium for 24h. Fully differentiated adipocytes were cultivated alone and in the presence of LPS (1 μ g/ml) for 24h.

5.2.3. Cytotoxicity assay

The possible cytotoxicity of treatments was routinely checked through the determination of the activity of the lactate dehydrogenase (LDH) released by the cells into the extracellular medium with a cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, DE) used according the manufacturer's instructions. The percentage of cytotoxicity was calculated using the maximum release of LDH activity obtained upon complete lysis of the 3T3-L1 differentiated adipocytes or RAW 264.7 macrophages incubated independently with 1 % (v/v) Triton X-100. For the coculture, the 100 % of LDH released corresponded to the sum of maximum LDH activity of 3T3-L1 and RAW cells, cultivated independently.

5.2.4. Assay of IL-6, CCL-2 and TNF α production

The concentrations of IL-6, CCL-2 and TNF α in the supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using mouse

IL-6, CCL-2 or TNF α kits (eBioscience, San Diego, CA) according to the manufacturer's protocol. The supernatants were diluted in order to reach measurable concentrations.

5.2.5. Adiponectin assay

The adiponectin secretion in culture medium was assessed by a commercially available sandwich ELISA kit (Millipore, Billerica, MA), according to the protocol instructions. Culture media, at day 8, were harvested and centrifuged. The supernatants were diluted in order to reach measurable concentrations.

5.2.6. Assay of nitric oxide release

Total nitrite concentrations in culture supernatants were determined using Cayman's nitrate/nitrite colorimetric assay kit (Cayman, Ann Arbor, MI) based on the Griess reaction. The Griess reagents convert nitrite into red-violet coloured water soluble azo compounds. The product absorbance was read at 540 nm and the total amount was calculated from the NaNO₂ standard curve.

5.2.7. Protein assay

After the 24h of mono- or coculture, cells were washed twice and harvested in lysis buffer. After one freezing/thawing, total protein content was measured using the BiCinchoninic Acid (BCA) Protein assay (Sigma) according to the manufacturer's protocol. The standard curve was realized with a standard solution of bovine serum albumin (BSA) (Sigma).

5.2.8. Staining procedure and adipocyte counting

3T3-L1 adipocytes were stained with DAPI (Sigma). The cells were first washed twice with PBS, then incubated with 4 % (v/v) formaldehyde during 30 min at 37°C and then permeabilized with a 1 % (v/v) triton-X-100 solution, added 3 times during 5 min. The cells were then washed with

PBS and DAPI (10 µg/ml) was added to 500 µl of PBS. After 10 min of incubation in the dark at 37°C, PBS was removed and replaced by fresh PBS. Then, microscopic pictures were taken. The detection is based on DAPI channel assay (SET 31000 DAPI (EX BP 360/40, BS LP 400, EM BP 460/50), which uses a 40x objective, a Nikon DS-Qi1Mc camera in combination with a 10x Nikon ECLIPSE TS100 microscope for image acquisition (Nikon, Brussels, BE). The parameters were optimized and fixed for all experiments (40x objective, gain of 1x, 100 ms exposure). Pictures were analyzed with the NIS-Element Software (Nikon). Three pictures were taken, with a random location of sampling. The area covered by the picture was always 0.25 mm² representing a number of cells varying between 115 and 130. The image analysis software automatically counted DAPI-stained nuclei.

5.2.9. Gene expression study

Total RNA was extracted from 3T3-L1 and RAW 264.7 cells using QIAzol reagent (Qiagen, Hilden, DE) according to the manufacturer's instructions. Quality and quantity of total RNA was assessed by Nanodrop spectrophotometry. Total RNA (1 µg) was reverse-transcribed using the Superscript III First-Strand Synthesis System (Invitrogen, Merelbeke, BE) following manufacturer's instructions. Then, qRT-PCR was performed in 96 well PCR plates (Bio-Rad, Nazareth Eke, BE), to estimate the mRNA abundance corresponding to either IL-6, CCL-2, iNOS, TNFα and adiponectin relatively to the housekeeping genes, *i.e.* β-actin, GAPDH and HPRT, used for normalization. A total of 5 µl cDNA with the appropriate dilution, 0.2 µl primer mix and 4.8 µl SYBRGreen SuperMix (Bio-Rad) were added to each well. The PCR amplifications were performed in a MyIQ2 thermocycler (Bio-Rad) as follows: 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. Melting curves were systematically assessed for quality control of the primers. Gene expression values were normalized to the geometric means of the expression values of β actin, GAPDH and HPRT and calculated on the bases of the comparative

cycle threshold (Ct) method ($2^{-\Delta\Delta C_t}$). The primers used were reported in **Table 5.1**.

Gene name	Forward primer	Reverse primer
β -actin	TAGACTTCGAGCAGGAGATGG	GGATTCCATACCCAAGAAGGA
GAPDH	GTGGAGATTGTTGCCATCAACG	CAGTGGATGCAGGGATGATGTTCTG
HPRT	ACATTGTGGCCCTCTGTGTG	TTATGTCCCCCGTTGACTGA
IL-6	ACTTCCATCCAGTTGCCCTTCT	GAATTGCCATTGCACAACCTCT
CCL-2	GTTGACCCGTAAATCTGAAGC	AAGGCATCACAGTCCGAGTCA
TNF α	TCCCCAAAGGGATGAGAAGTTC	TCATACCAGGGTTTGAGCTCAG
iNOS	ACACTCAGATCATCTTCTCAAAATTCTG	GTGTGGGTGAGGAGCACGTAGT
Adiponectin	GGAGAGAAGGGAGAGAAAGGTC	AACAGTGACGCGGGTCTCC

Table 5.1. Sequences of primers

5.2.10. Statistical analysis

All the results were expressed as means $\pm$ SEM. Data were analyzed by one-way ANOVA followed by Student post-hoc test to identify mean differences between all the treatments. Dunnett post-hoc test was performed to identify differences between treatments and positive control. P-values less than 0.05 were considered significant. Statistical analyses were performed using the JMP® 10 software (SAS Institute, Cary, NC).

5.3. Results

5.3.1. Macrophage activation

To verify whether RAW 264.7 macrophages could be activated and display a M1 profile, the cells were cultivated for different durations *i.e.* 0.5, 1, 2, 3, 4, 6 or 24h in the presence of LPS (1 $\mu\text{g/ml}$). After 24h, macrophages secreted significantly more NO than non LPS-stimulated macrophages (Data not shown). To determine the concentration of LPS that allows a good activation, 2×10^5 RAW 264.7 cells were treated with increasing concentrations of LPS ranging from 0.5 to 10 $\mu\text{g/ml}$ and two activation markers were followed, *i.e.* NO and TNF α (**Fig 5.2A**). All the results were expressed by mg of protein to reflect the total amount of cells. From the concentration of 1 $\mu\text{g/ml}$, the secretions of NO and TNF α reached a plateau. We fixed this concentration as the most effective and investigated whether the number of cells influences the extent of cell secretion (**Fig 5.2B**). NO release increased with the number of cell in contrast to that of TNF α that was not correlated to the number of cells since, when expressed in ng/mg cell protein, this secretion was constant whatever the amount of cells. None of these conditions affected the viability of RAW 264.7 cells as evaluated by LDH activity released in the culture medium (Data not shown).

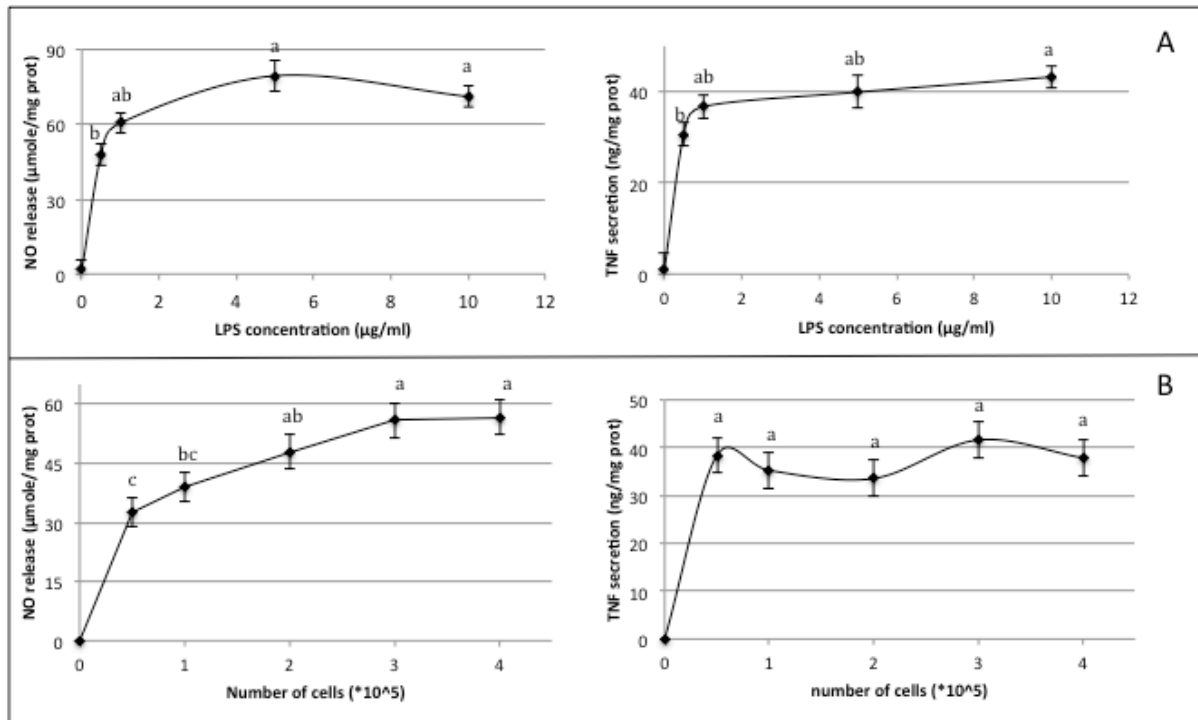


Fig 5.2. Activation of RAW264.7 macrophages after 24h. (A) Increasing concentrations of LPS were tested on RAW 264.7 cells (10^5 cells/well in 96 wells plate). NO and TNF α assays were performed on culture medium. (B) Effect of 1 μ g/ml of LPS after 24h on various numbers of RAW 264.7 cells. Means $\pm$ S.E.M, N=2 n=3 are given and compared to each other. Posts not sharing the same letter are significantly different ($p < 0.05$).

5.3.2. Secretion of pro-inflammatory molecules and adiponectin in coculture

We next investigated whether activated macrophages allowed to induce inflammation in the coculture system. First, adipocytes were cultivated during 8 days to acquire a fully differentiated adipocyte phenotype. The number of adipocytes per well was estimated by counting the nuclei stained with DAPI with the NIS software. The number of adipocytes in the 0,25 mm² area taken in picture was of 123.5 ± 7.4 cells. It was evaluated that in one well (3.8 cm² of surface) the number of adipocytes was $187\,000 \pm 700$. In order to respect the proportion of macrophage/adipocytes found *in vivo* ($\pm 60\%$), we chose to seed 2×10^5 macrophages per insert.

One day before the coculture, macrophages were activated with LPS (1 μ g/ml) in order to exhibit the inflamed profile before starting the coculture. At day 8, activated or non-activated macrophages were seeded in the upper

compartment of bicameral inserts above differentiated adipocytes in the lower one. As control, macrophages and adipocytes were also cultivated in monocultures. The culture media were harvested 24h after the beginning of the cocultures or the control monocultures.

Fig 5.3 presents the CCL-2, IL-6, TNF α and adiponectin secretions measured by ELISA. The secretions of macrophages and adipocytes cultivated alone were summed (Sum) and are compared to the coculture secretion (Coculture).

IL-6 and CCL-2 secretions in coculture are significantly higher when macrophages have been previously activated (Inflamed), as compared with the coculture with non-activated macrophages (Non inflamed).

Moreover, the significant differences between the sum and the coculture in inflamed conditions reveal that both types of cells were interacting. The adiponectin is only secreted by the adipocytes, its secretion in coculture was lower compared to the secretion of adipocytes cultivated alone. In the non inflamed condition, this difference appeared significant. Secretion of TNF α was not modified by the inflammatory or coculture conditions.

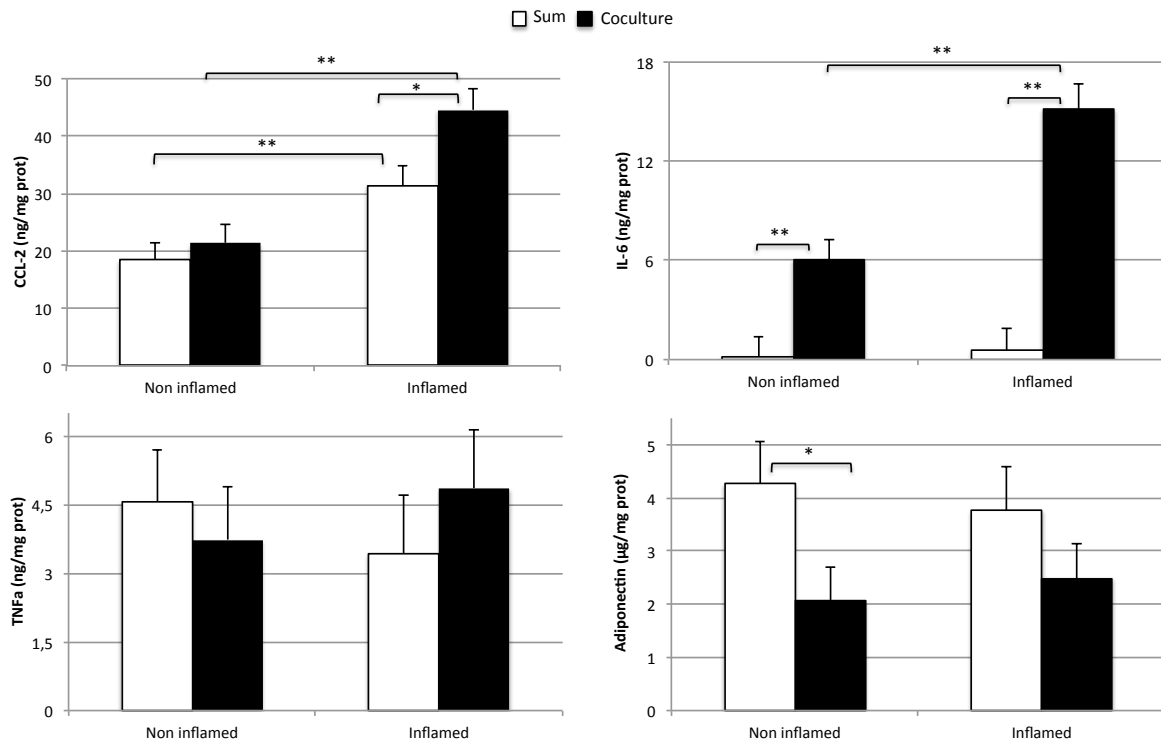


Fig 5.3. Effect of coculture on CCL-2, IL-6, TNF α and adiponectin secretions. The sum represents the addition of the secretion of adipocytes and that of macrophages, inflamed or not, upon separate cultivation. The inflamed conditions corresponding to macrophages preactivated with LPS during 24h before either the coculture or monoculture. Means $\pm$ S.E.M, N=3 n=3, are given and compared to each other (*p<0.05; **p<0.01).

5.3.3. Secretion of cytokine/chemokine by the two cell lines in inflamed conditions

In order to identify the main producer of IL-6 and CCL-2, macrophages and adipocytes were activated with LPS. Adipocytes as well as macrophages display membrane receptors able to recognize LPS, the Toll like receptor (TLR) 4 being responsible for the induction of the inflammatory induction via the NF- κ B pathway. Non-inflamed adipocytes and macrophages secreted very low quantities of inflammatory molecules. After the LPS incubation, the differentiated 3T3-L1 cells and macrophages increased their pro-inflammatory secretions (**Fig 5.4**). IL-6 and CCL-2 were more produced by the adipocytes than by macrophages, which could indicate that the main producers of these molecules in the coculture system are the adipocytes.

TNF α and adiponectin are only produced by macrophages and adipocytes, respectively.

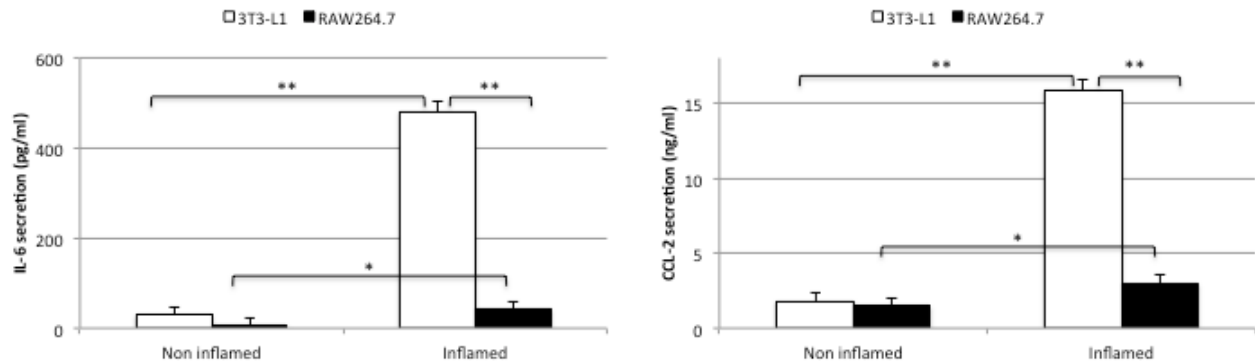


Fig 5.4. Secretion of adipocytes (3T3-L1) and macrophages (RAW264.7) cultivated alone. The inflamed cells were pretreated with LPS during 24h before the 24h of incubation with 10% FBS-DMEM medium. 3T3-L1 do not secrete TNF α . The secretions of TNF α by macrophages are the «sum» in Fig 5.2. The macrophages do not secrete adiponectin. The secretions of adiponectin by adipocytes are the «sum» in Fig 5.3. Means $\pm$ S.E.M, N=3 n=3 are given and compared to each other (*p<0.05; **p<0.01).

5.3.4. Effect of LPS-activation and coculture on gene expressions

To confirm the effect of coculture on the induction of inflammation, the transcription of pro-inflammatory and adiponectin genes was measured in adipocytes and macrophages. The effect of LPS on the same gene expressions was also investigated.

In adipocytes, all of the pro-inflammatory genes followed were overexpressed by the LPS treatment or the coculture. The adiponectin expression was decreased by the coculture but not by the LPS treatment (Fig 5.5).

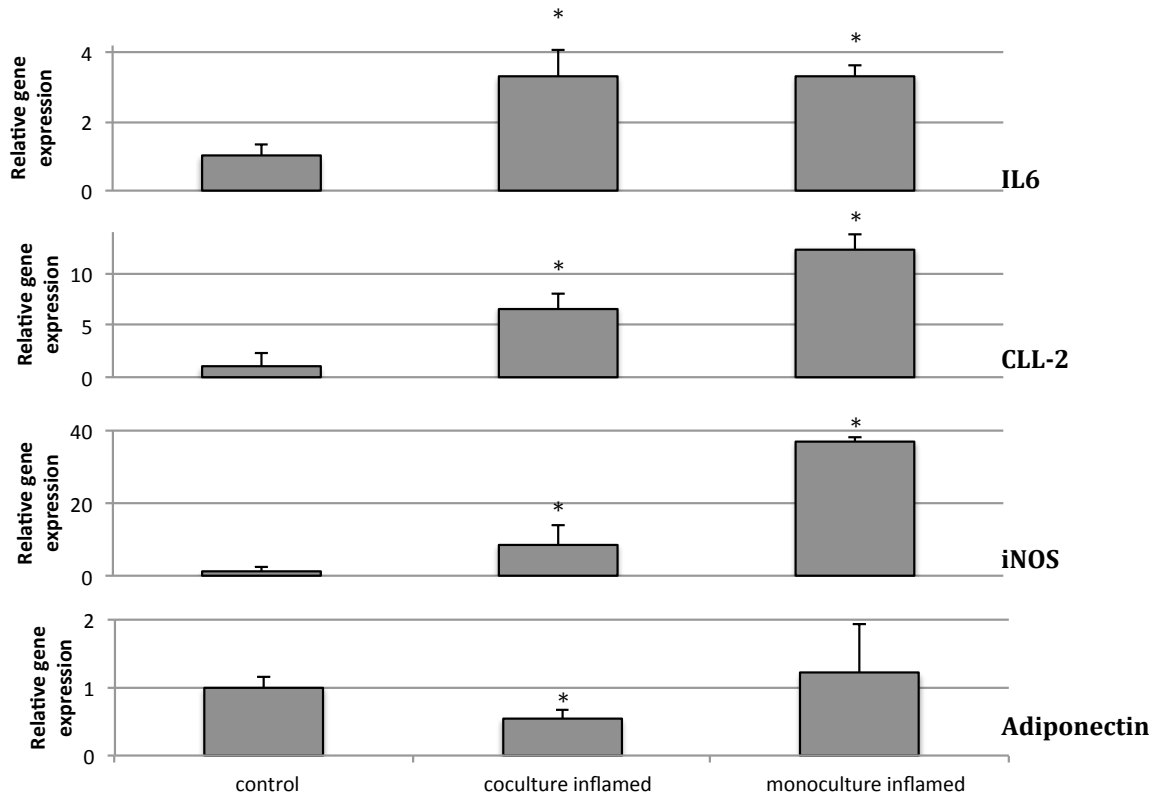


Fig 5.5. Pro-inflammatory gene expressions were analysed by real-time PCR using mRNA extracted from 3T3-L1 fully differentiated after 24h of culture. β -actin, GAPDH and HPRT were used as housekeeping genes and signals were normalized by the geometric mean of the three housekeeping genes and relative to control value. Data are presented as means $\pm$ S.E.M, N=3 n=1, are given and compared to the non inflamed corresponding condition (non inflamed coculture or non inflamed monoculture) (*p<0.01).

The same analyses were performed on macrophages (**Fig 5.6**). The treatment with LPS induced a drastic increased of the inflammatory gene expressions. The coculture increased the CCL-2 and TNF α gene expressions. However, no significant effect was observed for the iNOS gene expression. The expression of IL6 gene was too low to be detectable (N/A).

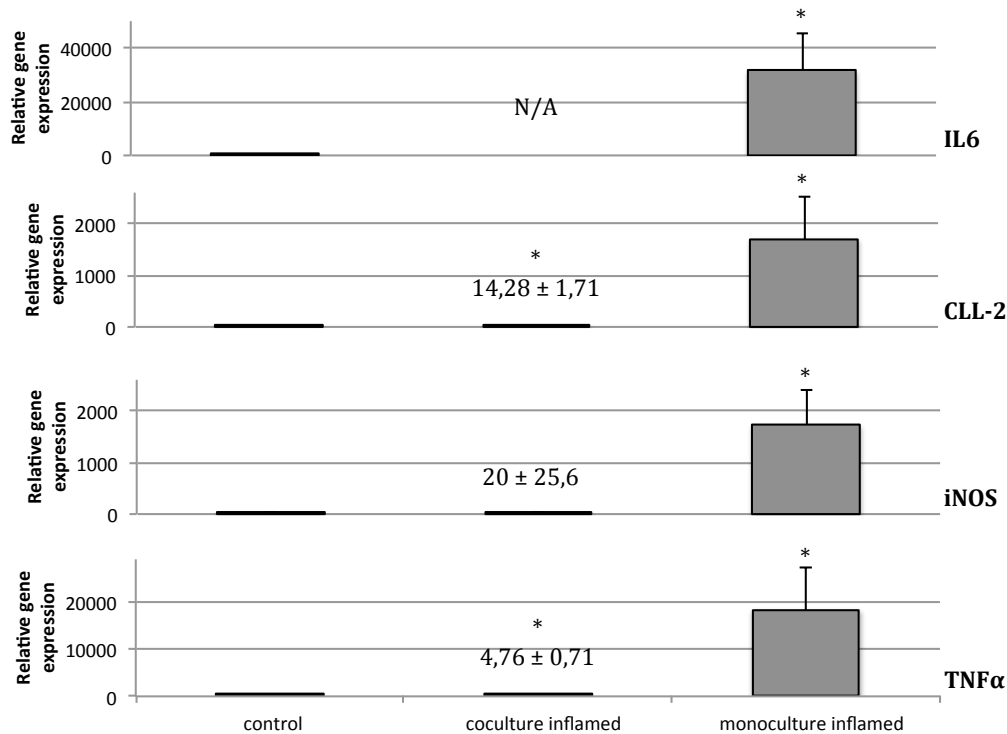


Fig 5.6. Pro-inflammatory gene expressions were analysed by real-time PCR using mRNA extracted from RAW after 24h of culture. β -actin, GAPDH and HPRT were used as housekeeping genes and signals were normalized by the geometric mean of the three housekeeping genes and relative to control value. Data are presented as means $\pm$ S.E.M, N=3 n=1, are given and compared to the non inflamed corresponding condition (non inflamed coculture or non inflamed monoculture) (*p<0.01).

5.4. Discussion

Obesity is characterized by a chronic low grade inflammation state, which is known to be due to adipose tissue secretions. Indeed, the adipocytes and infiltrating macrophages are the main source of pro-inflammatory circulating factors of obese individuals (CCL-2, IL-6 and TNF α).

The development of a model that simulates the inflammatory state of obese adipose tissue had already been done by Suganami *et al.* (2005)⁴. Their system consisted in a direct contact coculture between RAW 264.7 macrophages and differentiated 3T3-L1 adipocytes that mimics relatively well the conditions observed in the infiltrated adipose tissue. They showed that, as *in vivo*, the secretion of IL-6, TNF α and CCL-2 increased while that of adiponectin decreased. Similarly, the expression of IL-6 as TNF α and CCL-2 increased in coculture. In addition, they revealed that cells exposed to the conditioned medium from the other cell type presented an inflamed profile. This highlighted that soluble factors, in addition to contact between the cells, are important in the development of inflammation. They also demonstrated that the number of macrophages and the fat content of adipocytes are important factors that regulate inflammation intensity.

In our study, cocultures in bicameral inserts were performed. The inflammation appeared less important than in the contact coculture, but a significant level of pro-inflammatory secretions has been reached. Moreover, the bicameral coculture system has the advantage that cells can be harvested separately, allowing the study of gene expression in the two cell types, independently.

In adipose tissue of obese rodents, the macrophages can reach 60 % of the resident cells⁹. At the beginning of the coculture, macrophages represented 51.6 % of the total cell number. However, macrophages, unlike adipocytes, continued to proliferate and after 24h, the non activated RAW 264.7 macrophages increased their number by 50 %¹⁰. So, after 24h, in the non inflamed coculture, the proportion of macrophage reached 62 %. As the

RAW 264.7 treated with LPS proliferate less efficiently than the untreated cells¹⁰, the proportion was still maintained after 24h.

By activating previously the macrophages with LPS, we forced the cells to present the M1 pro-inflammatory profile before the coculture. *In vivo*, in the inflamed adipose tissue, the majority of macrophages present this M1 phenotype. The phagocytic cells have a pro-inflammatory behaviour, express the inducible nitric oxide synthase (iNOS) and TNF α . In addition, these macrophages carry cell surface markers (F4/80, CD11b, CD11c) that characterize the M1 profile¹¹. In this study, as revealed by the assay of NO and TNF α secretions, the macrophages have acquired the desired profile upon incubation with of 1 μ g/ml LPS (**Fig 5.2**).

Once the macrophages activated, they have been plated, for 24h, in the upper compartment of bicameral inserts containing 3T3-L1 adipocytes in their lower compartment. The activation of macrophages allowed to almost triple the secretion of IL-6 and CCL-2, as compared to coculture with non inflamed macrophages. Furthermore, the IL-6 secretion was higher than the sum of the secretions of both cell types cultivated independently (**Fig 5.3**). This indicates that both cells stimulated each other by their secreted molecules. For the CCL-2 secretion, this stimulation effect appears only in the inflamed conditions (**Fig 5.3**).

The secretion of adiponectin was reduced when adipocytes were cocultivated with macrophages. However, the decrease was only significant in the non inflamed coculture although a trend was also observed in the inflamed coculture. The decrease of adiponectin secretion in coculture is mainly due to the presence of TNF α secreted by macrophages¹². Indeed, this cytokine decreases the expression of adiponectin¹³, which was confirmed by the gene expression analysis in our work (**Fig 5.5 and 5.6**). As in our coculture system, after 24h of incubation, the level of TNF α was not different between the non inflamed and the inflamed conditions, it was not surprising to not observe differences in the secretion of adiponectin

between the non inflamed and the inflamed conditions. The LPS did not modify the adiponectin secretion and expression. LPS acts on both cells by binding on the TLR4. The activation of this receptor leads to the induction of pro-inflammatory gene expression¹⁴. However, previous study has shown that the activation of the adipocytes TLR4 tended to increase the adiponectin secretion and expression¹⁵.

Another group has already observed that TNF α secretion was not modified in bicameral coculture systems after 24h of coculture¹⁶. The contact between adipocytes and macrophages seems to be an important factor for macrophages TNF α overexpression. A recent study has revealed that inflammatory receptor 4-IBB in adipocytes and ligand 4-IBBL in macrophages were present in the membrane of the cells and mediated the bidirectional contact signalling¹⁷. The stimulation of these receptor/ligand enhanced TNF α , IL-6 and CCL-2 secretions in 3T3-L1 and RAW 264.7. The disruption of the interaction between 4-IBB/4-IBBL induced a partial decrease in the cytokines/chemokine secretions. However, even if in the bicameral coculture inflamed system that we developed, cell-cell contacts were not possible, IL-6, CCL-2 and adiponectin secretions were significantly modified as well as their expressions.

As revealed in **Fig 5.4**, the adipocytes were the main producers of IL-6 and CCL-2 in our system. For IL-6, it was strengthened by the undetectable level of IL-6 gene expression by macrophages in coculture. The 3T3-L1 adipocytes did not secrete detectable amount of TNF α and, in addition, we were unable to detect the TNF α gene expression by adipocytes. This observation is controverted; in most studies using 3T3-L1 adipocytes, the cells were able to produce detectable amount of TNF α ^{4,17}, although in adipose tissue, it is clear that the main production of this cytokine comes from the non fat cells^{18,19}.

Despite, the slight differences with the *in vivo* conditions, this coculture system incorporates the main characteristics found in an obese adipose tissue. By using this *in vitro* system, we will be able to study the effect of vegetal extracts on the adipose tissue inflammation by focusing on the paracrine and autocrine pathways. Moreover, we will investigate which of both cells are the targets of the extract by studying their gene expression independently.

5.5. References

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6. Pomegranate husk extract impacts on inflamed adipocytes and macrophages cultivated independently, but not on the inflammatory vicious cycle between adipocytes and macrophages

This chapter will present the effect of the pomegranate husk extract, punicalagin and ellagic acid, which already revealed effects on fat accumulation, on the inflammatory state of obese adipose tissue. Previous studies in our group on Caco-2 inflamed intestinal cells have shown that this extract reduced the inflammation, as well as punicalagin¹. In this chapter, we will focus on their potential anti-inflammatory effect on

¹ Hollebeeck, S. *et al.* Anti-inflammatory effects of pomegranate (*Punica granatum* L.) husk ellagitannins in Caco-2 cells, an in vitro model of human intestine. *Food and Function* **3**, 875–885 (2012).

inflamed macrophages and adipocytes cultivated independently and together in the *in vitro* coculture system described in chapter 5.

The result will be presented in the form of manuscript submitted for publication in “Journal of Nutritional Biochemistry” and entitled “Anti-inflammatory effect of a pomegranate husk extract on inflamed adipocytes and macrophages cultivated independently, but not on the inflammatory vicious cycle between adipocytes and macrophages”. The authors are Julie Winand and Yves-Jacques Schneider.

Abstract

Obese adipose tissues contain higher proportions of inflamed macrophages than the normal adipose tissue. Adipocytes and macrophages are known to secrete pro-inflammatory markers that establish the systemic inflammation leading to metabolic complications. CCL-2 secreted by hypertrophied adipocytes attracts and activates macrophages in the adipose tissue. These cells, in turns, secrete TNF α and other pro-inflammatory molecules. Pomegranate husk extract and its phenolic constituents, punicalagin and ellagic acid, have revealed anti-inflammatory effect. In this study, we used an *in vitro* coculture system of 3T3-L1 murine adipocytes and RAW264.7 macrophages to investigate the potential anti-inflammatory effects of these compounds on the vicious cycle between both cell types. The pomegranate husk extract presented an anti-inflammatory effect on inflamed cells cultivated independently, as suggested by a decrease of (i) CCL-2 secretion by both cell types; (ii) adipocytes IL-6 expression and secretion; and (iii) macrophage TNF α secretion. Nevertheless and surprisingly, no anti-inflammatory effect was observed in coculture. Punicalagin, at the same concentration as that found in the pomegranate extract, had a more potent effect than the extract and in coculture, it reduced significantly the IL-6 secretion. Ellagic acid decreased TNF α and CCL-2 macrophage secretion, CCL-2 adipocyte secretion and, in coculture, it reduced IL-6 secretion and expression by adipocytes. These results indicate that the pomegranate husk extract has an anti-inflammatory action on adipocytes and macrophages but seems to be unable to reduce the inflammatory vicious cycle between both cells. Its main ellagitannin, punicalagin, revealed a better effect on inflammation, suggesting that it will be a good candidate for more investigations.

6.1. Introduction

Obesity is a body state that concerns more than 11 % of the world population¹. This state is associated with low grade chronic inflammation that leads to the development of metabolic complications. In the case of obesity, the secretion of pro-inflammatory molecules by adipose tissue is upregulated². Among these potentially deleterious molecules, IL-6, TNF α and CCL-2 play crucial roles in the development of insulin resistance and cardiovascular complications. They are mainly secreted by two adipose tissue resident cells, *i.e.* adipocytes and macrophages. Thirty percent of the plasmatic IL-6 in obese patient is produced by the adipose tissue. This cytokine is thought to contribute to metabolic dysfunctions linked to obesity. CCL-2 is produced in high amount by hypertrophied adipocytes and attracts macrophages inside the adipose tissue³. Once inside the tissue, macrophages acquire an inflamed profile and secrete TNF α that will act in a paracrine way on adipocytes and macrophages and stimulates their pro-inflammatory secretions. Therefore, if the CCL-2 secretion is reduced, less even macrophages will be attracted and activated, which leads to a reduction of the inflammation.

Pomegranate is an ancient fruit that has already shown positive effects on health and particularly on the metabolic syndrome⁴. *In vitro* and *in vivo* studies have highlighted that this fruit contributes to the improvement of insulin sensitivity, cholesterol level, ... as well as anti-inflammatory effects⁴. These bioactive effects were often assigned to its most prevalent compound class, *i.e.* the ellagitannins. Among this family, punicalagin is very active and is found in high concentrations in the husk of the pomegranate and to a lesser extent in the juice. Punicalagin, as other ellagitannins, is partially catabolised to ellagic acid during its passage in the gastro-intestinal tract. However, punicalagin could also be found in the plasma after ingestion of high amounts of pomegranate extracts. Cerda *et al.* (2003) have demonstrated that after 10 days of pomegranate juice ingestion, punicalagin plasma concentration reached 27 μ M, while that of ellagic acid

reached only 1.6 μM ⁵. Indeed, ellagic acid is metabolised to urolithins by microflora and absorbed more efficiently under these more lipophilic forms⁶.

Based on the numerous positive effects of the pomegranate and its constituents, this study aimed at investigating their potential anti-inflammatory effects on the inflamed macrophages and adipocytes and on the interaction between the two cell types. We used a characterized pomegranate husk extract (PHE), particularly rich on punicalagin. The concentration used corresponded to 5 % of the daily dose that the suppliers recommended to take (*i.e.* 1 g). The concentrations of punicalagin and ellagic acid in this extract were determined by HPLC and the experiments were performed by using the corresponding concentrations.

6.2. Materials and methods

6.2.1. Materials and reagents

A hydroalcoholic Pomegranate (*Punica granatum* L.) husk extract (PHE) was prepared from Moroccan cultivars and provided by Stiernon S.A. (Ghislenghien, BE) under the form of a dry powder. The phenolic compounds previously identified in the extract by HPLC are gallic acid, punicalin, pedunculagin I, punicalagin, strictinin, ellagic acid-hexoside and ellagic acid. Punicalagin (95.35% purity) was purchased from Phytolab (Vestenbergsgreuth, DE); ellagic acid (> 90% purity) from Extrasynthese (Lyon, FR); Triton X-100, LPS, dexamethasone (DEX), 3-isobutyl-1-methyl-xanthine (IBMX) and insulin from Sigma-Aldrich (St Louis, MO); Dubelcco's Modified Eagle's Medium (DMEM), calf serum (CS), GlutaMax (100x), sodium pyruvate (100 mM), penicilin/streptomycin (100-100 µg/ml penicillin/streptomycin) and 0.05 % trypsin-0.02 % EDTA from Lonza (Verviers, BE) and fetal bovine serum (FBS) from PAA (Linz, AT).

6.2.2. Cell culture

RAW 267.4 murine macrophages (American Type Culture Collection, Manassas, VA) were subcultured in 25 cm² flasks (Corning, Badhoevedorp, NL) in DMEM containing 10 % (v/v) FBS, 1 % (v/v) antibiotics (v/v), 1 % (v/v) Na pyruvate and 1 % (v/v) GlutaMax.

The day before the coculture, macrophages were activated by 10 % FBS-DMEM medium supplemented with 1 µg/ml LPS. As control, the medium was replaced by fresh 10 % FBS-DMEM medium. After 24h of incubation, macrophages were harvested and resuspended in 10 % FBS-DMEM medium for either the coculture or control monoculture.

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were subcultured in 10 cm dish (Corning) in DMEM containing 10 % (v/v) CS, 1 % (v/v) antibiotics, 1 % (v/v), Na pyruvate and 1 % (v/v) GlutaMax. 70 % confluent cells were trypsinized and cultured in 12-wells culture plates for coculture assay (Corning). Two-day postconfluent 3T3-L1

preadipocytes, designated as Day 0, were differentiated with DMEM supplemented with 10 % (v/v) FBS, 1 % (v/v) antibiotics, 1 % (v/v) Na pyruvate, 1 % (v/v) GlutaMax and an adipogenic mixture containing 170 nM insulin, 50 μ M IBMX and 1 μ M DEX for 2 days. Cells were then maintained in the same 10 % FBS-DMEM medium with insulin (170 nM) for another 2 days. Afterwards, the culture medium was replaced every 2 days by 10 % FBS-DMEM medium for four more days. Eight days after differentiation induction, fully differentiated 3T3-L1 adipocytes having accumulated large lipid droplets, were used for coculture experiments (day 8). Both cell types were cultured at 37°C under a humidified, 10 % (v/v) CO₂ atmosphere.

Coculture in bicameral inserts was performed by plating RAW 264.7 macrophages (activated or not, 2×10^5 cells) in the upper compartment of 0.4 μ m pore size inserts (Corning) and placing them in 12 wells plate containing the 8 days differentiated 3T3-L1 adipocytes, for 24h or 48h.

As controls, macrophages (activated or not) were also seeded in 12 wells plate in 10 % FBS-DMEM medium for 24h or 48h. Fully differentiated adipocytes were cultivated alone and in the presence of LPS (1 μ g/ml) for 24h or 48h.

PHE and the phenolic compounds were added in the culture medium for the 24h or 48h of coculture/monoculture. PHE was solubilized in Hank's balanced saline solution (HBSS) (Lonza) at the concentration of 2.5 mg/ml and added to the culture medium at the final concentration of 66 μ g/ml corresponding to 4 μ M of punicalagin and 24 μ M of ellagic acid. Punicalagin was solubilized in 25 % (v/v) methanol at 50 mM and added to the culture medium at the final concentration of 4 μ M. Ellagic acid and resveratrol were solubilized at 50 mM in DMSO and respectively added to the culture medium at 24 and 25 μ M, respectively. Controls with appropriate vehicles were also realized.

6.2.3. Cytotoxicity assay

The possible cytotoxicity of treatments was routinely checked through the determination of the activity of the lactate dehydrogenase (LDH) released by the cells into the extracellular medium with a cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, DE) used according the manufacturer's instructions. The percentage of cytotoxicity was calculated using the maximum release of LDH activity obtained upon complete lysis of the 3T3-L1 differentiated adipocytes or RAW 264.7 macrophages incubated independently with 1 % (v/v) Triton X-100. For the coculture the 100 % of LDH release corresponded to the sum of maximum LDH activity of 3T3-L1 and RAW cells cultured independently.

6.2.4. Assay of IL-6, CCL-2 and TNF α secretion

The concentrations of IL-6, CCL-2 and TNF α in the cell supernatants were determined by selective ELISA for mouse IL-6, CCL-2 and TNF α (eBioscience, San Diego, CA) according to the manufacturer's protocol. The supernatants were diluted in order to reach measurable concentrations.

6.2.5. Adiponectin assay

The adiponectin secretion in culture medium was assessed by a commercially available sandwich ELISA kit (Millipore, Billerica MA), according to the protocol instructions. Culture media, at day 8, were harvested and centrifuged. The supernatants were diluted in order to reach measurable concentrations.

6.2.6. Assay of nitric oxide release

Total nitrite concentrations of supernatant were determined using Cayman's Nitrate/Nitrite colorimetric Assay Kit (Cayman, Ann Arbor, MI) based on the Griess reaction. The Griess reagents convert nitrite into red-violet coloured a water soluble azo compound. The product absorbance was

read at 540 nm and the total amount calculated from the NaNO_2 standard curve.

6.2.7. Protein assay

After the 24h of mono- or coculture, cells were washed twice and harvested in lysis buffer. After one freezing, total protein content was measured using the BiCinchoninic Acid (BCA) Protein Kit assay (Sigma) according to the manufacturer's protocol. The standard curve was realized with a standard solution of bovine serum albumin (BSA) (Sigma).

6.2.8. Gene expression study

Total RNA was extracted from 3T3-L1 and RAW 264.7 cells using QIAzol reagent (Qiagen, Hilden, DE) according to the manufacturer's instructions. Quality and quantity of total RNA was assessed by Nanodrop spectrophotometry. Total RNA (1 μg) was reverse-transcribed using the Superscript III First-Strand Synthesis System (Invitrogen, Merelbeke, BE) following manufacturer's instructions. Then, qRT-PCR was performed in 96 well PCR plates (Bio-Rad, Nazareth Eke, BE), to estimate the mRNA abundance corresponding to either IL-6, CCL-2, iNOS or $\text{TNF}\alpha$ relatively to the housekeeping genes, *i.e.* β -actin, GAPDH and HPRT, used for normalization. A total of 5 μl cDNA with the appropriate dilution, 0.2 μl primer mix and 4.8 μl SYBRGreen SuperMix (Bio-Rad) were added to each well. The PCR amplifications were performed in a MyIQ2 thermocycler (Bio-Rad) as follows: 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. Melting curves were systematically assessed for quality control of the primers. Gene expression values were normalized to the geometric means of the expression values of β actin, GAPDH and HPRT and calculated on the bases of the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta\text{Ct}}$). The primers used were reported in **Table 6.1**.

Gene name	Forward primer	Reverse primer
β -actin	TAGACTTCGAGCAGGAGATGG	GGATTCCATACCCAAGAAGGA
GAPDH	GTGGAGATTGTTGCCATCAACG	CAGTGGATGCAGGGATGATGTTCTG
HPRT	ACATTGTGGCCCTCTGTGTG	TTATGTCCCCCGTTGACTGA
PPAR γ	CAGTGATATCGACCAGCTGAAC	GGTGATTGTGCCGTTGTCTTTC
Adiponectin	GGAGAGAAGGGAGAGAAAGGTC	AACAGTGACGCGGGTCTCC
IL-6	ACTTCCATCCAGTTGCCTTCT	GAATTGCCATTGCACAACTCT
CCL-2	GTTGACCCGTAAATCTGAAGC	AAGGCATCACAGTCCGAGTCA
TNF α	TCCCCAAAGGGATGAGAAGTTC	TCATACCAGGGTTTGAGCTCAG
iNOS	ACACTCAGATCATCTTCTCAAATTTCG	GTGTGGGTGAGGAGCACGTAGT

Table 6. 1. Sequences of primers genes used in qRT-PCR

6.2.9. Statistical analysis

All the results were expressed as means $\pm$ SEM. Data were analysed by one-way ANOVA followed by Dunnett post-hoc test to identify mean differences between treatment and positive control. P-values less than 0.05 were considered significant. Statistical analyses were performed using the JMP® 10 software (SAS Institute, Cary, NC).

6.3. Results

6.3.1. Effect of pomegranate husk extract and its main phenolic constituents on IL-6, CCL-2, TNF α and NO secretions by RAW 264.7 macrophages

To determine whether the PHE, punicalagin and ellagic acid decreased the pro-inflammatory molecules secretion, RAW 264.7 macrophages, pre-stimulated with LPS, were incubated in the presence or absence of 66 $\mu\text{g/ml}$ PHE, 4 μM punicalagin or 24 μM ellagic acid during 24 or 48h. None of these compounds affected cell viability as evaluated by LDH activity released in the culture medium (Data not shown). The TNF α secretion was reduced by all the treatment after 24 and 48h, the most important effect being observed after 48h of incubation. Similar effects were observed on the CCL-2 secretion. However, the IL-6 secretion was not affected by the treatments (**Fig 6.1A**). After 24h of incubation with the vegetal compounds, only punicalagin decreased significantly the NO release (**Fig 6.1B**)(similar results after 48h, Data not shown).

In order to further investigate the effects of these compounds on the activation of macrophages, RAW 264.7 cells were plated in the presence or absence of PHE, punicalagin or ellagic acid for one hour. Then, 1 $\mu\text{g/ml}$ LPS was added for 24h. Two markers of the activation state were followed; NO and TNF α (**Fig 6.2**). Resveratrol was used as reference. This stilbene, as expected⁷, reduced the NO release by activated macrophages in a dose dependant manner, as well as TNF α secretion, but only at 150 μM . PHE reduced slightly NO and TNF α secretion. Punicalagin and ellagic acid only affected the NO production at 12 and 72 μM , respectively.

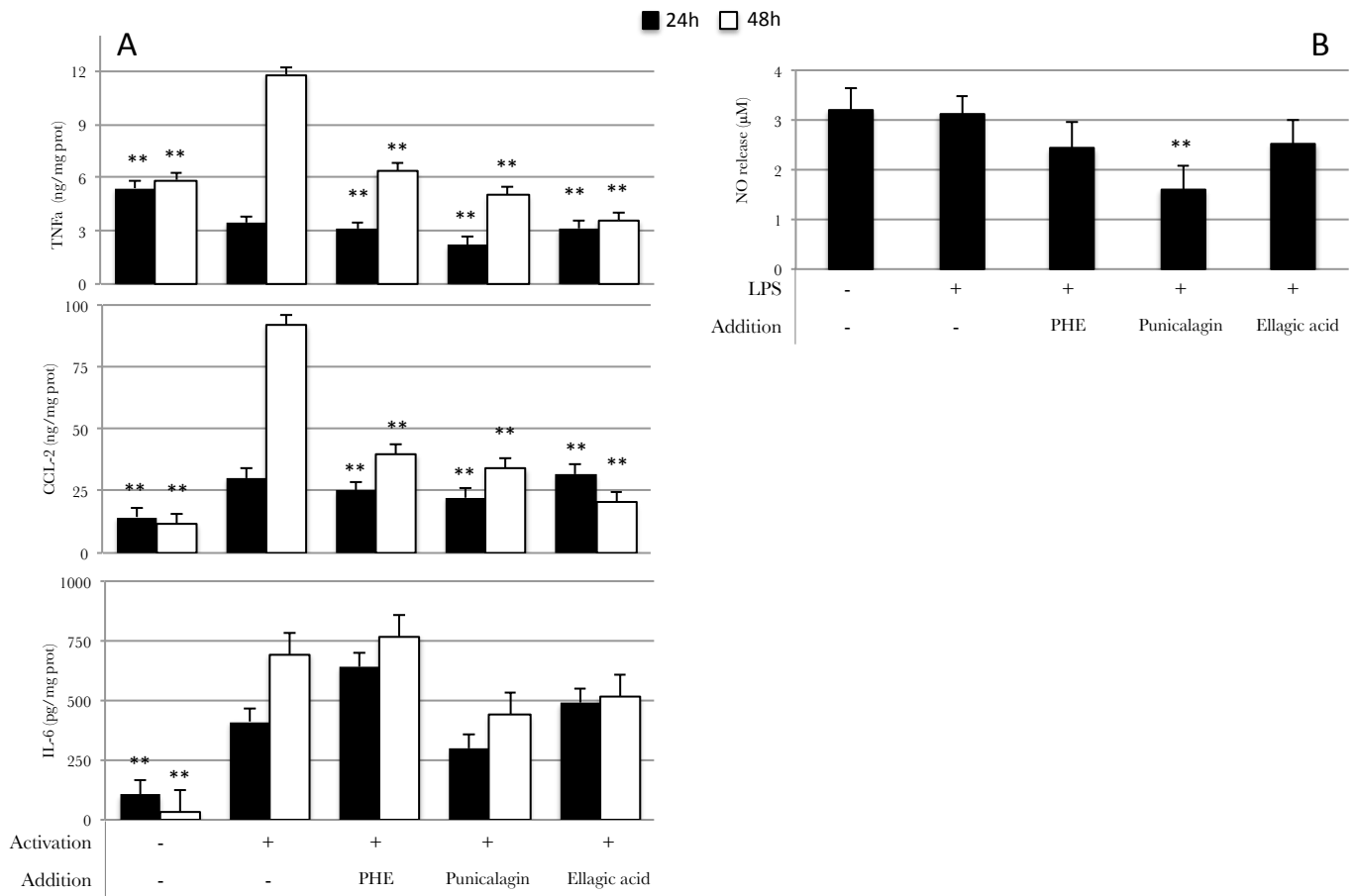


Fig 6.1. Effect of PHE, punicalagin and ellagic acid on TNF α , CCL-2 and IL-6 secretions (A) and NO release (B) by macrophages pretreated or not with LPS. Macrophages preactivated or not were plated and cultivated during 24h or 48h in the presence of PHE (66 μ g/ml), punicalagin (4 μ M), ellagic acid (24 μ M). Culture media were then harvested and pro-inflammatory parameters measured. (A) TNF α , CCL-2 and IL-6 secretions were assessed by ELISA and compared to the LPS treated control. (B) NO release was measured by NO assay on medium of 24h of culture and compared to the LPS treated control. Means $\pm$ SEM are given, N = 3; n = 3, (** p<0.01).

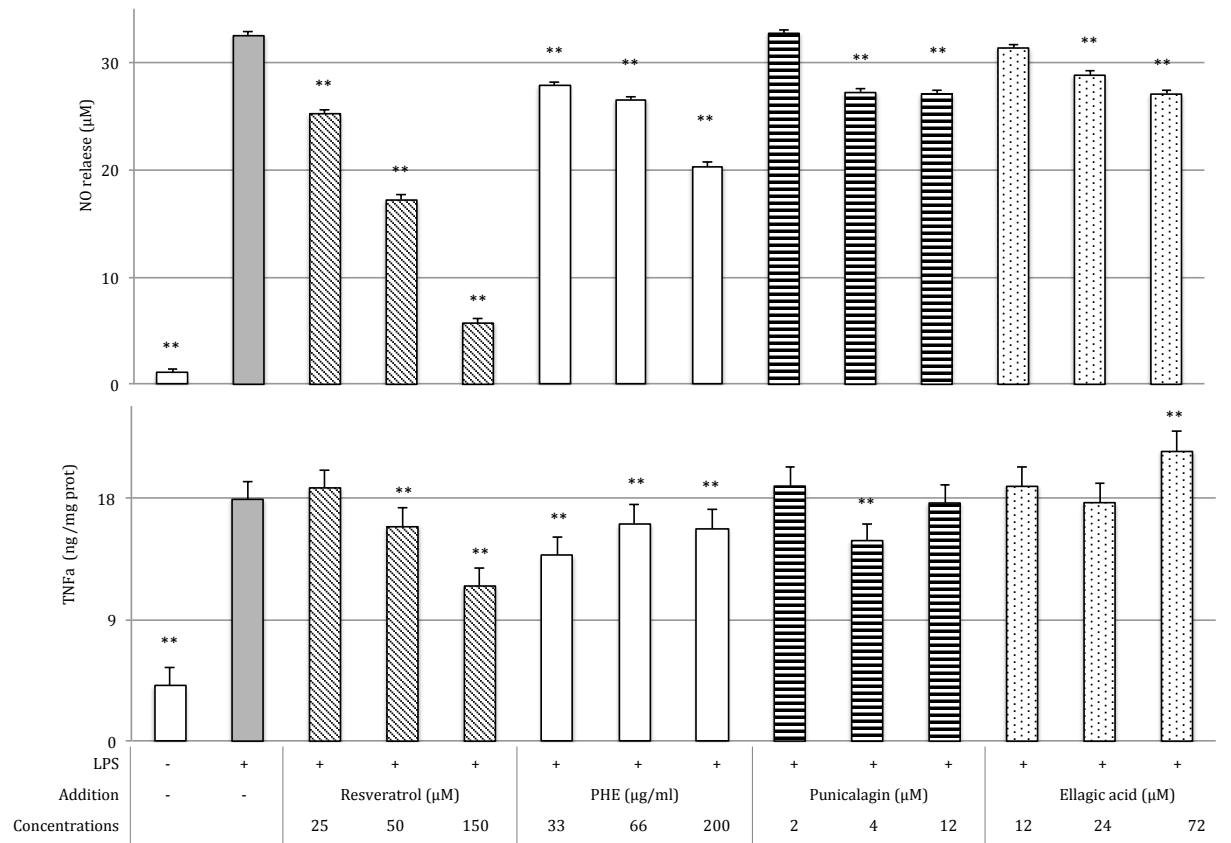


Fig 6.2. Effect of PHE, punicalagin, ellagic acid and resveratrol on TNF α and NO production by macrophages activated by LPS. Macrophages were incubated with various concentrations of the different compounds After 1h, LPS was added to the culture medium for 24h. Culture media were then harvested and TNF α and NO secretions measured and compared to the LPS treated controls. Means $\pm$ SEM, N = 3; n = 3 are given, (* p<0.05; ** p<0.01).

6.3.2. Effect of pomegranate husk extract and its main phenolic constituents on IL-6, CCL-2, adiponectin and NO productions by 3T3-L1 adipocytes activated by LPS

The adipocytes, as macrophages, express the Toll like receptor (TLR) 4 that binds the LPS, which triggers the NF- κ B cascade inducing the expression of pro-inflammatory genes⁸. 3T3-L1 cells were exposed during 24 or 48h to PHE, punicalagin or ellagic acid in the presence or absence of LPS. The treatment with LPS alone increased drastically the secretion of CCL-2 and IL-6, but did not modify the secretion adiponectin and the NO release (**Fig**

6.3). PHE, punicalagin and ellagic acid reduced more importantly the CCL-2 secretion after 48h of incubation, by decreasing its secretion of about 65, 40 and 50 %, respectively. After 24h, PHE and punicalagin reduced the IL-6 secretion by 72.5 and 95.0 %, respectively. Punicalagin even reduced IL-6 release at a level below the control one. The adiponectin secretion was not modified by the LPS treatment or the incubation with PHE, punicalagin or ellagic acid. NO production was not affected by the different treatments (similar results after 48h, Data not shown).

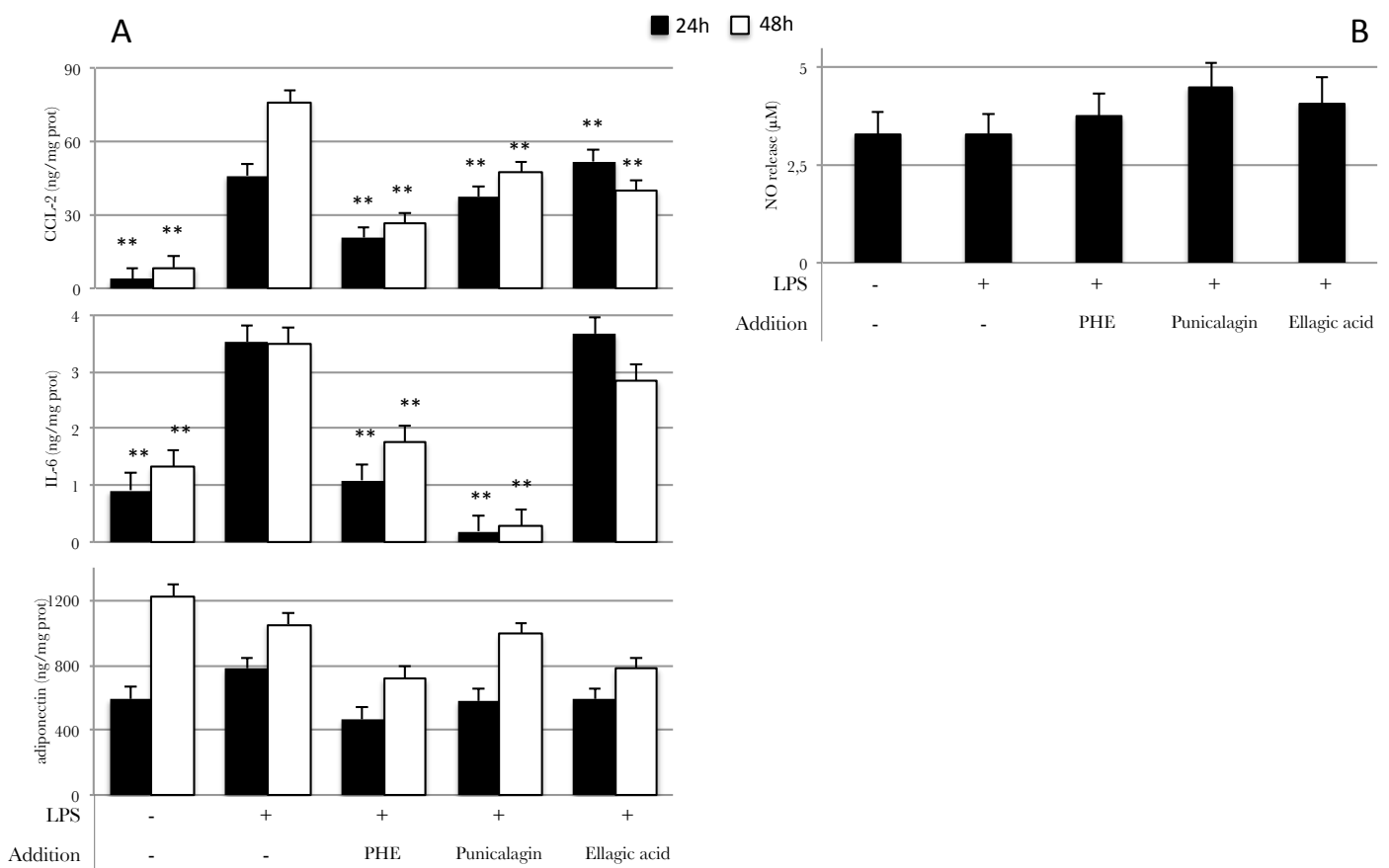


Fig 6.3. Effect of PHE, punicalagin or ellagic acid on CCL-2, IL-6 and adiponectin secretions (A) and NO release (B) by 3T3-L1 adipocytes activated or not by LPS. Adipocytes differentiated for 8 days were treated during 24h or 48h with 66 μg/ml PHE, 4 μM punicalagin 24 μM ellagic acid in the presence of LPS. As controls, adipocytes were incubated in the presence or absence of LPS. Culture media were then harvested and pro-inflammatory parameters and adiponectin were measured. (A) CCL-2, IL-6 and adiponectin secretions were assessed by ELISA and compared to the LPS treated control. (B) NO release was measured by NO assay on medium of 24h of culture and compared to the LPS treated control. Means ± SEM, N = 3; n = 3 are given, (** p < 0.01).

6.3.3. Effect of pomegranate husk extract and its main phenolic constituents on IL-6, CCL-2, TNF α , adiponectin and NO productions by 3T3-L1 adipocytes and RAW 264.7 activated macrophages in coculture

To verify whether the compounds exert an anti-inflammatory effect upon interactions between macrophages and adipocytes, non-inflamed and inflamed cocultures were performed. Macrophages, activated or not, were plated on bicameral insert above the differentiated adipocytes. PHE, punicalagin or ellagic acid were added to the culture medium during the coculture. After 24h or 48h, media and cells were harvested.

The pre-activation of macrophages with LPS significantly increased the secretions of IL-6 and CCL-2 in inflamed coculture as compared to the coculture with macrophages non exposed to LPS. Secretions of TNF α were, however, unaffected by the inflammatory state of macrophages (**Fig 6.4**). PHE, punicalagin and ellagic acid increased TNF α secretions after 48h by more than 100 % as compared to control conditions. The only anti-inflammatory effects observed upon incubation with the tested compounds were slight decrease of CCL-2 secretion after incubation with the three tested compounds and a decrease of the IL-6 secretion 48h after ellagic acid incubation. The compounds did not have any effect on adiponectin and NO production in coculture (Data not shown).

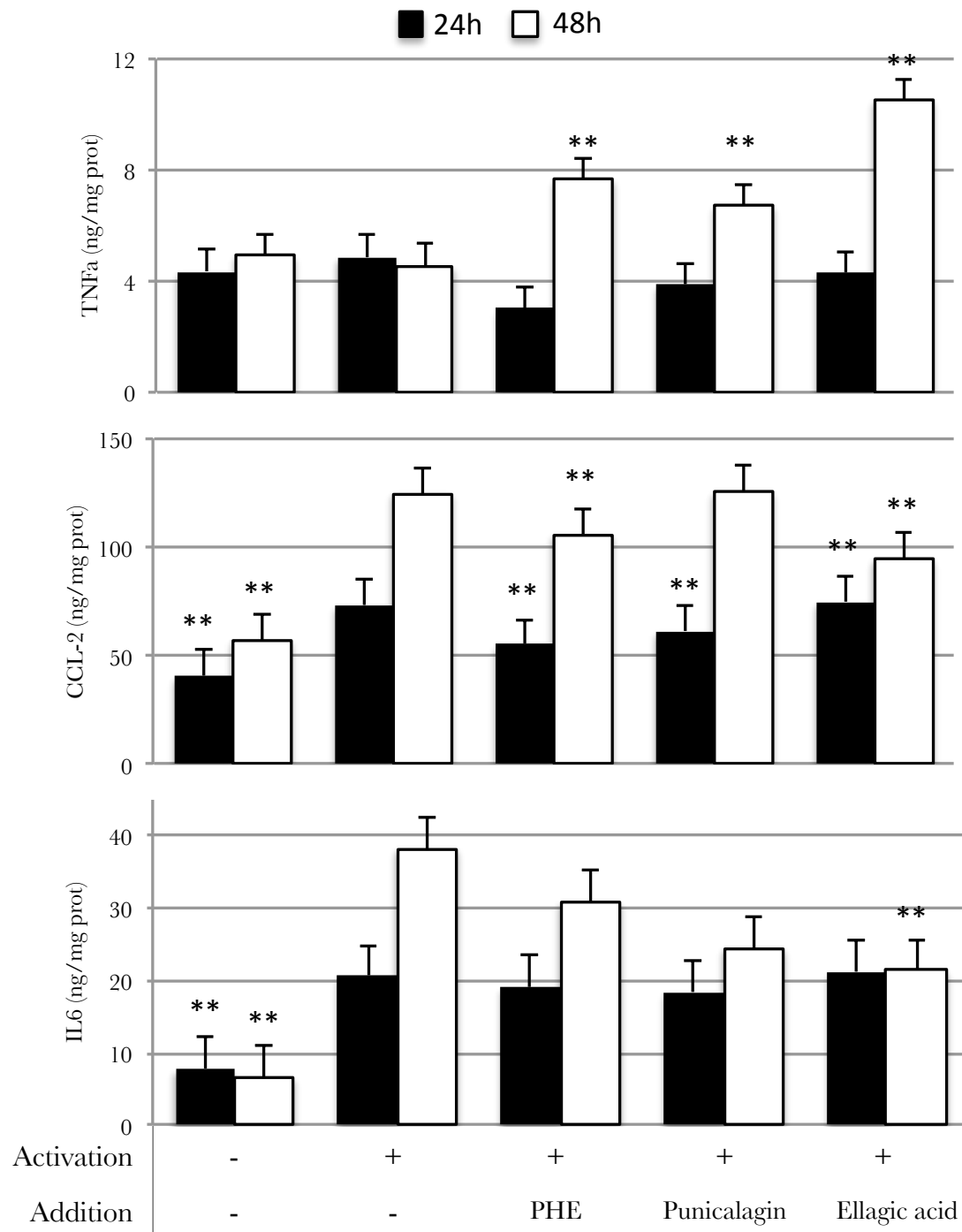


Fig 6.4. Effect of PHE, punicalagin and ellagic acid on TNF α , CCL-2 and IL-6 secretions in coculture of 3T3-L1 adipocytes and macrophages preactivated with LPS. 3T3-L1 adipocytes differentiated for 8 days were cocultivated with macrophages pretreated with LPS, for 24h or 48h in the presence or absence of 66 μ g/ml PHE, 4 μ M punicalagin or 24 μ M ellagic acid. As control adipocytes were cocultivated with macrophages pretreated with LPS or not. Culture media were then harvested and pro-inflammatory parameters assessed by ELISA and compared to the LPS treated controls. Means $\pm$ SEM, N = 3; n = 3 are given, (* p<0.05; ** p<0.01).

6.3.4. Effect of pomegranate husk extract and its main phenolic constituents on IL-6, CCL-2, TNF α and NO gene expression in RAW 264.7 activated macrophages

Fig 6.5 resumes the effect on the tested compounds on pro-inflammatory gene expressions. RAW 264.7 cells were treated with PHE, punicalagin or ellagic acid during 1h, then LPS was added for the next 6 hours. The transcript levels of the genes were determined by qRT-PCR. The gene expressions were normalized to geometric means of references genes *i.e.* HPRT, GAPDH and β -actin, and expressed in fold ratio of the activated cells. The activation increased the expression, as compared to basal expression (control). None of the treatments showed a significant effect on the inflammatory gene expression.

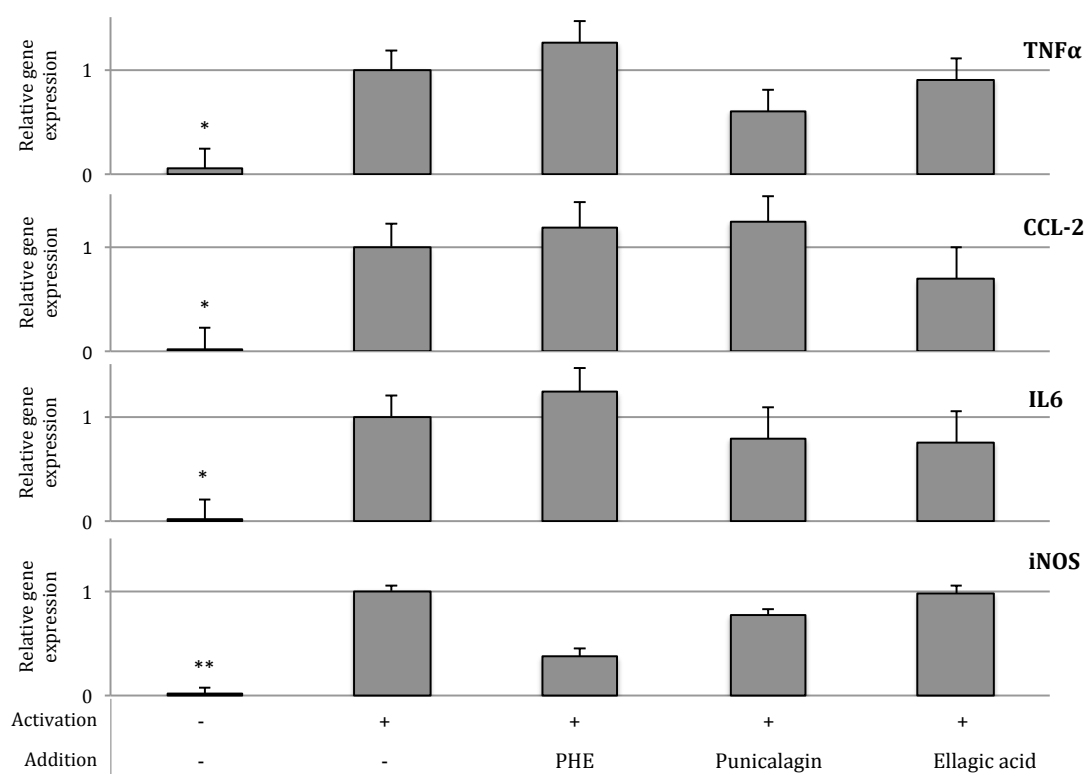


Fig 6.5. Relative gene expressions in macrophages pre-activated with LPS after 24h of treatment with 66 μ g/ml PHE, 4 μ M punicalagin or 24 μ M ellagic acid. Inflammation markers were analysed by qRT-PCR and normalized by the geometric mean of the 3 reference genes and relative to activated control value. Means $\pm$ SEM, N=3 n=1 are given. (* $p < 0.05$; ** $p < 0.01$)

6.3.5. Effect of pomegranate husk extract and its main phenolic constituents on IL-6, CCL-2, TNF α , NO, adiponectin and PPAR γ gene expressions in 3T3-L1 activated with LPS or after 24h of coculture

3T3-L1 cells were incubated with PHE, punicalagin or ellagic acid in the presence or absence of LPS. After 24h, cells were harvested and total RNA was isolated. qRT-PCR revealed that LPS significantly increased the expression of IL-6, CCL-2 and iNOS expression, as compared to control expression by a factor of 3.3, 12.4 and 37, respectively (**Fig 6.6**). LPS does not affect the expression of PPAR γ and adiponectin. The incubation with PHE reduced the expression of IL-6 by about 30%, which is consistent with the ELISA results. Punicalagin showed a drastic down-regulation of IL-6 and CCL-2 expressions. The ellagitannin reduced the IL-6 expression under the level of non-inflamed 3T3-L1 as already observed for the cytokine secretion. In addition, it up-regulated PPAR γ expression. Ellagic acid did not have any effect.

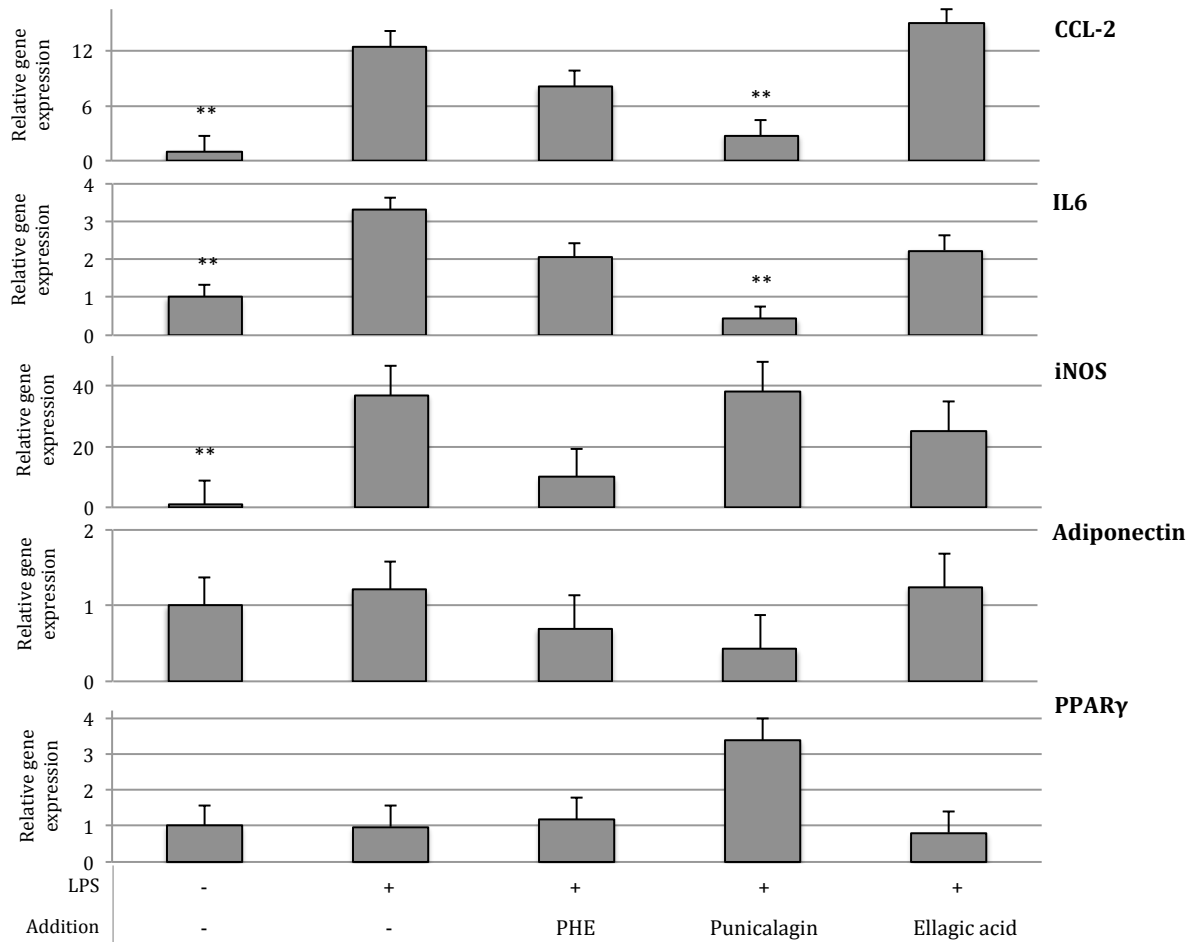


Fig 6.6. Relative gene expressions in adipocytes activated with LPS after 24h of treatment with 66 µg/ml PHE, 4 µM punicalagin or 24 µM ellagic acid. Inflammation markers, adiponectin and PPARγ were analysed by qRT-PCR and normalized by the geometric mean of the three reference genes and relative to non activated control value. Means ± SEM are given and compared to the activated control, N=3 n=1. (* p< 0.05; ** p<0.01).

The coculture upregulated the expression of IL-6, CCL-2 and iNOS in adipocytes although, in a less extent than LPS did (**Fig 6.7**). PHE and punicalagin had no impact on the expression of pro-inflammatory genes of 3T3-L1 cells in coculture. However, ellagic acid down-regulated the IL-6 expression, which is correlated with the reduction of IL-6 secretion in coculture treated with ellagic acid (**Fig 6.4**)

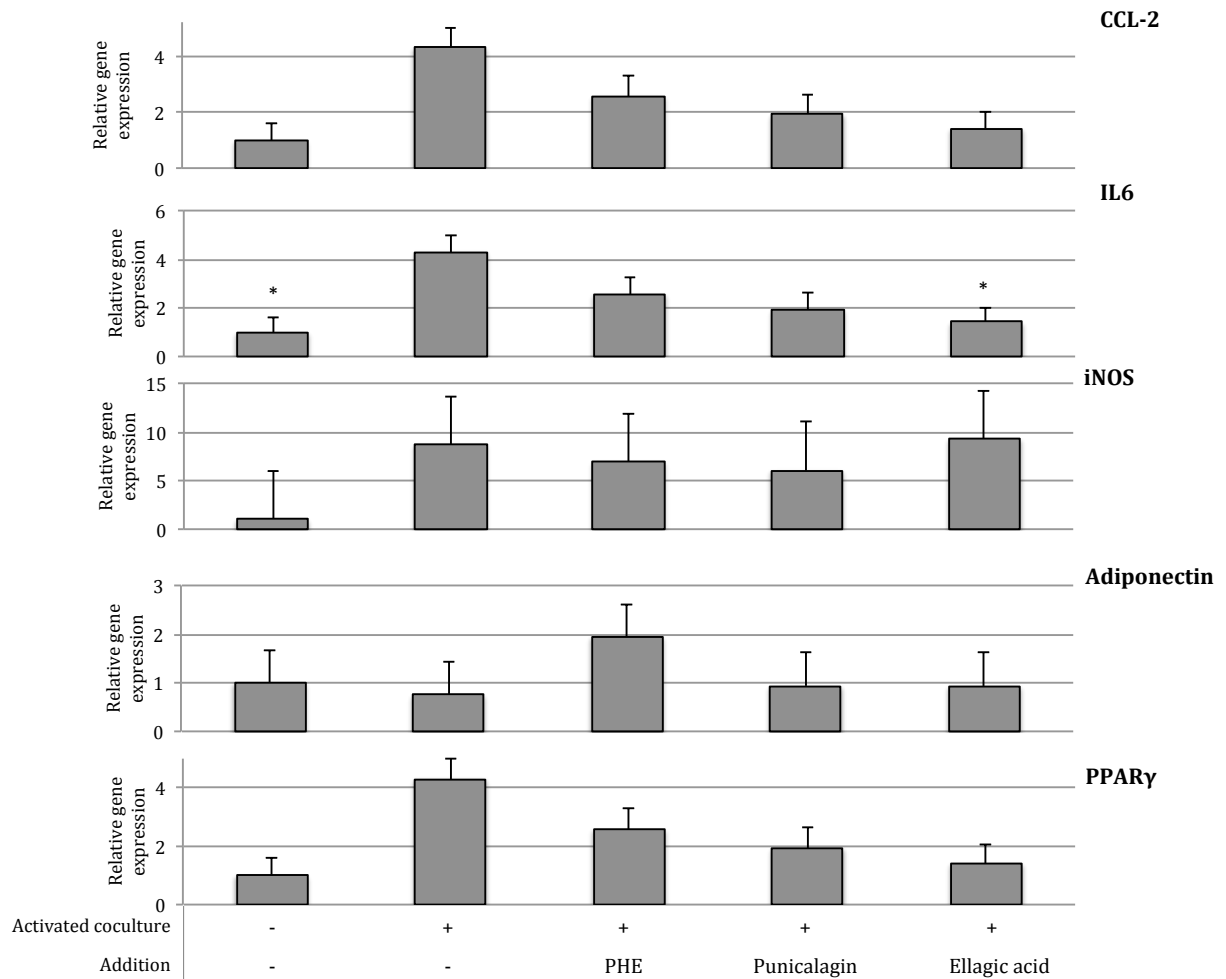


Fig 6.7. Relative gene expressions in adipocytes cocultivated with macrophages during 24h in presence or absence of 66 μ g/ml PHE, 4 μ M punicalagin or 24 μ M ellagic acid. As control, coculture with non activated macrophages was performed. Inflammation markers, adiponectin and PPAR γ were analysed by qRT-PCR and normalized by the geometric mean of the three reference genes and relative to non activated control value. Means $\pm$ SEM are given and compared to the activated control, N=3 n=1. (* p < 0.05; ** p < 0.01).

6.4. Discussion

In this work, we have shown that pomegranate husk extract (PHE) and its main phenolic constituent, *i.e.* punicalagin, decreased the secretion of pro-inflammatory markers by inflamed macrophages and adipocytes, when cultivated separately. However, surprisingly, they were not any more able to maintain this effect upon their coculture, which suggests that they could not act on inflammatory vicious cycle established between both cell types. Pomegranate has already demonstrated its health benefits in obesity as reviewed by Medjakovis & Jungbauer (2012)⁴. This effect is attributed to punicalagin, an ellagitannin present in high concentration in its husk. Although the bioavailability of this phenolic compound remains disputed, *in vivo* effects were observed. Upon pomegranate ingestion of various part of the pomegranate fruit (seed, husk, flower...), benefits were reported on insulin sensitivity, body weight, cholesterol level...⁹. In our group, Hollebeeck *et al.* (2012) reported on a decrease of intestinal inflammation and demonstrated that PHE and punicalagin reduced cytokines secretion and expression by Caco-2 inflamed cells¹⁰. Other studies have revealed anti-inflammatory effects of pomegranate, punicalagin or ellagic acid in different cases but their effect on adipose tissue inflammation has never been studied.

As obesity is characterized by an inflammation state maintained by the secretions from the adipose tissue, trying to reduce the deleterious interaction between adipocytes and macrophages seems to be a good target. Macrophages attracted by the secretion (*e.g.* CCL-2) of hypertrophied adipocytes infiltrate the adipose tissue and maintain a chronic inflammatory state by interacting with adipocytes. An *in vivo* study by Baranowski *et al.* (2012) has shown that flaxseed oil decreased of CCL-2 secretion of the adipose tissue resulting in a reduction of TNF α released by macrophages¹¹. So reducing the CCL-2 secretions could lead to an improvement of the inflammatory state.

We reported that PHE, punicalagin and ellagic acid reduced the CCL-2 secretion by inflamed adipocytes and macrophages (**Fig 6.1** and **Fig 6.3**). Moreover, PHE, punicalagin and ellagic acid, to a lower extent, could reduce the activation of macrophages induced by LPS. This endotoxin acts on TLR4 present in the membrane of macrophages, which induces inflammatory pathways. It is known that the free fatty acids, released in high amounts by adipocytes in case of obesity, are also able to act on this receptor and be therefore responsible to an increase in TNF α secretion¹². We showed that after already 24h of treatment, PHE, punicalagin and ellagic acid were able to reduce the TNF α secretion by activated macrophages. This may indicate that the extracts could be able to reduce the existing inflammation state by (i) reducing the CCL-2 secretion by macrophages and adipocytes that normally attracts new macrophages inside the tissue and (ii) decreasing the TNF α secretions of macrophages.

However, when PHE, punicalagin or ellagic acid are added at the beginning of the coculture, they were not able to reduce the secretion of TNF α and moreover they worsened it. One explanation may be the induction of adipocyte lipolysis by PHE and ellagic acid that will increase the free fatty acid in the medium leading to an induction of TNF α expression.

The IL-6 secretion was drastically increased by LPS stimulation or coculture in our experimental conditions. *In vitro* studies have already highlighted that adipocytes secrete more IL-6 than macrophages^{13,14}. This cytokine induces insulin resistance *in vitro*¹⁵, which is correlated with obesity induced insulin resistance in adipose tissue and liver *in vivo*¹⁶. IL-6 also activates the production of the C-reactive protein (CRP) by the liver; the presence of IL-6 and CRP at high plasmatic concentrations is a predictive factor for the development of type 2 diabetes². Adipocytes isolated from human adipose tissue secreted IL-6 although it was demonstrated that other non identified adipose tissue cells secrete the bulk of IL-6¹⁷. So, decreasing its production by the adipose tissue could be a good alternative to reduce the obesity impact on metabolic functions. In this work, we revealed that, after 48h of incubation, punicalagin could decrease the IL-6

secretion by adipocytes cultivated alone or in coculture. Its effect was even more powerful in coculture. The gene expression analysis revealed that punicalagin decreased the IL-6 expression in adipocytes cocultivated or activated with LPS.

We finally highlighted that the ellagitannin acts significantly on PPAR γ expression. This factor is implicated in adipogenesis and in adipocyte functions. Its upregulation is correlated with improvement in insulin resistance and inflammatory state linked to obesity¹⁸. Actually, the thiazolidinediones (TZD), a well-known PPAR γ ligand family, are antidiabetic drugs that promote the formation of small adipocytes that are more insulin sensitive than bigger ones¹⁹. This ligand also increases the expression of adiponectin²⁰. Finally, TZD is also correlated with a decrease of TNF α and resistin production, two molecules linked to the development of insulin resistance²⁰. In this study, no significant effects on adiponectin secretion and expression were shown although a slight increase of the expression was observed.

This model has focused on the impact of PHE, punicalagin and ellagic acid on the paracrine-autocrine pathway in the adipose tissue. However, it was reported that contact between macrophages and adipocytes induced more important inflammatory secretions. It is mediated by membrane receptor interaction between the two cells; the 4-1BB/4 receptor and its ligand 4-1BBL. The receptor 4-1BB/4 is upregulated in adipocyte in case of obesity and induces overexpression of IL-6, CCL-2 and TNF α by these cells. The same effect is observed in macrophages¹⁴. It would be interesting to investigate the effect of these compounds on the direct interaction between both cells.

To conclude, PHE, punicalagin and ellagic acid have revealed good anti-inflammatory effects on adipocytes and macrophages, cultured independently. This positive effect was, however, partially lost in coculture, which was correlated with the gene expression. Indeed the most important effects were observed after LPS stimulation of monocultures. However, the

anti-inflammatory effects brought out in this work are a clue to explain some of the *in vivo* effects of pomegranate on obesity complications^{21,22}

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7. Potential anti-inflammatory effect of three apple extracts on adipocyte and macrophage interaction in an *in vitro* coculture model

This chapter will present the last result part, which reports the effect of the three apple extracts on the inflammation simulated in the *in vitro* system of inflamed adipose tissue. As in the previous chapter, the extract will be added in the coculture system and their anti-inflammatory effects will be assessed.

Abstract

Adipose tissues of obese people are characterized by a low grade inflammatory state. The hypertrophied adipocytes increase their secretion of chemokine (*e.g* CCL-2) that attracts macrophages inside the tissues. Once in the adipose tissues, macrophages are activated and secrete pro-inflammatory molecules maintaining a chronic inflammatory state. This inflammation may spread in the entire body and contribute to the development of metabolic disorders associated with obesity. Apples are commonly consumed fruits that present potential beneficial health effects. We have investigated the anti-inflammatory effects of three different apple extracts on the inflammation of the adipose tissue simulated *in vitro*. Phloretin and phlorizin, two dihydrochalcones found in the extracts, were also tested, at similar concentrations than those found in the extracts, for their potential effect on inflammation. 3T3-L1 adipocytes were cocultivated with LPS activated RAW 264.7 macrophages for 24h in the presence or absence of three different apple extracts or phloretin or phlorizin. The extracts reduced the inflammatory secretions (IL-6, CCL-2 and TNF α) of adipocytes and macrophages cultivated independently and together. These decreases were correlated with a decrease in corresponding gene expressions. The three extracts were also able to reduce the nitric oxide production of macrophages treated with LPS. Nevertheless, we highlighted that the presence of phloretin or phlorizin should not be responsible for the anti-inflammatory effects since the two dihydrochalcones failed to reduce the inflammatory secretions. This work confirms that apple extracts present potential beneficial effect on the development of inflammation in obese adipose tissue that could contribute to prevent the obesity related metabolic disorders. Moreover, the anti-inflammatory effect was not due to phloretin and phlorizin but rather to other compounds or to additive/synergic effects of the whole extracts.

7.1. Introduction

Obesity is characterized by a chronic low grade inflammatory state maintained by the adipose tissue¹. Indeed, in addition to its role in energy storage, the adipose tissue secretes different regulatory molecules. In a healthy state, these molecules, called adipokines, contribute to the metabolism regulation. In case of weight gain, adipocytes enlarge and their secretions are modified. They release more free fatty acids (FFAs) and inflammatory molecules (CCL-2, IL-6) that promote insulin resistance. In addition, macrophages that become present in high number in the obese adipose tissue secrete IL-6, CCL-2, TNF α and NO. The macrophage and adipocyte secretions maintain the inflammatory state of each other.

Numerous evidence has indicated that these secretions are related to obesity complications (type 2 diabetes, atherosclerosis...)^{2,3}. The thiazolidinediones (TZDs), an anti-diabetic drug family, activate the peroxisome proliferator-activated receptor (PPAR) γ , which acts as a key master regulator for the expression of many genes involved in adipose tissue functions. In this way, TZDs decrease IL-6 and TNF α secretion and increase that of adiponectin, an adipokine that regulates the metabolism and improve insulin sensitivity⁴.

Some evidence has shown that natural compounds such as polyphenols are able to decrease the inflammatory state of the adipose tissue. *In vitro* experiments in coculture system have revealed that oligomerized grape seed polyphenols⁵, naringenin chalcone, ... are able to reduce the inflammatory secretion and interaction between the two cells types. Other phytochemicals have also shown interesting effects *in vivo*, *i.e.* green tea catechins, which increased adiponectin and insulin level in type 2 diabetic patients after 12 weeks of exposure⁶, grape seed procyanidins, which reduced IL-6, TNF α and C reactive protein (CRP) expression in adipose tissue of Zucker rats fed with high fat diet⁷... Numerous other studies have proved that fruits, vegetables and other natural compounds found in a normal human diet could have positive effects on the development of

obesity and related complications. Among them, apples have the reputation to be good for health for hundred years. These fruits are consumed in many forms; simply eating raw, in juice, after cooking, ... Their regular consumption seems linked to a decreased risk of cancer⁸, reduced oxidation level of low-density lipoprotein⁹... Several *in vitro*^{10,11,12} and *in vivo*^{13,14} studies have confirmed these effects and identified compounds in the fruit that have high beneficial health potential¹⁵. The phenolic compounds could confer the antioxidant power to the apple¹⁶. They are present mainly in the peel and the total content is higher than in most other classically consumed fruits¹⁷.

Among the phenolic compounds found in apples, phloretin-2-*O'*-glucoside, named phlorizin (PZ) is a dihydrochalcone present principally in the peel. According to the variety, its concentration can reach 16 mg in an apple (150g, 300 mg polyphenols in total)¹⁸. Phlortein (PT), the unglycosylated form of PZ is found in plasma after apple consumption. PT but not PZ has shown anti-inflammatory effects on inflamed RAW264.7 murine macrophages¹⁹. Moreover, whole apple extracts are able to reduce pro-inflammatory secretions and expressions of inflamed intestinal cells *in vitro*^{20,21}. Najafian *et al.* (2012) have highlighted *in vivo* evidence of the effect of PZ on negative parameters linked to obesity. This polyphenol is able to reduce the body weight of diabetic rats and improve their lipid plasma profile¹⁴.

In this study, we have tested the anti-inflammatory potential of three different extracts of whole apple on LPS-activated macrophages and adipocytes cultivated independently and together in a bicameral coculture system. The composition in polyphenols of the extracts has been characterized and PT and PZ have been tested at similar concentrations found in the extracts.

7.2. Materials and methods

7.2.1. Materials and reagents

Three extracts of whole apple fruit *i.e.* AE1, AE2 and AE3 were prepared from *Malus domestica* Borkh, an unknown variety and *Malus pumila* Mill., respectively and provided by Stiernon S.A. (Ghislenghien, BE) as a dry powder. Their phenolic compounds composition is resumed in **Table 7.1**. Phlorizin and Phloretin were purchased from Sigma-Aldrich (St Louis, MO); Triton X-100, LPS, dexamethasone (DEX), 3-isobutyl-1-methyl-xanthine (IBMX) and insulin from Sigma-Aldrich; Dubelcco's Modified Eagle's Medium (DMEM), calf serum (CS), GlutaMax (100x), sodium pyruvate (100 mM), penicillin/streptomycin (100-100 µg/ml penicillin/streptomycin) and 0.05 % trypsin-0.02 % EDTA from Lonza (Verviers, BE) and fetal bovine serum (FBS) from PAA (Linz, AT).

	AE1	AE2	AE3
Total polyphenol content	> 80%	> 90%	> 40%
Catechin	0,4	24,2	2,9
Caffeic acid	1,3	1,1	0
Chlorogenic acid	27,9	107,8	10,9
Coumaric acid	0,7	1,9	0
Quercetin	0,3	2,8	0,5
Phlorizin	54,6	110,8	83
Epicatechin	2,6	41	4,2
Quercitrin	8,3	5,7	0,2
Isoquercitrin	5,5	0,7	0,1
Rutin hydrate	3,1	0,4	0,3
Phloretin	0,04	2,7	0,1
Hyperoside	13,1	4,4	0,2
Avicularin	12,3	1,5	0,18

Table 7.1. Concentration of polyphenols (µg/mg of extract) after filtration

7.2.2. Cell culture

RAW 267.4 murine macrophages (American Type Culture Collection, Manassas, VA) were subcultured in 25 cm² flasks (Corning, Badhoevedorp, NL) in DMEM containing 10 % (v/v) FBS, 1 % (v/v) antibiotics, 1 % (v/v) Na pyruvate and 1 % (v/v) GlutaMax.

The day before the coculture, macrophages were activated by 10 % FBS-DMEM medium supplemented with 1 µg/ml of LPS. As control, the medium was replaced by fresh 10 % FBS-DMEM medium. After 24h of incubation, macrophages were harvested and resuspended in 10 % FBS-DMEM medium for either the coculture or control monoculture.

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were subcultured in 10 cm dish (Corning) in DMEM containing 10 % (v/v) CS, 1 % (v/v) antibiotics, 1 % (v/v) Na pyruvate and 1 % (v/v) GlutaMax. 70 % confluent cells were trypsinized and cultured in 12-well culture plates for coculture assay (Corning). Two-days postconfluent 3T3-L1 preadipocytes, designated as Day 0, were differentiated with DMEM supplemented with 10 % (v/v) FBS, 1 % (v/v) antibiotics, 1 % (v/v) Na pyruvate, 1 % (v/v) GlutaMax and an adipogenic mixture containing 170 nM insulin, 50 µM IBMX and 1 µM DEX for 2 days. Cells were then maintained in the same 10 % FBS-DMEM medium with insulin (170 nM) for another 2 days. Afterwards, the culture medium was replaced every 2 days by FBS-DMEM for four more days. Eight days after differentiation induction, fully differentiated 3T3-L1 adipocytes having accumulated large lipid droplets, were used for coculture experiments (day 8). Both cell types were cultured at 37°C under a humidified, 10 % (v/v) CO₂ atmosphere.

Coculture in bicameral inserts was performed by plating RAW 264.7 macrophages (activated or not, 2x10⁵ cells) in the upper compartment of 0.4 µm pore size insert (Corning) and placing them in 12 wells plate containing the 8 days differentiated 3T3-L1 adipocytes for 24h.

As control, macrophages (activated or not) were also seeded in 12 well plates in FBS-DMEM for 24h. Fully differentiated adipocytes were cultivated alone, in the presence or absence of 1 µg/ml LPS for 24h. Apple extracts and

the phenolic compounds were added in the culture medium for 24h of coculture/monoculture. Extracts were directly solubilized in the culture medium at appropriate concentrations. Phlorizin and phloretin were solubilized in DMSO at 50 mM and added to the culture medium to reach the appropriate concentration. Controls with appropriate vehicles were also realized.

7.2.3. Cytotoxicity assay

The possible cytotoxicity of treatments was routinely checked through the determination of the activity of the lactate dehydrogenase (LDH) released by the cells into the extracellular medium with a cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, DE) used according the manufacturer's instructions. The percentage of cytotoxicity was calculated using the maximum release of LDH activity obtained upon complete lysis of the 3T3-L1 differentiated adipocytes or RAW 264.7 macrophages incubated independently with 1% (v/v) Triton X-100. For the coculture the 100% of LDH release corresponded to the sum of maximum LDH activity of 3T3-L1 and RAW cells cultured independently.

7.2.4. Assay of IL-6, CCL-2 and TNF α secretion

The concentrations of IL-6, CCL-2 and TNF α in the cell supernatants were determined by selective ELISA for mouse IL-6, CCL-2 and TNF α (eBioscience, San Diego, CA) according to the manufacturer's protocol. The supernatants were diluted in order to reach measurable concentrations.

7.2.5. Adiponectin assay

The adiponectin secretion in culture medium was assessed by a commercially available sandwich ELISA kit (Invitrogen, Merelbeke, BE), according to the protocol instructions. Culture media, at day 8, were harvested and centrifuged. The supernatant was diluted in order to reach measurable concentrations.

7.2.6. Assay of nitric oxide release

Total nitrite concentrations in supernatants were determined using Cayman's Nitrate/Nitrite colorimetric Assay Kit (Cayman, Ann Arbor, MI) based on the Griess reaction. The Griess reagents convert nitrite into a red-violet coloured water soluble azo compound. The product absorbance was read at 540 nm and the total amount calculated from the NaNO_2 standard curve.

7.2.7. Protein assay

After the 24h of monoculture/coculture, cells were washed twice and harvested in lysis buffer. After one freezing, total protein content was measured using the BiCinchoninic Acid (BCA) Protein Kit assay (Sigma) according to the manufacturer's protocol. The standard curve was realized with a standard solution of bovine serum albumin (BSA) (Sigma).

7.2.8. Gene expression study

Total RNA was extracted from 3T3-L1 and RAW 264.7 cells using QIAzol reagent (Qiagen, Hilden, DE) according to the manufacturer's instructions. Quality and quantity of total RNA was assessed by Nanodrop spectrophotometry. Total RNA (1 μg) was reverse-transcribed using the Superscript III First-Strand Synthesis System (Invitrogen) following manufacturer's instructions. Then, qRT-PCR was performed in 96 well PCR plates (Bio-Rad, Nazareth Eke, BE), to estimate the mRNA abundance corresponding to either IL-6, CCL-2, iNOS or $\text{TNF}\alpha$ relatively to the housekeeping genes, *i.e.* β -actin, GAPDH and HPRT, used for normalization. A total of 5 μl cDNA with the appropriate dilution, 0.2 μl primer mix and 4.8 μl SYBRGreen SuperMix (Bio-Rad) were added to each well. The PCR amplifications were performed in a MyIQ2 thermocycler (Bio-Rad) as follows: 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. Melting curves were systematically assessed for quality

control of the primers. Gene expression values were normalized to the geometric means of the expression values of β actin, GAPDH and HPRT and calculated on the bases of the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$). The primers used were reported in **Table 7.2**.

Gene name	Forward primer	Reverse primer
β -actin	TAGACTTCGAGCAGGAGATGG	GGATTCCATACCCAAGAAGGA
GAPDH	GTGGAGATTGTTGCCATCAACG	CAGTGGATGCAGGGATGATGTTCTG
HPRT	ACATTGTGGCCCTCTGTGTG	TTATGTCCCCCGTTGACTGA
PPAR γ	CAGTGATATCGACCAGCTGAAC	GGTGATTTGTCCGTTGTCTTTC
Adiponectin	GGAGAGAAGGGAGAGAAAGGTC	AACAGTGACGCGGGTCTCC
IL-6	ACTTCCATCCAGTTGCCTTCT	GAATTGCCATTGCACAACCTCT
CCL-2	GTTGACCCGTAAATCTGAAGC	AAGGCATCACAGTCCGAGTCA
TNF α	TCCCCAAAGGGATGAGAAGTTC	TCATACCAGGGTTTGAGCTCAG
iNOS	ACACTCAGATCATCTTCTCAAAATTTCG	GTGTGGGTGAGGAGCACGTAGT

Table 7.2. Sequences of primer genes used in qRT-PCR

7.2.9. Statistical analysis

All the results were expressed as means $\pm$ SEM. Data were analysed by one-way ANOVA followed by Dunnett post-hoc test to identify mean differences between treatment and positive control. P-values less than 0.05 were considered significant. Statistical analyses were performed using the JMP® 10 software (SAS Institute, Cary, NC).

7.3. Results

7.3.1. Effect of apple extracts, PT and PZ on IL-6, CCL-2 and TNF α secretion by RAW 264.7 macrophages

Macrophages were previously activated with LPS during 24h. The medium was then replaced by a LPS free medium in the presence or absence of the apple extracts or the pure polyphenols. After 24h, media were harvested and pro-inflammatory markers measured. The secretions of IL-6 and CCL-2 were increased of 14 and 1.3 fold, respectively after the LPS activation, as compared to non-activated macrophage secretions. In contrast, TNF α secretion was decreased 24h after LPS activation. The AE1 and the AE2, reduced the secretions of the three cytokines/chemokines. The AE3 reduced the secretion of TNF α and CCL-2 but presented a weak effect on the IL-6 secretion (**Fig 7.1**).

When the extracts were added at the same time as LPS during 24h, we observed that the three extracts were able to reduce the NO release. AE1 at 0.3 mg/ml reduced the NO release by 50 %, AE2 (0.1 mg/ml) and AE3 (0.1 mg/ml) by 30 % (**Fig 7.2**).

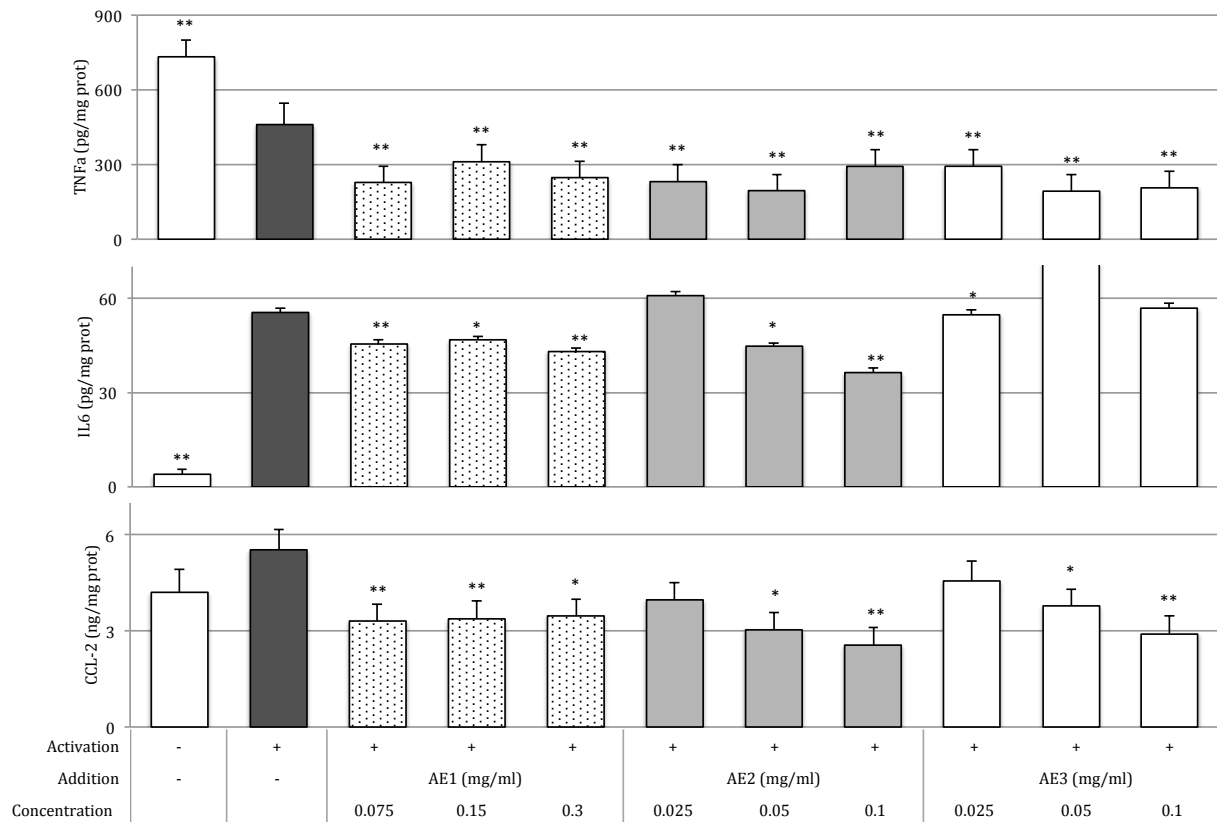


Fig 7.1. Effects of AE1, AE2 and AE3 on TNF α , IL-6 and CCL-2 secretions by macrophages pretreated with LPS. Macrophages preactivated were plated and cultivated during 24h in the presence of the apple extracts at various concentrations. Culture media were then harvested and pro-inflammatory parameters measured. TNF α , IL-6 and CCL-2 secretions were assessed by ELISA and compared to the LPS treated control. Means $\pm$ SEM are given, N = 3; n = 3, (* p<0.05; ** p<0.01).

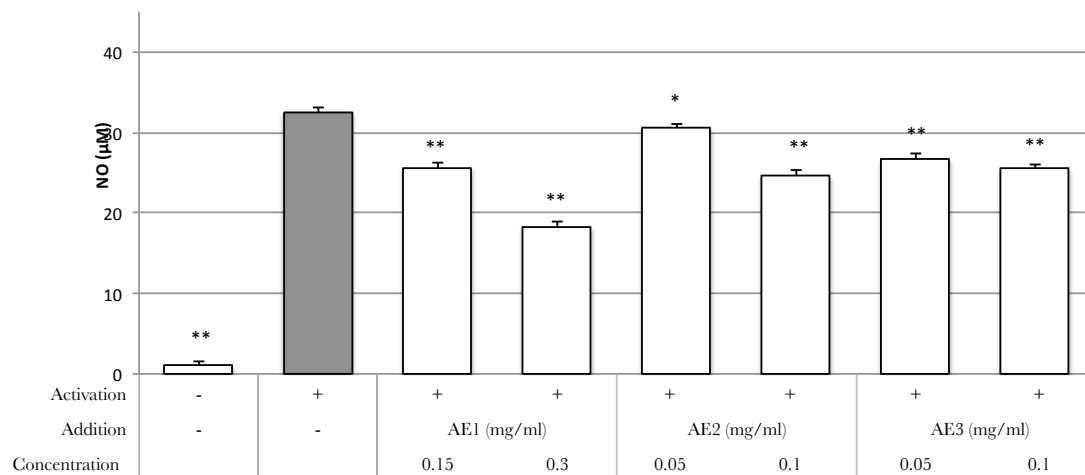


Fig 7.2. Effects of AE1, AE2 and AE3 on NO release by macrophages activated with LPS. Macrophages were plated in a 96 wells plates and cultivated during 24h in the presence or absence of LPS and the apple extracts at various concentrations. Culture media were then harvested and NO releases measured. NO releases were assessed and compared to the LPS treated control. Means $\pm$ SEM are given, N = 3; n = 3, (* p<0.05; ** p<0.01).

Surprisingly, PT and PZ revealed a pro-inflammatory effect and almost doubled the secretion of TNF α and increased slightly the IL-6 release (**Fig 7.3**).

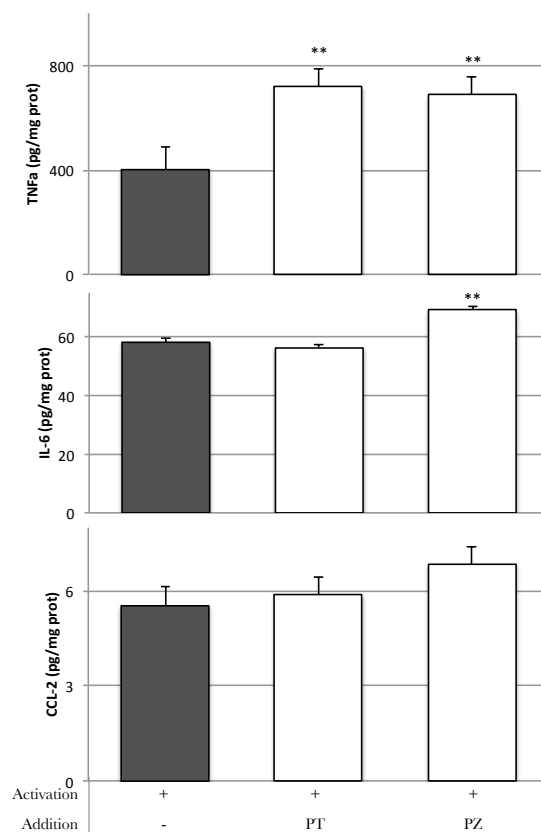


Fig 7.3. Effects of PT and PZ on TNF α , IL-6 and CCL-2 secretions by macrophages pretreated with LPS. Macrophages preactivated were plated and cultivated during 24h in the presence of PT (5 μ M) and PZ (50 μ M). Culture media were then harvested and pro-inflammatory parameters measured. TNF α , IL-6 and CCL-2 secretions were assessed by ELISA and compared to the LPS treated control. Means $\pm$ SEM are given, N = 3; n = 3, (** p<0.01).

7.3.2. Effect of apple extracts, PT and PZ on IL-6, CCL-2 and adiponectin secretions by 3T3-L1 adipocytes activated by LPS

Fully differentiated 3T3-L1 cells were incubated during 24h with 10 % FBS-DMEM medium supplemented with LPS and containing or not the apple extracts or the polyphenols at intermediate concentrations. The LPS

supplementation increased the IL-6 and the CCL-2 secretions by 17 and 4.5 fold, respectively (**Fig 7.4**). The 3T3-L1 cells did not secrete detectable amount of $\text{TNF}\alpha$. AE1 (0.15 mg/ml) reduced the CCL-2 secretion of LPS activated adipocytes to the same level of non-activated cells. This extract also halved the secretion of IL-6; AE2 (0.05 mg/ml) halved the secretion of both pro-inflammatory molecules; AE3 (0.05 mg/ml) only reduced the IL-6 and CCL-2 secretions by 30 %. Both polyphenols presented a lower effect than the extracts; PT (2.5 μM) did not affect the secretions whereas PZ (25 μM) reduced slightly the CCL-2 secretion and halved the IL-6 secretion. The secretion of adiponectin was not modified by the LPS treatment. AE1 and AE2 halved the secretion of the adipokine. The same effects were observed in non activated adipocytes (Data not shown).

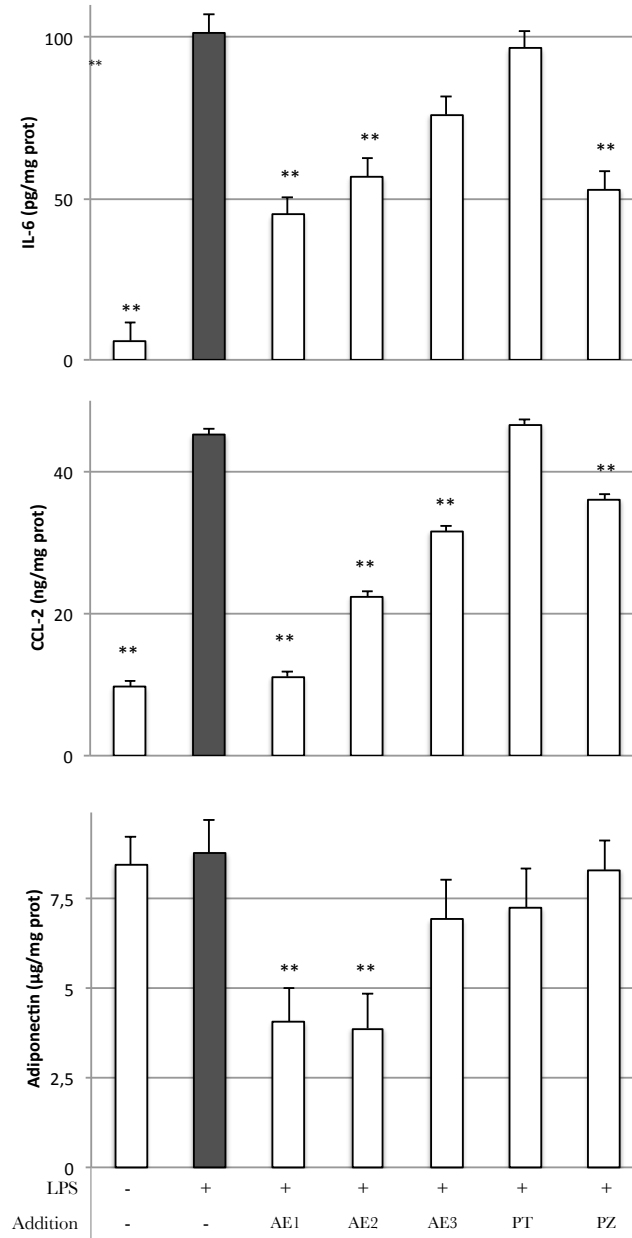


Fig 7.4. Effects of AE1, AE2, AE3, PT and PZ on CCL-2, IL-6 and adiponectin secretions by 3T3-L1 adipocytes activated by LPS. Adipocytes differentiated for 8 days were treated during 24h with AE1 (0.15 mg/ml), AE2 (0.05 mg/ml), AE3 (0.05 mg/ml), PT (2.5 µM) or PZ (25 µM) in the presence of LPS. As controls, adipocytes were incubated in the presence or absence of LPS. Culture media were then harvested and pro-inflammatory parameters were measured by ELISA and compared to the LPS treated control. Means $\pm$ SEM are given, N = 3; n = 3, (** p<0.01).

7.3.3. Effect of apple extracts, PT and PZ on IL-6, CCL-2, TNF α secretions by 3T3-L1 adipocytes and RAW 264.7 activated macrophages in coculture

The effects of the AEs and the polyphenols were investigated in the bicameral coculture system. As already observed on cells cultivated independently, AE1 reduced the secretion of TNF α , IL6 and CCL-2 (**Fig 7.5**). Moreover, this reduction was more apparent than that detected in the monoculture. This was also the case for AE3 that presented a weak effect on the monoculture whereas in the coculture system, its effect is more marked for the three measured parameters. AE2 did not significantly reduced the CCL-2 secretion but showed an effect on TNF α and IL-6 releases.

The pure polyphenols did not affect the pro-inflammatory secretions in coculture except PZ that decreased the TNF α secretion (**Fig 7.6**). None of these treatments affected the secretion of NO and adiponectin in coculture (Data not shown).

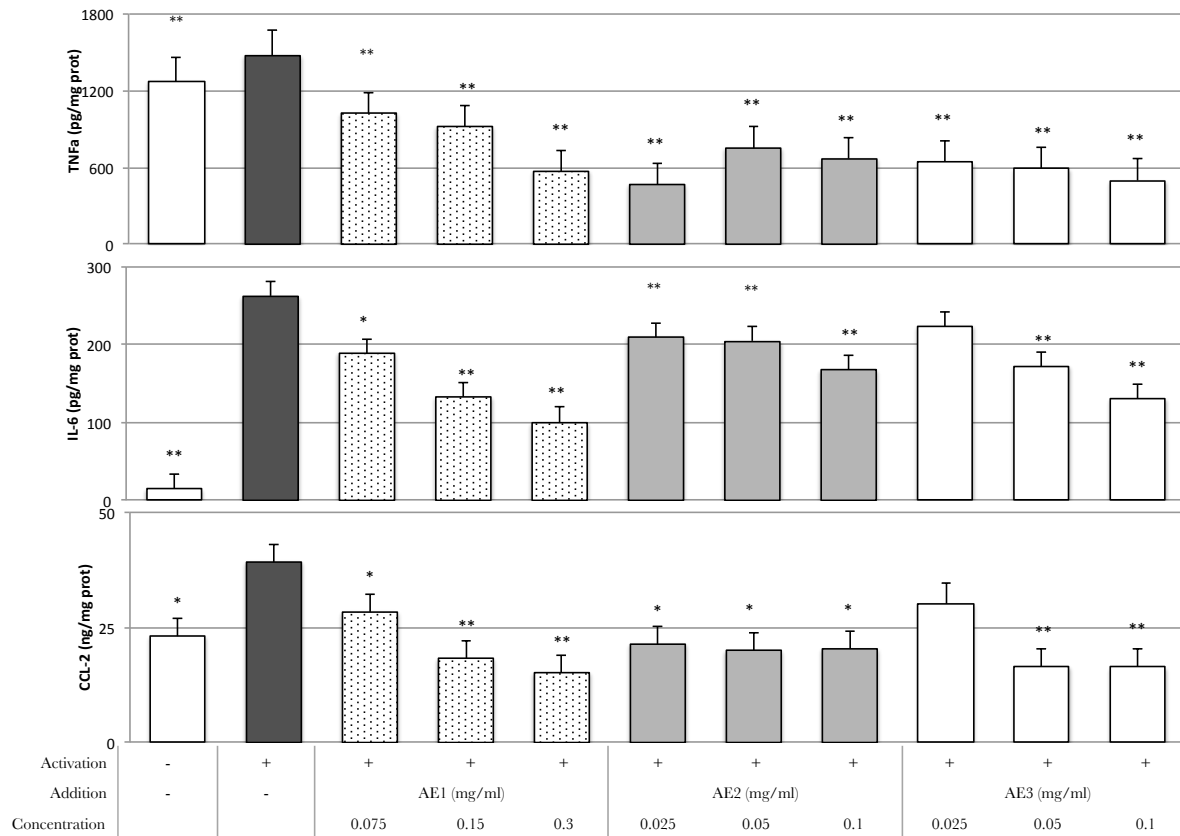


Fig 7.5. Effects of AE1, AE2, AE3 on TNF α , CCL-2 and IL-6 secretions in coculture of 3T3-L1 adipocytes and macrophages preactivated with LPS. 3T3-L1 adipocytes differentiated for 8 days were cocultivated with macrophages pretreated with LPS for 24h in the presence or absence of the extracts at various concentrations. As control adipocytes were cocultivated with macrophages pretreated with LPS or not. Culture media were then harvested and pro-inflammatory parameters assessed by ELISA and compared to the LPS treated controls. Means $\pm$ SEM are given, N = 3; n = 3, (*p<0.05; **p<0.01).

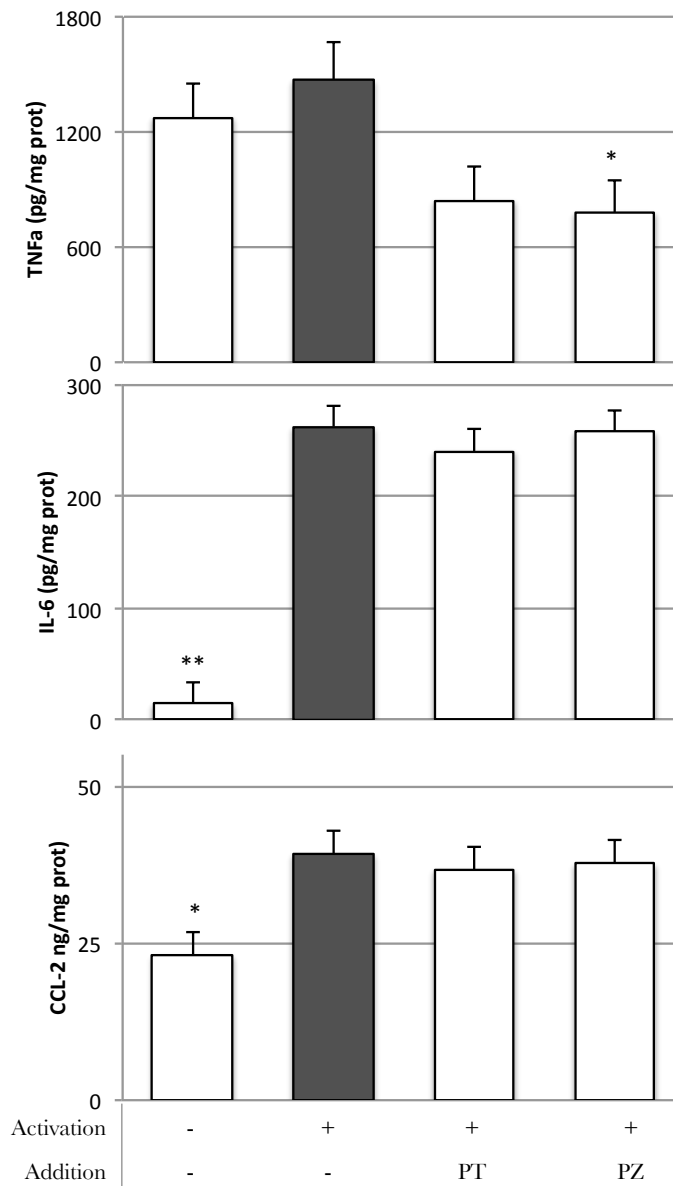


Fig 7.6. Effects of PT and PZ on TNF α , CCL-2 and IL-6 secretions in coculture of 3T3-L1 adipocytes and macrophages preactivated with LPS. 3T3-L1 adipocytes differentiated for 8 days were cocultivated with macrophages pretreated with LPS for 24h in the presence or absence of the polyphenols. As control adipocytes were cocultivated with macrophages pretreated with LPS or not. Culture media were then harvested and pro-inflammatory parameters assessed by ELISA and compared to the LPS treated controls. Means $\pm$ SEM are given, N = 3; n = 3 (*p<0.05; ** p<0.01).

7.3.4. Effect of apple extracts, PT and PZ on the expression of gene related to the inflammatory state in RAW 264.7 macrophages and 3T3-L1 adipocytes activated with LPS

After 24h of monoculture, cells were harvested in QIAzol reagent in order to extract the mRNA and analyse the expression of IL-6, CCL-2, iNOS and TNF α . For the adipocytes, the expressions of adiponectin and PPAR γ were also measured.

In macrophages, LPS increased the expression of inflammatory genes such as TNF α , IL-6 CCL-2 and iNOS, however this increase was not significant for the TNF α gene expression (**Fig 7.7**). The AE2 and AE3 reduced the IL-6 expression until an undetectable level and PT reduced significantly the expression of this cytokine. AE1 and PZ tended also to decrease the IL-6 expression in macrophages although the effect was not significant. The CCL-2 expression was reduced by all the compounds although no significant effect was measured. Finally, no effect was observed on the expression of TNF α and iNOS mRNA in macrophages excepted AE3 that increased the expression of iNOS.

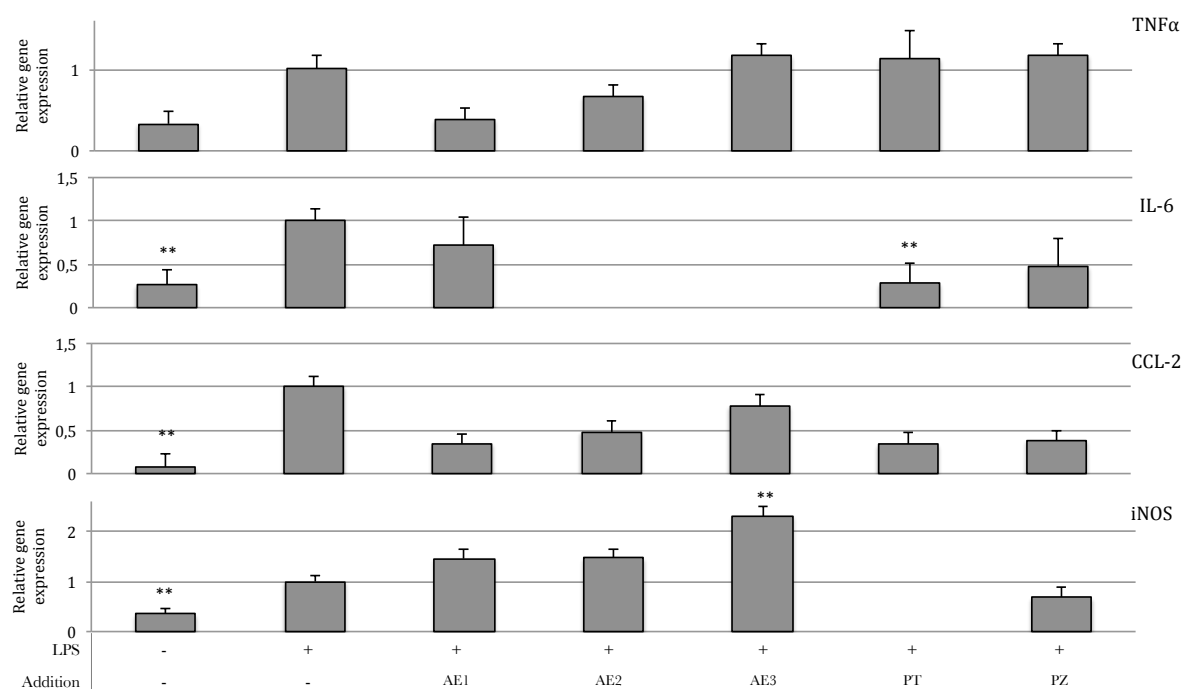


Fig 7.7. Relative gene expressions in macrophages activated with LPS after 24h of treatment with AE1 (0.15 mg/ml), AE2 (0.05 mg/ml), AE3 (0.05 mg/ml), PT (2.5 μ M) or PZ (25 μ M). Inflammation markers were analysed by qRT-PCR and normalized by the geometric mean of the three reference genes and relative to non activated control value. The IL-6 gene expression after the treatment with AE2 and AE3 was too low to be detected, as well as the expression of iNOS after PT treatment. Means $\pm$ SEM are given and compared to the activated control, N=3 n=1 (* $p < 0.05$; ** $p < 0.01$).

In adipocytes, the LPS did not significantly increase the expression of the inflammatory genes and it had no effect on the expression of PPAR γ and adiponectin after the 24h of incubation (**Fig 7.8**). The extracts did not show any effect on the inflammatory gene expressions. AE1 and AE3 induced an increase of the expression of PPAR γ and adiponectin.

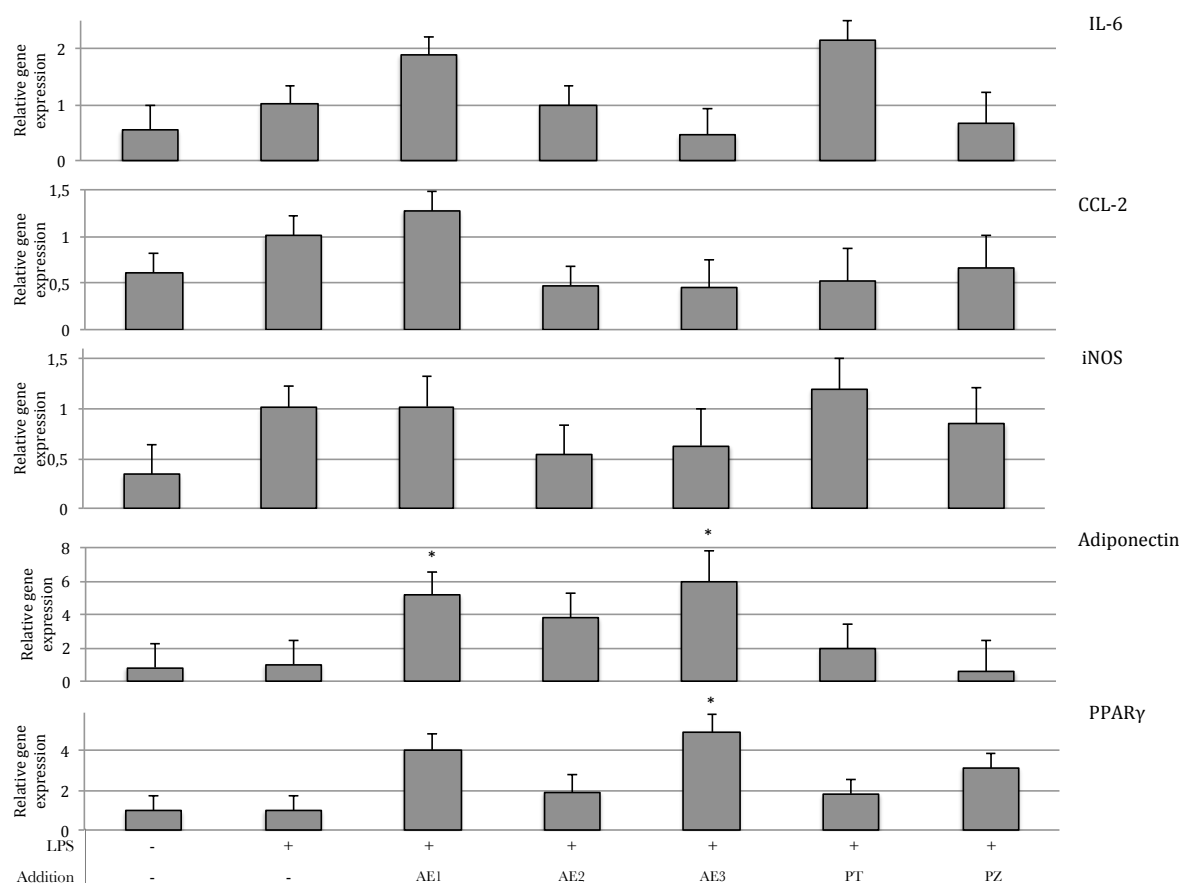


Fig 7.8. Relative gene expressions in adipocytes treated during 24h with LPS in the presence of absence of AE1 (0.15 mg/ml), AE2 (0.05 mg/ml), AE3 (0.05 mg/ml), PT (2.5 μ M) or PZ (25 μ M). Inflammation markers were analysed as well as adiponectin and PPAR γ by qRT-PCR and normalized by the geometric mean of the three reference genes and relative to non activated control value. Means $\pm$ SEM are given and compared to the activated control, N=3 n=1 (* p<0.05; ** p<0.01).

7.3.5. Effect of apple extracts, PT and PZ on the expression of gene related to the inflammatory state in RAW 264.7 macrophages and 3T3-L1 adipocytes after 24h of coculture

The coculture affected significantly the related inflammatory gene expression in both cell types. In macrophages cocultivated with adipocytes, AE1, PT and PZ reduced the CCL-2 expression (**Fig 7.9**). PZ was also responsible for a decrease in TNF α expression whereas AE3 increased it slightly. However, none of these effects were significant. IL-6 mRNA was not detectable in the macrophages samples.

Concerning the adipocytes cocultivated during 24h with the LPS-activated macrophages, we observed that AE1, AE3, PT and PZ reduced the expression of all the pro-inflammatory genes followed, *i.e.* IL-6, CCL-2 and iNOS (**Fig 7.10**). The IL-6 expression appeared to be significantly down regulated by AE1 and PT. PT also reduced drastically the expression of CCL-2 until a level corresponding to a real down-regulation. PPAR γ expression was significantly decreased by PT.

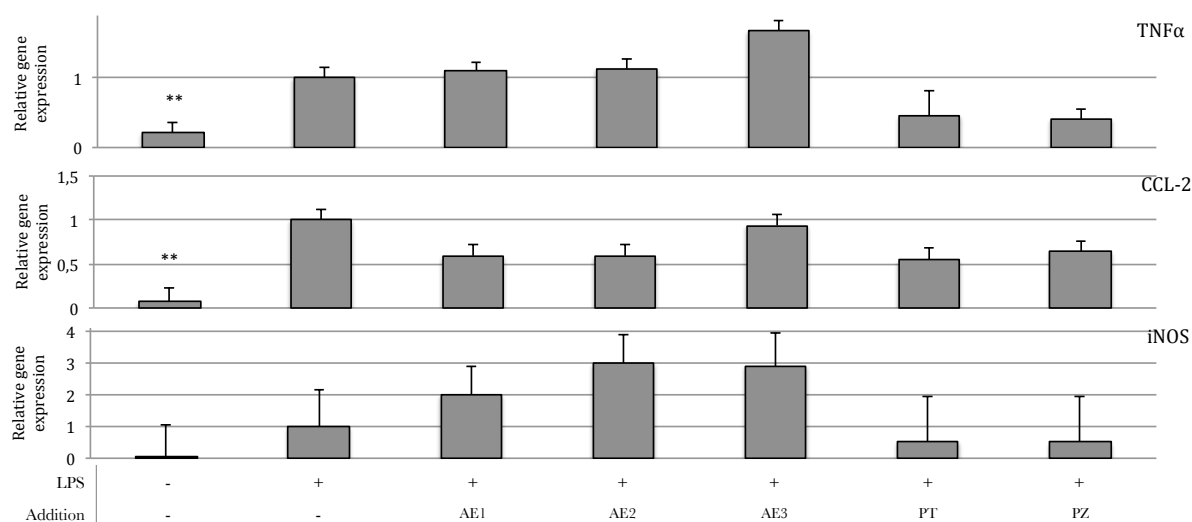


Fig 7.9. Relative gene expressions in macrophages after 24h of coculture in the presence or absence of AE1 (0.15 mg/ml), AE2 (0.05 mg/ml), AE3 (0.05 mg/ml), PT (2.5 μ M) or PZ (25 μ M). Inflammation markers were analysed by qRT-PCR and normalized by the geometric mean of the three reference genes and relative to non activated control value. Means $\pm$ SEM are given and compared to the activated control, N=3 n=1, (* $p < 0.05$; ** $p < 0.01$).

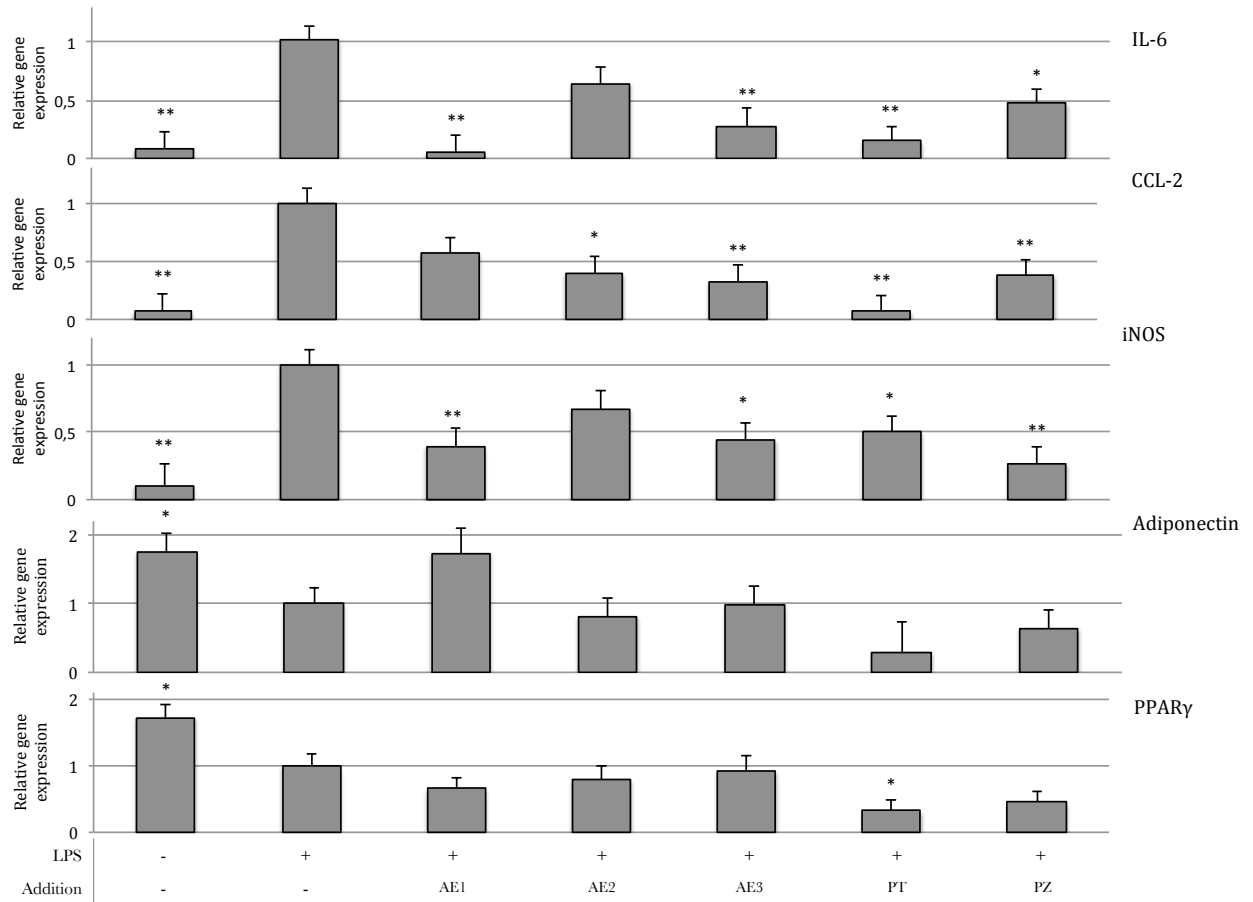


Fig 7.10. Relative gene expressions in adipocytes after 24h of coculture in the presence or absence of AE1 (0.15 mg/ml), AE2 (0.05 mg/ml), AE3 (0.05 mg/ml), PT (2.5 μ M) or PZ (25 μ M). Inflammation markers, adiponectin and PPAR γ were analysed by qRT-PCR and normalized by the geometric mean of the three reference genes and relative to non activated control value. Means $\pm$ SEM are given and compared to the activated control, N=3 n=1, (* $p < 0.05$; ** $p < 0.01$).

7.4. Discussion

It has been clearly reported that obesity is linked to an inflammatory state that contribute to the development of metabolic disorders²². The reduction of the pro-inflammatory secretions by the adipose tissue is an interesting approach to prevent or reduce the health negative impact of obesity such as insulin resistance²³. Apples have already demonstrated some beneficial effects on inflammation. An *in vitro* study on human intestinal or immune cells has highlighted that an apple extract, as well as two polyphenols found in apple, *i.e.* PT and procyanidin B₂, reduced the expression of inflammatory genes by acting on the NF- κ B pathway²¹. In addition, apples may have positive effect on obesity development as shown in a study from Bocqué *et al.* (2013) revealing that the consumption of apple extract by rat fed with high fat sucrose diet reduced the body weight gain and the total fat mass. The extract also improved the insulin resistance of the animals, as compared to the control group²⁴. These observations suggested that apples could affect the inflammatory state associated with obese adipose tissue.

For this study, we chose to test three apple extracts prepared from different varieties. AE1 concentration used in the experiments was three fold higher than those of AE2 and AE3 in order to reach PZ concentration normally found after apple ingestion. AE1 extract contained 5% of PZ although AE2 and AE3 contained 15% of PZ.

We have shown that the three apple extracts tested decreased the inflammatory state of both macrophages and adipocytes. However, the magnitude of their effects was different. It appeared that AE1 had the most powerful effect on adipocyte inflammatory secretions (IL-6, CCL-2) (**Fig 7.4**). As adipocytes secreted more IL-6 and CCL-2 than macrophages did, it was not surprising to observe that AE1 was the most efficient extract in coculture to reduce the IL-6, CCL-2 as well as TNF α secretions (**Fig 7.5**). AE2 is the extract that contains the higher amount of polyphenols (>90%) compared to AE1 (>80%) and AE3 (>40%). Its effect on inflammation, although interesting, was weaker than the effect of the two other extracts at

the same concentrations. So, it appeared that the polyphenol content did not really impact on the anti-inflammatory potential.

AE3 was the extract presenting the best effect on the TNF α secretion by macrophages cultivated alone and in coculture by halving it even at a low concentration of 0.025 mg/ml (**Fig 7.1** and **Fig 7.5**). As this cytokine is a key pro-inflammatory mediator in obesity, reducing its secretion could be a good approach to prevent insulin resistance. Indeed, TNF α , secreted by macrophages in adipose tissue, acts on adipocytes by increasing the amount of released FFAs and by decreasing their adiponectin secretion²⁵. Both of these actions contribute to the development of insulin resistance associated with obesity. In addition, AE3 increased adipocyte adiponectin expression after 24h of incubation (**Fig 7.8**). However, no effect was observed on the secretion. Taken together, these observations made of AE3 a potential candidate for the prevention of obesity complications.

Surprisingly, AE1 and AE2 halved the adiponectin secretion of LPS-treated adipocytes (**Fig 7.4**) while the gene expression was increased. The same effect was observed in untreated adipocytes (Data not shown). At this time, we are unable to explain this observation. More investigations have to be done in order to verify whether after 48h the level of the secreted adipokine becomes higher or whether the extracts interacted directly with the protein.

In order to verify if the two polyphenols, *i.e.* PT and its glucoside conjugated, PZ, which represent from 33 (AE2) to 81 (AE3) % of the total phenolic compounds, could be the constituents responsible for the anti-inflammatory effect of the apple extracts, they were tested as pure compounds in the *in vitro* systems. We did not observe really significant effect of these polyphenols at the same concentrations than those found in the extracts. A previous study had however demonstrated that PT at a high concentration (50 μ M) reduced IL-6 gene expression in 3T3-L1 adipocytes¹⁰. Moreover, Chang *et al.* (2012) reported that PT but not PZ

reduced the IL-6, TNF α and NO release by activated RAW 264.7 macrophages²⁶. But as in the study of Hassan *et al.* (2010), the tested concentrations were higher than those used in this study and far from plausible physiological concentrations. At a concentration closer to ours (3 μ M), Chang *et al.* (2012) have shown that PT did not reduce the inflammatory secretions²⁶.

Whatever, the polyphenols PT and PZ are most probably modified *in vivo* by the digestion process. The lactase phlorizin hydrolase, present in the membrane of intestinal cells, hydrolyses PZ into PT²⁷. PT is then conjugated to glucuronides and/or sulphate or could also be degraded by the microflora. So free forms of PZ and PT are not observed in the plasma, except after ingestion of high doses²⁸.

This work highlighted that, according to the cultivar, variations in the anti-inflammatory effects of apple extracts on the adipose tissue could differ. However apples, at least for the three extracts that have been tested, seem to reduce the inflammation state of adipocytes and macrophages as well as the inflammatory vicious cycle existing between both cell types. We also demonstrated that the observed effect of the extract was not entirely due to the polyphenols PT and PZ. Other active compounds could be quercetin and its glycosylated forms, which are mainly present in the AE1 (16 μ M in 0.15 mg/ml of AE1). Indeed, quercetin has already proved anti-inflammatory effect on adipocytes exposed to macrophages conditioned medium²⁹ and on LPS stimulated macrophages³⁰. AE2 was richer in catechin and epicatechin (19 %) than the two other extracts (2 and 7 % in AE1 and AE3, respectively), however, only a few studies have investigated the potential anti-inflammatory effect of catechins. More investigations have to be done to identify the active compounds in the extracts and/or to evidence potential additive or even synergistic effects linked to the simultaneous presence of various phenolic compounds, as well as other substances, in the apple extracts.

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8. General discussion and conclusion

This work aimed at investigating, on the one hand, the interactions between adipocytes and macrophages within the adipose tissue and, on the other hand, to evaluate potential beneficial effects of vegetal extracts from apple and pomegranate. For that purpose, we set up a coculture system consisting of adipocytes and macrophages able to mimic, as close as possible, the inflammatory conditions existing in the adipose tissue of the obese patient. These people present a chronic low grade inflammation state in their adipose tissue that is strongly linked to the development of metabolic disorders such as insulin resistance, cardiovascular complications ... It is known that the inflammatory mediators secreted by this tissue come mainly from the adipocytes and the macrophages. The phagocytic cells are attracted and activated inside the adipose tissue through secretions from the hypertrophied adipocytes. Once in the adipose tissue, the macrophages maintain an inflammatory vicious cycle with the surrounding adipocytes and both cells maintain the chronic inflammatory state in the adipose tissue that may spread in the whole body and induce or maintain metabolic disorders.

8.1. The coculture system

Our system of coculture was able to reproduce at least partially the inflammation of the adipose tissue. Indeed when macrophages previously activated were added in the bicameral insert above the adipocytes, the secretion and expression of two pro-inflammatory factors, *i.e.* IL-6 and CCL-2, were significantly higher than those calculated from the sum of monocultures of adipocytes and macrophages as well as from the coculture of non inflamed cells. Our experimental approach was based on the model of Suganami *et al.* (2005) who were the first to develop a coculture system with adipocytes and macrophages in order to simulate the inflammatory state of adipose tissue¹.

The coculture used by Suganami *et al.* allowed the contact between the two cell types. They showed that this system allowed to increase the CCL-2

secretion more than 10 fold when compared to the sum of the monoculture secretions¹. In our case, the secretion increased only by 1.5 fold and the preactivation of macrophages with LPS impacted only slightly on this secretion (2 fold). So, our coculture system did not allow to reach the CCL-2 secretion level obtained when the cells are in direct contact. However, the increase of the CCL-2 secretion that we obtained was significant and sufficient to measure a potential impact of the different treatments. Concerning the TNF α , the contact coculture system induced a 3 fold increase. In our case, no significant effect on TNF α secretion was obtained, indicating that the contact between adipocytes and macrophages could be important to induce the secretion of this cytokine.

Among others, some critical points have to be discussed:

- ✓ It appeared that contact between adipocytes and macrophages played a role in the inflammatory induction process. Indeed, a recent evidence highlighted that a couple of receptor-ligand, *i.e.* 4-IBB and 4-IBBL, in the membrane of adipocytes and macrophages, respectively, induced the secretion of IL-6, CCL-2 and TNF α by the activation of the NF- κ B and MAPK pathways (**Fig 8.1**)². However, although the disruption of the 4-IBB/4-IBBL interactions decreased the cytokines/chemokine secretions, the authors did not observe a total suppression of the marker secretion (slight reductions of 33, 22 and 11 % of the secretions of IL-6, CCL-2 and TNF α , respectively). This indicated that other receptor/ligand couples but also soluble factors contributed to the induction of the inflammatory secretions. Other studies using conditioned media have revealed that soluble factors are able to induce and maintain the inflammatory secretions^{1,3,4}; TNF α as well as adiponectin are two well-known soluble molecules that act on the secretion of adipocytes and macrophages, respectively^{5,6,7}.

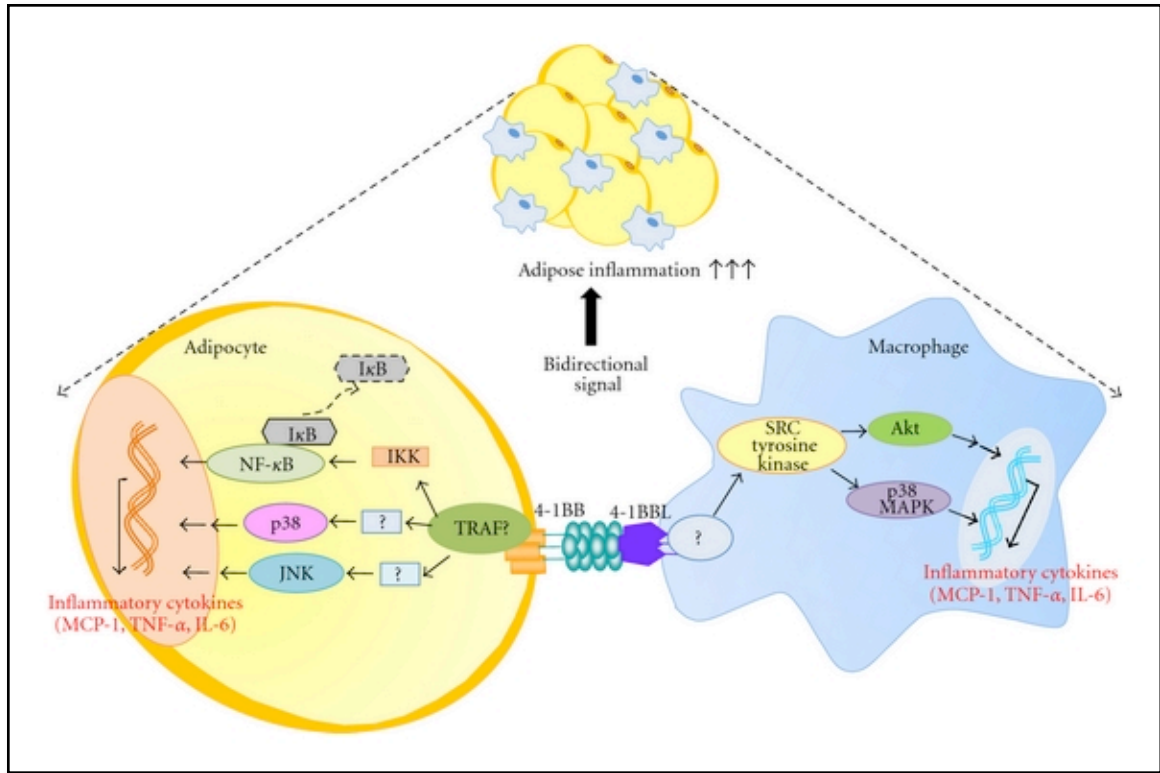


Fig 8.1. Interaction between adipocytes and macrophages by the receptor/ligand couple 4-1BB/4-1BBL leading to the activation of pro-inflammatory cascades. TNF α , CCL-2 and IL-6 are released by both cell types in response to bidirectional signalling. Tu *et al.* (2012)

So even though our coculture system did not allow the contact between the cells, as it occurs *in vivo*, it allowed to increase significantly the pro-inflammatory secretion of both cell types. This high level of secretion was reached thanks to the pre-activation of the macrophages in order to better simulate the *in vivo* obese conditions where macrophages present mainly the M1 activated profile⁸.

- ✓ In addition to the “non contact” difference, the coculture only used two different types of cells whereas numerous other cells are present in the adipose tissue and may contribute to the inflammatory state. T cells, preadipocytes and other cells are also present in the tissue and secrete molecules that influence the inflammatory state (**Fig 8.2**)⁹. CD8⁺ T cells, like macrophages, accumulate in the adipose tissue of obese patient. CD8 deficient mice did not show significant increase of

macrophage infiltration under a high fat diet and presented lower expression level of cytokines compared to control mice¹⁰. Natural killer T (NKT) cells and eosinophils are also involved in the adipose tissue inflammation^{11,12}. Whatever the *in vitro* system used, it would be always far from the *in vivo* reality.

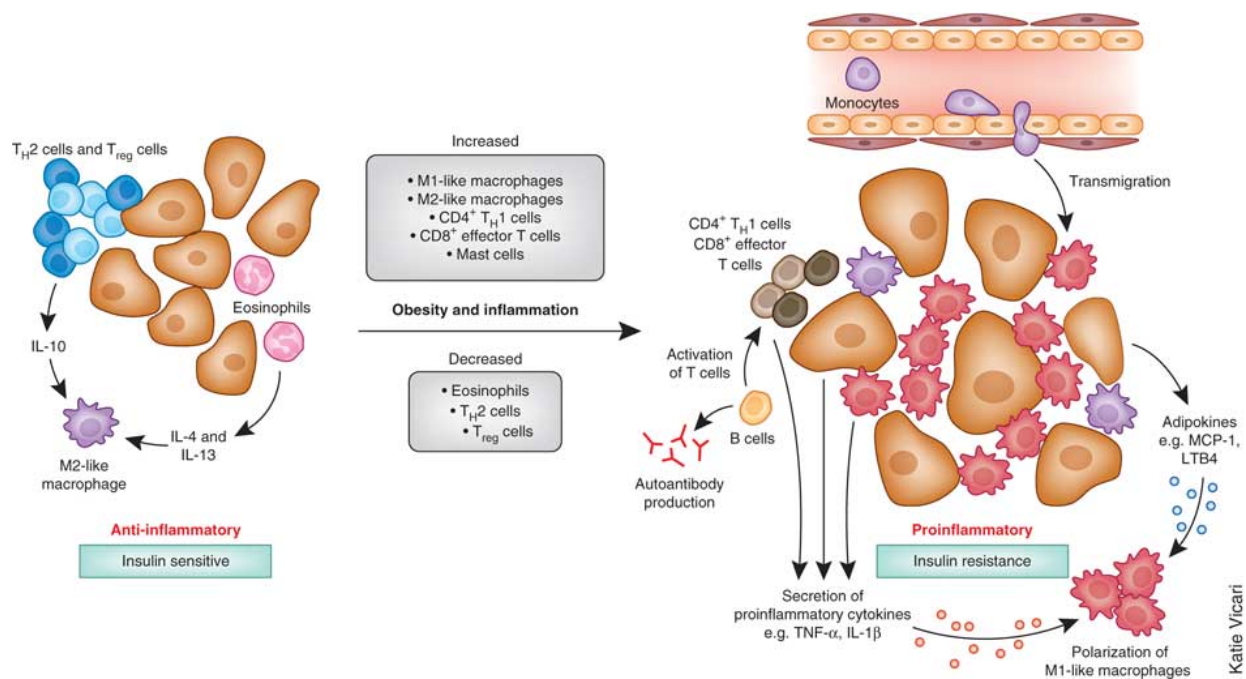


Fig 8.2. Obesity development and adipose tissue modifications. Cell infiltration and activation associated with obesity. In the lean state, the main cells composing the adipose tissues are the adipocytes, the M2 macrophages, the eosinophils and the T_H2 and T_{reg} cells. The eosinophils maintain the anti-inflammatory state by secreting IL-4 and IL-13. In case of obesity, the hypertrophied adipocytes secrete chemoattractive signals that attract monocytes and activate them, inducing their polarization until M1 pro-inflammatory macrophages. These M1 macrophages induce a decrease in the T_{reg} and eosinophil populations and an increase in the T_H1 and $CD8^+$ population cells. All these cells contribute to the inflammatory secretions. Osborn *et al.* (2012)¹³

- ✓ Finally, the coculture was set up with murine cells, which could present differences with the human cells. Indeed, for example, in mouse, the adipokine resistin is secreted exclusively by adipocytes whereas in human it is secreted mainly by the macrophages¹⁴. Other differences between murine and human adipocytes or macrophages have been reported: (i) murine adipocytes are more sensitive to induction of apoptosis, (ii) murine adipocytes synthesize fatty acid mainly from glucose whereas human adipocytes do it in a fewer

extent¹⁵, (iii) human macrophages seems to express differently the iNOS¹⁶... These differences among others are important and should be considered.

Other models of coculture using human cell have already been used¹⁷. Coculture of human primary adipocytes with human primary macrophages or macrophage cell lines. Even if the utilization of human primary adipocytes allows to be closed to the reality, their availability is limited. Indeed, the sampling technique, the preparation and the variability between individuals make this type of culture very complicated and expensive.

A human preadipocyte cell line characterized in 2001 has the ability to differentiate in adipocytes. These cells were extracted from the SVF of the SCAT of an infant suffering of the Simpson-Golabi-Behmel syndrome (SGBS) and were called the SGBS adipocyte cell line. These cells could be differentiated with a cocktail similar to the one used with 3T3-L1 and keep their differentiation ability until the passage 30¹⁸. Keuper *et al.* (2011) have tested coculture of SGBS adipocytes with THP-1 human macrophage cells¹⁷. The THP-1 macrophage cell line is the most described and used cell line. These cells are first in a monocyte state and have to be differentiated in macrophages. When cocultivated with human SGBS adipocytes, they induce, as expected, the pro-inflammatory secretions and inhibit the adipocyte differentiation.

Although these numerous drawbacks should be taken into account, the model presented advantages.

- ✓ It involved the two cell types that are the most abundant in the obese adipose tissue and that secrete the bulk of the pro-inflammatory molecules affecting the metabolism¹⁹.
- ✓ It allowed us to distinguish the paracrine/autocrine effects from the contact effect.

- ✓ Suganami *et al.* (2007) have shown that the inflammatory pathways in the bicameral coculture system are induced in the same way than in the contact system²⁰.

Despite its limitations, the system used in this work has allowed us to investigate the potential effect of vegetal extract on the deleterious interactions between the adipocytes and the macrophages by focusing on the autocrine/paracrine interactions.

8.2. Adipogenesis and lipid accumulation, controversial effects

The other part of this work was to investigate the potential effect of the different extracts on adipogenesis and triglyceride accumulation. As exposed, the effects observed were opposite according to the extracts used, *i.e* the apple extracts tended to reduce the triglyceride accumulation whereas the pomegranate extract increased the amount of triglycerides stored in adipocytes (hypertrophy). However, both fruit extract tended to reduce the number of differentiated adipocytes (hyperplasia) although PT increased the number of differentiated cells. All of these effects, although opposed, could present positive effects on obesity development. However, as the control of metabolism is very complex, these results have to be put into perspectives.

- ✓ The TZD antidiabetic drug has the ability to increase the number of adipocytes²¹, activate PPAR γ ²², increase adiponectin secretion²³, which allow to improve the insulin sensitivity. These facts prove that increasing the number of adipocytes could be a good way to restore the insulin sensitivity. Indeed, as the number of adipocytes is higher, the amount of triglycerides stored by cells is reduced. Small adipocytes are more insulin sensitive than big adipocytes²⁴, moreover, they secrete less pro-inflammatory cytokines than bigger ones²⁵. An interesting fact is that TZD promotes adipocyte

differentiation specifically in the SCAT and not in the VAT, which is associated with insulin resistance¹⁵. As the SCAT responds better to insulin signaling, increasing the number of adipocytes in this tissue allows to restore more effectively the FFA plasma level.

- ✓ However, in case of obesity prevention, as the number of adipocytes could not be decreased, it seems not interesting to increase the number of adipocytes since it would allow to store more easily energy excess. Indeed, it is known that ex-obese people (who have more adipocytes than lean people) re-gain weight faster than people who have never been obese²⁶. In conclusion, in normal condition, reducing the level of new differentiated adipocytes could prevent a potential weight gain but, in case of obesity, it appears important to increase the number of adipocytes in order to improve metabolic complications.
- ✓ Concerning the adipocytes sizes, numerous evidences highlight a correlation between the size and the pro-inflammatory secretions. Big adipocytes secrete more pro-inflammatory molecules than smaller ones^{25,27,28} and have a better ability to release FFA but also to catch it from the circulation²⁹. Again, depending on the localization of the adipose tissue (SCAT or VAT), the adipocyte morphology will impact more or less on the pro-inflammatory state and FFA plasma level.

8.3. Pomegranate husk extract and its main phenolic constituents

As already mentioned, the choice of the pomegranate husk extract was not trivial as this extract already revealed anti-inflammatory effects in previous studies on human Caco-2 cells in a model of the intestinal barrier³⁰. This study made in our group was consolidated by other works revealing a potential anti-inflammatory effect of pomegranates^{31,32}. In addition to its

effects on inflammation, the pomegranate had also revealed some potential effects on obesity prevention³³.

The results obtained during this work have highlighted *in vitro* evidences explaining previous *in vivo* observations:

- The pomegranate husk extract reduced the number of differentiated adipocytes to about 60% upon exposure at the early stage of the differentiation process. This indicated that pomegranate could reduce the hyperplasia that occurs during the development of obesity. A recent *in vivo* study on mice fed with high fat diet supplemented with a pomegranate husk extract (6 mg/day) during 4 weeks, revealed that the PHE tended to decrease the amount of visceral and subcutaneous adipose tissue and significantly decreased the epididymal adipose tissue proportion as compared to the mice fed only with the high fat diet³⁴. So, PHE clearly could influence the fat storage *in vitro* but also *in vivo*.
- In addition, our *in vitro* experiments showed that the adipocyte phenotype is modified under the action of PHE and the cells present bigger droplets than normal adipocytes at the same stage. This indicates that the extract could promote hypertrophy of adipocytes, which is not specially a good point since bigger adipocytes secrete proportionally more pro-inflammatory cytokines than smaller adipocytes²⁵. However, it would favor a more important storage in adipocytes, preventing the development of new adipocytes in order to face the request of storage.
- A recent work has shown that 3T3-L1 cells differentiated in the presence of PHE during 8 days presented less lipid accumulation due to an inhibitory effect on the fatty acid synthase³⁵.
- PHE decreased the pro-inflammatory secretions and expression in adipocytes and to a lesser extent in macrophages *in vitro*. This is correlated with its effect to reduce the expression of COX-2 and IL-1 β in the visceral adipose tissue of mice fed with high fat diet³⁴.

Punicalagin, the main ellagitannin found in pomegranate, is present in higher amount in the husk than in the flesh of the fruit. The PHE used in this work is particularly rich in this polyphenol (9% w/w)³⁰. Punicalagin presented characteristics that made it a good candidate as an active molecule to fight obesity complications.

- In a previous work, we showed that this polyphenol inhibited the pancreatic lipase activity³⁶. This property could induce a reduction of the lipid digestion and absorption.
- More recently, Wu *et al.* (2013) have revealed that punicalagin, as PHE and ellagic acid, inhibited the fatty acid synthase activity with an IC₅₀ of 4.5 μ M (all substrates confounded)³⁵. This inhibitory effect came out as a reduction of lipid accumulation in 3T3-L1 adipocytes differentiated in the presence of these extracts. At similar concentration to ours, punicalagin and ellagic acid reduced the lipid content by 60%, as compared to control lipid content. As the FAS starts to synthesize triglycerides from day 3³⁷, it is normal that in our case, the lipid accumulation was unaffected.
- Punicalagin, but not ellagic acid, increased the number of small lipid droplets in adipocytes aged of 15 and 20 days, as compared with control adipocytes at the same stage. Moreover, punicalagin decreased the number of differentiated cells.
- After 24h, punicalagin, and ellagic acid to a lesser extent, was able to reduce the secretion and expression of IL-6 and CCL-2 by adipocytes activated with LPS and also in coculture. This indicates that a longer exposure to this vegetal extracts probably significantly decreased the expression of these genes. The effect of punicalagin and ellagic acid on macrophages secretions was also more marked than the effect of PHE.
- As punicalagin and ellagic acid belong to the hydrolysable tannins family, it is probable that they lead to protein precipitation. For the FAS, this was not the case³⁵. Hollebeeck *et al.* (2012) have shown that punicalagin directly interacted with IL-6³⁰. However, the ratio

between the concentrations of punicalagin and IL-6 used in the test was very different to the one found in these experimental conditions. In our experimental conditions, punicalagin, either PHE or ellagic acid, did not interact with the cytokines/chemokine (IL-6, TNF α and CCL-2) (Data not shown).

In conclusion, these observations, made of the pomegranate husk extract a good candidate to reduce the lipid accumulation in case of obesity. This, correlated with its clear inhibitory effects on pro-inflammatory adipocyte secretions, indicates that PHE could prevent obesity complications by acting principally on adipocyte functions. Among the polyphenols contained in this extract, punicalagin has shown powerful effects on inflammation of adipose tissue as well as on adipogenesis.

8.4. Apple extracts, phloretin and phlorizin

Apples, inversely to pomegranates, are present in many varieties and forms in our supermarkets making its daily consumption easier. The variety of polyphenols found in apples and their concentrations are higher than those found in other currently consumed fruits in Europe³⁸. Although numerous good health effects are attributed to apples, less is known about their mechanisms of action. Among these effects, some evidences showed that apples may act on metabolic syndrome development³⁹ but no *in vitro* experiments allowed to explain this effect.

Observations made in this work, as well as previous evidence, made of apples good candidates to prevent and fight against obesity development and complications:

- The three extracts decreased inflammatory secretions of macrophages and adipocytes cultivated independently and together. Most of these observations were correlated with a decrease of the corresponding gene expression. However, depending on the cultivar the effects are more or less pronounced.

- The AE1 and AE2, acted on adipogenesis by decreasing the number of differentiated adipocytes and the triglycerides accumulation. These two extract slowed down the lipid accumulation as compared to control adipocytes.
- These effects are not due to the dihydrochalcones PT and PZ. Other polyphenols present in the extracts could probably contribute more significantly to the effects of apples.
- Among the three extracts that were tested, AE1 cumulated a lot of powerful effects, *i.e.* an important decrease of inflammation, adipogenesis and lipid accumulation. Compared with the two other extracts, AE1 contains a higher amount of quercetin glycosides; *i.e.* quercitrin, isoquercitrin, hyperoside, rutin hydrate and avicularin that reached 33% of the total phenolic content (against 4.5% and 1.4% for AE2 and AE3 respectively). These quercetin derivatives present a high antioxidant activity corresponding to 27.5% of the total antioxidant power of apple polyphenols⁴⁰. The glycosylated forms of quercetin have already proved *in vitro* anti-inflammatory effect on macrophages by decreasing the NO and the PGE₂ release^{41,42,43}. Moreover, isoquercetin has already shown an inhibitory effect of adipogenesis in 3T3-L1 cells by activating the Wnt/ β -catenin signalling pathway⁴⁴. The powerful effect of AE1 could be explained by the presence of these flavonoids.
- The other extracts AE2 and AE3 presented also good effects that could be assimilated to the same or other phenolic compounds. It is also important to mention that synergic effects between polyphenols could exist. For example, when resveratrol and quercetin are used together, they decrease the lipid accumulation in 3T3-L1 cells by 68.6 % as compared to 9.4 and 15.9 % when resveratrol and quercetin are used alone respectively. The same synergistic effect is observed on apoptosis of the 3T3-L1 cells⁴⁵.
- Phloretin and phlorizin are known to inhibit intestinal glucose absorption⁴⁶.

- Previous experiments in our laboratory have revealed that quercetin inhibited the pancreatic lipase activity leading to a decrease of triglyceride digestion³⁶.

The effects already observed *in vivo* after apple consumption⁴⁷, found the some explanations in the results obtained in this work. By reducing the inflammatory secretions of macrophages and by acting on adipocyte functions, apples could have a better potential profile than pomegranates. Moreover, the potential impact on digestive functions may also contribute to the prevention of obesity development. Indeed, PT and PZ are known to inhibit intestinal glucose absorption by acting on GLUT2⁴⁸ and seems to reduce the activity of digestive enzymes such as amino-peptidase⁴⁶, maltase⁴⁶ or pancreatic lipase⁴⁹. Moreover, other polyphenols present in apples, *i.e.* quercetin and its derivatives, also impact on some of these enzymes^{36,48}. So, the apple consumption could reduce the digestion/assimilation of sugar or lipid leading to a reduction of calorie absorption.

8.5. Potential effect on inflammatory transcription factors

Although the effect on the inflammatory gene expressions indicates that the PHE or AEs could interfere with inflammatory pathways, we did not investigate during this work the potential action of PHE or AEs on the well-known inflammatory transcription factors *i.e.* NF- κ B, ERK, JNK and p38. Numerous studies using the same coculture system of adipocytes and macrophages have highlighted that natural compounds could decrease the inflammatory secretions by acting on these transcription factors in macrophages and/or in adipocytes:

- by inhibiting the phosphorylation of JNK, luteolin was able to decrease the expression and secretion of pro-inflammatory molecules by macrophages in coculture⁵⁰;

- by acting on the phosphorylation of ERK1/2, the paeoniflorin inhibited the lipolysis of adipocytes in coculture resulting in a decrease of TNF α secretion by macrophages⁵¹;
- oligomerized grape seed polyphenols decreased the NF- κ B transcriptional activity in adipocytes and the phosphorylation of ERK1/2 in the cocultivated cells leading to a decrease of pro-inflammatory secretions⁵².

These evidences indicate that, on the one hand, various transcription factors could be the target of the active molecules present in vegetal compounds and that, on the other hand, the vicious cycle could be broken by acting on only one cell type. In the bicameral system used in our work, the NF- κ B and MAPK (ERK, JNK and p38) pathways are also activated as reported by Suganami *et al.* (2007). So we could postulate that the different extracts used in this work and having an effect on the inflammatory secreted molecules act on at least one of these pathways. Numerous evidences strengthen this assumption:

- A polyphenol-rich pomegranate extract has already shown inhibitory effect on (1) the activation of MAPK by decreasing the phosphorylation of JNK and ERK and (2) the activation of NF- κ B by inhibiting the degradation of I κ B in basophilic human cell line KU812⁵³;
- A study on KBM-5 human myeloid cell highlighted that a pomegranate juice, punicalagin but not ellagic acid interfered with the NF- κ B DNA binding⁵⁴;
- Previous studies in our group, on human Caco-2 cells, have reported that various polyphenols, and among them ellagic acid and quercetin, could decrease the signalling NF- κ B activation pathway⁵⁵;
- Phloretin, as well as procyanidin B₂, reduced the NF- κ B activity in human colon DLD-1 cells, human leukemia T-cell Jurkat and in human monocytic leukemia MonoMac6 cells⁵⁶;

- An apple peel extract inhibited the ERK and JNK activity in epidermal JB6/AP/ κ B transformed murine cells⁵⁷.

We could postulate that the extracts used in this work act on these pathways, which should result in a decrease in the expression of the inflammatory genes *in vitro*.

8.6. Bioavailability and bioaccessibility

As a model of adipose tissue, our cells were always exposed to the pure filtrated extract. Obviously, *in vivo*, the ingested extract will never arrive, as it is, until the adipose tissue.

Sadly, the **bioaccessibility**, which is related to the food matrix, and therefore the **bioavailability** of the whole extracts used, are unknown. Nevertheless, for PHE, a previous study on its fate during digestion had been done by Hollebeeck *et al.* (2012) indicating that punicalagin is partially transformed in ellagic acid⁵⁸. The impact of digestion on other phenolic compounds has not been investigated, as well as the impact of other parameters such as enterocyte transformations and microflora actions. However, as mentioned earlier, Neyrinck *et al.* (2012) have shown that mice fed with a PHE presented modifications of adipose tissue functions (inflammatory profile and weight)³⁴ indicating an effect of the extract on the adipose tissue. Only a few other *in vivo* studies have focused on the impact of pomegranate directly on adipose tissue functions. But the effects of pomegranate derivative consumption on the metabolic disorder exists clearly⁵⁹. So, the pomegranate, and more particularly the pomegranate husk extract, is a vegetal product presenting a high potential to prevent the obesity development and related complications.

Concerning the apple extracts, one study, using rats fed with high fat diet, has shown that the consumption of an apple extract (0.7 g/kg rat, per day) decreased the weight gain and the total adipose tissue weight. The extract also decreased the level of circulating leptin compared with the group fed

only with high fat diet³⁹. This study, although using a high amount of extract, provided evidence that apple compounds act on the adipose tissue. The bioavailability of some polyphenols found in apple has already been studied. For example, it is known that quercetin is readily bioavailable⁶⁰ while procyanidins are large molecules virtually not bioavailable⁶¹. In addition, phenolic compounds undergo metabolization that adds moiety to their natural free structure. Glucoronidation, sulphatation, methylation are different modifications that occur after the ingestion of the polyphenols. Only PT has been identified in its free form in the plasma⁶². Furthermore, it is known that these modifications change the potential effect of the polyphenols although it was reported that, at the cellular level, the conjugated molecules could be deconjugated into their free forms⁶³.

A study comparing the antioxidant function of pomegranate juice to apple juice in humans revealed that, even if the pomegranate juice polyphenol content was five fold higher than in the apples, the two juices presented the same level of antioxidant capacity in plasma⁶⁴. *In vitro*, the pomegranate juice showed five fold more antioxidant activity compared with apple juice. This may indicate that the apple polyphenols could be more bioaccessible and bioavailable than the pomegranate polyphenols.

8.7. Conclusion

To conclude, this work has allowed to strengthen recommendations for fruits and vegetable consumptions. We highlighted that the apple and pomegranate extracts, in addition to contribute to the amelioration of the quality of diet, could potentially interfere with the weight gain and/or its harmful effects.

By developing a coculture system able to mimic the inflamed obese adipose tissue and using *in vitro* adipocyte cell line, we have been able to:

- show the mechanism whereby PHE and punicalagin could prevent the obesity development and complications by acting on adipogenesis and reducing inflammatory secretions of inflamed adipocytes
- demonstrate that apple extracts present anti-inflammatory potent effects combined with anti-adipogenic effects making of this commonly consumed fruit a good candidate to contribute to the prevention of obesity development and complications

Even if these first investigations have obviously to be confirmed by *in vivo* studies, pomegranates and apples could be good candidates to contribute to counter obesity development and complications. A daily consumption of these fruits could improve, at least partially, the body state. The substitution of high calorie content snacks with apples or pomegranates will first reduce the calorie intake because of their low calorie content and their action on digestive enzymes, secondly bring important nutrients such as vitamins, fibers,... and thirdly, suggested in this work, influence cell functions and improve body conditions. In this work we have highlighted effects on adipose tissue functions but other studies have shown that apples or pomegranates impact on cancer development, inflammatory bowel disease, diabetes...

So, the regular consumption of these natural extracts could potentially improve the obesity development and/or complications. However more investigations have to be done in order to strengthen these first results. The last chapter will propose future experiments to embellish this work.

8.8. References

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9. Perspectives

Although the obesity development could be prevented by improving food intakes and increasing energy expenditure, its occurrence increases each year. This problem concerns all the worldwide population, rich and poor countries, adult and children ... Except for some acute cases where surgical approaches provide significant results, most existing drugs do not really reveal long term effects as this “fatty” state present many metabolic component interacting with each other. So targeting an unique part of the metabolic chain appears to fail almost systematically. It is the reason why the development of a “multi-level” approach to counter the obesity appears very important.

In this work, we have shown that natural extracts of fruits, *i.e.* pomegranate and apple, have the power to modify, improve, interfere with molecular deregulation that occurs in obese adipose tissue. These *in vitro* experiments, in addition to the *in vivo* existing studies, proved that natural products have the ability to improve human health.

The results obtained in this study open up new perspectives in the prevention of the obesity development.

9.1. Additional *in vitro* investigations

Concerning the global effect of the different extracts that have been tested during this work (anti-adipogenic effect combined with anti-inflammatory one), it would be interesting to investigate **the long term effect of the extracts**.

Indeed, in this work, by adding the extracts at the same time that the beginning of the coculture or during the four first days of the differentiation, we only focused on the effect at this specific moment. However, as already shown by other works, an incubation during all the differentiation process (8 days) could favour more pronounced effect on adipocytes functions¹. This type of treatment would correspond to a daily

ingestion of the extracts during a long period compared to a ponctual ingestion that we simulated in our work.

The experiment would consist in the addition of the extracts during the differentiation process until their coculture with macrophages. This could confirm our first results postulating the preventive effects on obesity complications.

Another important aspect followed by many *in vitro* studies is the **impact on lipolysis**. Indeed, TNF α secreted by macrophages induces lipolysis by the adipocytes. The release of FFAs in high amount contributes to the development of insulin resistance and maintains the inflamed state of surrounding macrophages. As observed in our work, the treatment with PHE or apple extracts at the early stage of the differentiation modified the lipid distribution, which could impact on the exchange of FFAs between the cells and the extracellular environment. In order to verify the impact of the extracts on the lipolysis rate of adipocytes, it would be interesting to stimulate differentiated adipocytes, pre-treated with the extracts, with TNF α in order to quantify the lipolysis rate and the amount of glycerol released in the culture medium.

Adipocytes of obese tissues are also characterized by an **insulin resistance** induced by the TNF α . Experiments should be performed to investigate whether the extracts could improve this insulin resistance. The experiment would consist in measuring the 2-deoxi-D-[1- ^{3} H] glucose (2-DOG) uptake induced by insulin upon treatment with TNF α in the presence or absence of the extracts. TNF α , by increasing the insulin resistance, induces a decrease of 2-DOG uptake. As pomegranate and apple extract have already shown effects on insulin sensitivity of peripheral tissues, we could expect that they will increase the 2-DOG uptake as compared to the control condition^{2,3}.

As already mentioned above, the coculture system that was used presented limits, as the cells did not **establish contact** between each other. So, it will

be relevant to verify if the anti-inflammatory effect observed in the indirect coculture system would still be observed in a coculture system wherein the cells would be in a direct contact. Recent work has showed that CD11b MicroBeads system allows to isolate macrophages (CD11b⁺ cells) from adipocytes cultivated in a contact coculture system. This could permit to determine the gene expressions of both cell separately while verifying if the extracts impact on one of the cells or both⁴.

Another strategy would consist in working with **adipose tissue explants** from obese mice. These *ex vivo* conditions present the advantage that they would include all the cell types that are present and contribute to the inflammatory conditions. The explant utilisation should allow a closer approach of the *in vivo* situation and to verify if the anti-inflammatory effects are confirmed.

As also mentioned in this work, the extracts tested were always added in the medium in their “pure” form. However, it is clear that if these compounds are ingested, they will undergo the digestion process. For the PHE, Hollebeeck *et al.* (2012) have clearly highlighted that punicalagin is degraded during the *in vitro* digestion and that this degradation is actually mainly due to the alkaline conditions encountered in the duodenal phase⁵. Moreover, it is known that ellagic acid may be further modified by the microflora and that the metabolites, urolithin A, B, C or D, are found in the plasma after pomegranate juice consumption⁶. According to the studies, the ellagic acid and punicalagin may be present in the plasma after the consumption of a pomegranate juice but never at high concentrations^{6,7}. The ***in vitro* digestion model** developed by Hollebeeck *et al.* (2012)⁸ should be used to digest the different extracts, which would be then added in the coculture system. This would be a first hint of the residual effect of the extracts after the digestion.

As the microflora degradation also impact on the extract composition and residual activity, it would be interesting to add a microflora digestion step

to the digestion model. It would consist in incubate the extract, previously digested, with a fecal sample from a human healthy subject⁹.

Or, the SHIME, for Simulator of Human Intestinal Microbial Ecosystem, which is a validated dynamic model of the complete gastrointestinal tract, would be used to perform all the steps of the digestion. Indeed, in addition to the gastric and duodenal steps developed by Hollebeeck *et al.* (2012), this system offers an *in vitro* adapted microbial community, which would be easier controlled than the microflora of human subject.

Obviously, all these additional *in vitro* experiments could be discuss because they are still very far from the *in vivo* reality. But they have the benefit to be easy set up and to allow a first quick screening of the extracts, as well as a better mechanistic approach.

9.2. *In vivo* investigations

The pomegranate husk and the apple extracts were provided in the form of a dry powder, which would make easy their formulation to facilitate ingestion, after addition in a food matrix or dissolution in water. Previous works using the same kind of extract have been done by adding the extract in the water or in the diet of mice¹⁰.

It would be interesting to fed mice or pigs, for instance, with a high fat diet supplemented or not with the PHE or apple extracts by respecting plausible amount of ingested extracts. In order to investigate the potential effect on obesity development of the extracts numerous factors would be followed:

- body weight and total fat mass
- insulin resistance could be evaluated by a hyperinsulinemic euglycemic clamp experiment¹¹.
- glycemia, insulinemia, level of circulating adiponectin and leptin

- other plasma biomarkers (cholesterol, triglycerides, HDL, LDL, CCL-2, CRP, IL-6). Cholesterol, triglycerides and HDL levels could be measured by enzymatic techniques. Then, the LDL level could be deduced by using the Friedewald formula^{12,13}. CCL-2, IL-6 and CRP are molecules easily detectable by ELISA or RIA.
- adipose tissue functions
 - the presence of M1 activated macrophages could be revealed by using immunohistochemistry techniques. After a section in a frozen adipose tissue, mounting on slide and fixation, an incubation with appropriate antibodies (against F4/80) would be performed. The secondary antibody coupled with a peroxidase would be used in order to visualize the macrophages
 - the number and size of adipocytes could be determined by ten μm thick sections of different adipose tissues. A microscope coupled with a camera would allow to make image analysis¹⁴.
 - the triglyceride content of adipocytes and their repartition
the expression of IL-6, TNF α , CCL-2, adiponectin in adipose tissue. Total RNA could be extracted from visceral adipose tissue in order to follow the expression of these parameters.

As the first goal of the Delhaize Chair was to further evaluate experimentally approaches to prevent obesity by the combination of diet rich in polyphenols with physical exercises, the consumption of such natural compounds combined with physical exercises would be the last study closing this multi-disciplinary project. The laboratory of Pr Marc Francaux has already started this investigation with the pomegranate husk extract. Some studies have already proved that exercise training inhibits inflammation in adipose tissue of mice fed with a high fat diet. Mice running 60min/day during 16 weeks did not show significant differences in body weight or fat mass as compared with the control group. However, the number of infiltrating and activated macrophages as well as the expression

of inflammatory genes in adipose tissues were clearly lower than that in the control group¹⁵. We would expect that the supplementation with one of the extracts used in this work would bring more important impacts.

9.3. References

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