Relazione del Collegio dei Docenti del Dottorato di Ricerca in
Ambiente, Salute e Processi Ecosostenibili
Facoltà di Ingegneria, Dipartimento di Modellistica per l'Ingegneria
Università della Calabria
XXVII Ciclo

Nell'ambito del Corso di Dottorato di Ricerca in "Ambiente, Salute e Processi Ecosostenibili", la dr.ssa **Anita Schella** ha svolto una ricerca riguardante la verifica degli effetti salutistici dall'assunzione di un nuovo preparato a base di olio d'oliva extravergine , sotto la direzione del prof. Sergio Mazzulla dal titolo "SPREAD BIO OIL INTAKE AND THE LOWER LIKELIHOOD OF BEING OBESE, HYPERTENSIVE AND DIABETIC".

La ricerca è stata realizzata principalmente presso i laboratori del DIBEST dell'Unical, e per circa un anno presso la School of Pharmacy, Department of Nutrition, Food Science and Physiology (CAF), della University of Navarra Pamplona, (Spain).

Il lavoro svolto dalla dr.ssa **Anita Schella** è inserito in un progetto PON sviluppato presso il DIMES che riguarda la formulazione di un olio dalla caratteristica di spalmabilità. L'allieva ha studiato sia teoricamente che sperimentalmente i possibili effetti salutistici per verificarne l'effetto benefico nel caso di soggetti obesi, diabetici and ipertensivi. Le prove sperimentali sono state eseguite su ratti assoggettati a diete ricche di questo nuovo prodotto reso disponibile dal DIMES, evidenziandone l'attività vasodilatatoria sulle arteriole mesenteriche.

Lo studio sperimentale è stato condotto con grande dovizia e attenzione, necessarie per le sue potenziali ricadute, che richiedono una particolare accuratezza.

Il lavoro risulta esaustivo dell'argomento, con una buona ricerca bibliografica ed eseguito con estrema cura, e i risultati ottenuti aprono ad ulteriori possibili sviluppi ed interpretazioni.

L'intero lavoro di ricerca approfondisce tematiche e metodiche non trattate nei corsi fondamentali e molto interdisciplinari, per cui la dr.ssa **Anita Schella** ha dovuto impegnarsi per apprendere queste tecniche mostrando un buon grado di autonomia e capacità di apprendere.

La dr.ssa **Anita Schella** ha partecipato alle attività dei diversi laboratori che ha frequentato acquisendo dimestichezza anche in altri settori lontani dalla sua formazione di base, mettendo in evidenza buone doti e capacità scientifiche, oltre che buone qualità relazionali, che le hanno consentito una facile interazione con tutti i gruppi di ricerca non solo del laboratorio in particolare con quelli dell'Università di Navarra con cui ha instaurato un solido contatto. Come conseguenza di questa sua attività ha pubblicato su riviste scientifiche alcuni dei risultati sperimentali ottenuti.

Il Collegio dei Docenti, visto l'impegno profuso e la qualità della sua attività, esprime un giudizio pienamente favorevole ai fini dell'ammissione della dr.ssa **Anita Schella** all'esame finale per il conseguimento del titolo di Dottore di Ricerca in "Ambiente Salute e Processi Ecosostenibili".

il Coordinatore del Collegio
Prof. Bruno de Cindio

Addì, 29 novembre 2014

UNIVERSITÀ DELLA CALABRIA

UNIVERSITY OF CALABRIA

Department of Biology, Ecology and Earth Sciences

PhD School "Life Sciences"

PhD in "Health, Ambient and Eco-sustainable Processes"

UNIVERSITY OF NAVARRA SUPPORTING

XXVII CYCLE

EXTRA VIRGIN OLIVE OIL INTAKE AND THE LOWER LIKELIHOOD OF BEING OBESE, HYPERTENSIVE AND DIABETIC

BIO/09

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ABBREVIATIONS

ADIPORs	Adiponectin Receptor
AKT	Protein Kinase B
AMPK	AMP-Activated Protein Kinase
ANGPTL2	Angiopoietin Like Protein 2
Apo A-I	Apoprotein AI
Apo B-I	Apoprotein BI
APPL1	Adaptor protein, Phosphotyrosine interaction, PH domain and Leucine zipper containing 1
ATLG	Adipose Triglyceride Lipase
ATP	Adenosine Triphosphate
BAT	Brown Adipose Tissue
BMI	Body Mass Index
BP	Blood Pressure
C/EBPβ	CCAAT/Enhancer Binding Protein beta
C1q	Complement C1q subcomponent
CAV1	Caveolin 1
CCL2	Chemokine Ligand 2
CD36	Cluster of Differentiation 36
CD91	Cluster of Differentiation 91
COMT	catechol-O-Methyl-Transferase
CRP	C-Reactive Protein
CXCL5	CXC-Chemokine Ligand 5
DG	Diglyceride
EAT	Exercise-Induced Activity
eNOS	endothelial Nitric Oxide Synthase
ERKs	Extracellular Signal-Regulated Protein Kinases
EVOO	Extra virgin olive oil
FA	Fatty Acid
FAO	Fatty Acid Oxidation

FAOSTAT	Food and Agriculture Organisation of the United Nations
FASN	Fatty Acid Synthase
FDA	Food and Drug Administration
FFA	Free Fatty Acid
GDP	Guanosine Diphosphate
GI	Gastrointestinal
GLUTs	Glucose Transporters
GPCRs	G-Protein-Coupled Receptors
GTP	guanosine triphosphate
HBS	Hexosamine biosynthesis
HDL-C	High-Density Lipoprotein
HFD	High-Fat Diet
HIFs	Hypoxia-Inducible Factors
HMW	High–Molecular Weight
HOCXC9	Homeobox 9
HSL	Hormone-Sensitive Lipase
HPLC	High-performance liquid chromatography
ILs	Interleukins
NOS2	inducible Nitric Oxide Synthase
IOOC	International Olive Oil Council
IRS-1	Insulin Receptor Substrate 1
LCN2	Lipocalin 2
LDL-C	Low-Density Lipoprotein
LDLR	Low density lipoprotein receptor
LG	Lipogenesis
LMW	Low–Molecular Weight
LP	Lipolysis
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemoattractant Protein-1
MCTs	Monocarboxylate Transporters
Med Diet	Mediterranean Diet
MEKs	Mitogen-Activated Protein Kinase
MGL	Monoacylglycerol Lipase

MMP-9	Matrix metallopeptidase 9
MMW	Middle–Molecular Weight
MUFA	Mono Unsaturated Fatty Acids
NAMPT	Nicotinamide Phosphoribosyltransferase
NEAT	Non Exercise-Induced Activity
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
NPRA	Atrial Natriuretic Peptide Receptor
OPO	Pomace Olive Oil
p38MAPK	p38 Mitogen-Activated Protein Kinases
PAI-1	Plasminogen Activator Inhibitor 1
PDO	Protected Designation of Origin
PGE-2	Prostaglandin E2
PGI	Protected Geographical Indication
PGI2	Prostaglandine
p-HPEA	p-Hydroxyphenil ethanol
p-HPEA-EDA	Dialdehydic form of decarboxymethyl etenolic acid linked to p-HPEA
PI3K	Phosphatidylinositide 3-Kinases
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKCs	Protein kinases C
PKs	Protein kinases
PPARGC1A	Peroxisome Proliferator-Activated Receptor γ Coactivator 1α
PPARs	Peroxisome Proliferator-Activated Receptors
PTEN	Phosphatase and Tensin Homolog
PUFA	Polyunsaturated Fatty Acids
PYY	Peptide Tyrosine Tyrosine
RAF	protein-serine/threonine kinase
Ras	Rat Sarcoma
RBP-4	Retinol-Binding Protein 4
RER	Rough Endoplasmic Reticulum
ROO	Refined Olive Oil
ROS	Reactive Oxygen Species
RXR	Retinoid X Receptor
SAT	Subcutaneus Adipose Tissue

SER	Smooth Endoplasmic Reticulum
SFA	Saturated Fatty Acid
sFRP5	Secreted Frizzled-Related Protein 5
SGLT1	Sodium-Glucose Trasporter
SHOX2	Short Stature Homeobox 2
SNP	Single-Nucleotide Polymorphism
SOCS	Suppressor of Cytokine Signaling S
SOS	Son of Sevenless
SR-A	Steroid receptor RNA Activator
SREBP-1c	Sterol Regulatory Element Binding Protein-1c
T2D	Type 2 Diabetes
TBX15	T-Box Transcription Factor 15
TFA	Trans Fatty Acid
TFAM	Transcription Factor A, Mitochondrial
TGs	Triglycerides
TLR	Toll-Like Receptors
TNF	Tumor Necrosis Factor
UCP1	Uncoupling Protein-1
UNESCO	United Nations Educational, Scientific and Cultural Organization
USA	United States of America
VAT	Visceral Adipose Tissue
VCAM1	Vascular Cell Adhesion Molecule 1
VOO	Virgin Olive Oil
WAT	White Adipose Tissue
WHO	World Health Organization
WNT5a	Protein Wnt-5a
ZIC1	Zinc Finger Protein 1
β-Ars	β-adrenoreceptors

CHAPTER 1
ADIPOSE TISSUE DYSFUNCTION IN OBESITY

1.1 Introduction

Obesity is a worldwide phenomenon which affects people from different cultural and economic backgrounds (WHO 2014), who tend to eat more fat and sugar, while reducing their intake of complex carbohydrates and dietary fibers. Globally, there has been an increase both in the amount of people consume and sedentary works. On the other hand, the modes of transportation and the increasing urbanization typically resulting in decreased of physical activity (Dean et al. 2014). In 2008 the WHO estimated that more than 35% adults aged 20 and over were overweight and 11% obese (Fig. 1). However in 2011 more than 40 million children under the age of five were overweight, with a raising to 2.1 billion in 2013 (Ng et al. 2014). Are considered overweight people who have a BMI between 25.0 and 29.9 and obese whose have a BMI of 30.0 (Table 1) (Anthes 2014).

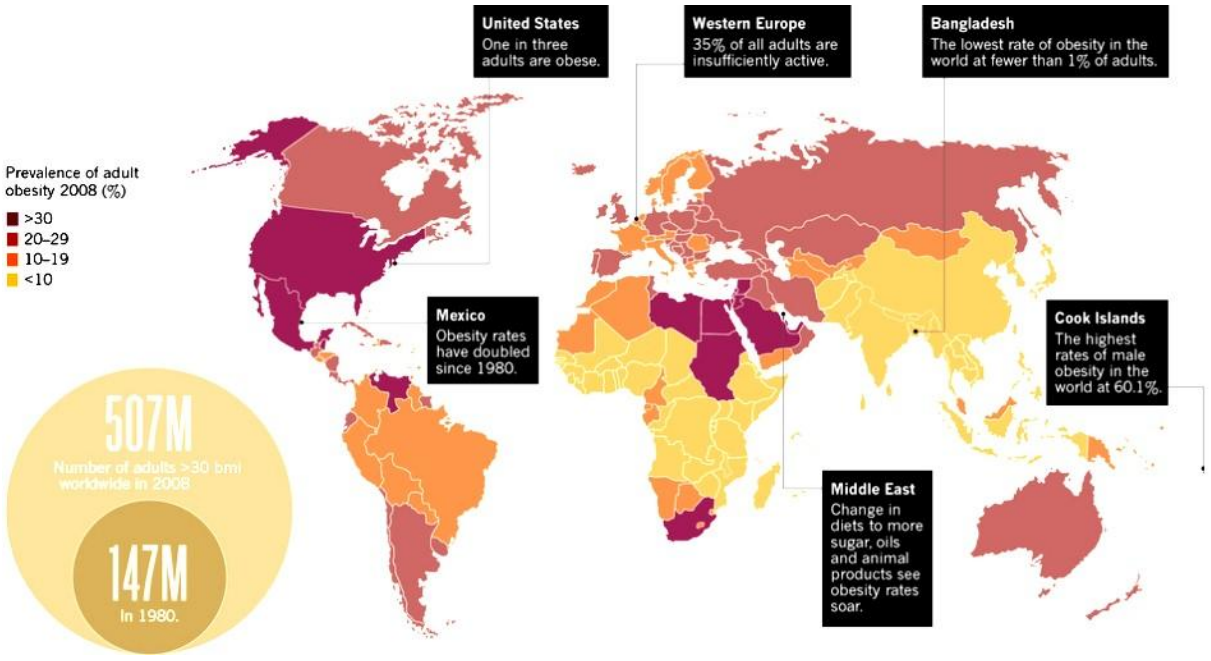


Figure 1: Global Spread. World Health Organization estimation about aged-related overweight and obese individuals in 2008. Modified from Scully (Scully 2014).

Overweight	Obese
BMI 25.0 – 29.0 (kg/m)	BMI 30 (kg/m)

Table 1: Definition of overweight and obese people according with the BMI. Adapted from Anthes (Anthes 2014).

Like a multifactorial chronic disease, obesity is characterized by hypertrophy and hyperplasia of adipose tissue, as a result of imbalance energy intake versus energy expended (Chatzigeorgiou et al. 2014).

The energy intake is regulated by appetite (Kalra et al. 1999). During fasting, the regulation of food intake is mainly achieved by altering the expression of neurotransmitters such as norepinefrin, endorphin and pancreatic peptides (neuropeptide Y and peptide YY) . Upon food intake, in order to reduce food intake or induce satiety, suppressor appetite like dopamine, seroronin, calciton, cholecystokinin, and glucagon are secreted (Panickar 2013). The basal metabolism, the minimum energy expenditure required to maintain vital function, is ranged around 65% and 75% of the total energy expenditure (Suarez 2012). Its increase is closely associated to the percentage of lean mass that consume more energy, and decreases with age while increases with physical exercise (Suarez 2012).

The energy expended is regulated by basal metabolism, thermogenesis and physical activity (Astrup et al. 1997). Thermogenesis, the heat production which occurs primarily in BAT and skeletal muscle in response to the diet, is induced by activation of β3-ARs (Ricquier 2002; Arch 2008) and of AMPK signal pathway that stimulates the FA into mitochondria (Dulloo 2011). Physical activity, any movement of muscle mass that results in energy expenditure (Hills and Byrne 2004), can be divided into EAT and NEAT (Levine 2004). NEAT, ranged from 15-50 % of total calories (Levine et al. 2005), concerns activities which are physical but fall outside of exercise, such as pacing, fiddling, typing, talking, standing, and other occupational activities performed at work or school. Moreover, exercise is defined as vigorous activity, scheduled and repetitive in order to maintain or improve physical health (Hills and Byrne 2004).

Evidences have been suggested about the involvement of genetic factors (nutrigenomics and epigenetics) into appetite/satiety regulation in obesity susceptibility (El-Sayed Moustafa and Froguel 2013). It has been shown that nutrient intake can also influence gene expression (Bouchard and Ordovas 2012), providing a possible justification at the molecular level of how

nutrients and other food components interact with the genes (Marti et al. 2005). Among the different mechanisms that may be involved in the interindividual differences occurring in obesity, epigenetics has recently emerged (F. I. Milagro et al. 2013). They are defined as heritable changes in gene expression which occur without DNA sequences modifications, include DNA methylation, histone modifications and several types of micro RNAs (Fermin I. Milagro et al. 2011). In this context, epiobesigenes, such as PPARGC1A, PPARγ, SOCS1/SOCS3, TNFα and CAV1, have proposed (Campion, Milagro, and Martinez 2009).

The expansion in scientific understanding of genetics, cellular and socio-economic influences has not translated into sustainable solutions for its prevention and treatment (Dean et al. 2014). Since the large variability in individuals' responses, some leading scientists in the field presented an holistic approach for tackling obesity, the so called healthy weight management throughout life (Dean et al. 2014), a weight-loss treatment in term of genetic and epigenetic research, optimization of energy expenditure, food intake behaviour and lifestyle interventions.

Currently, obesity which is associated with comorbidities such as T2D, cardiovascular diseases and hypertension (Fig. 2) (Dean et al. 2014), it's become one of the leading threats to public health worldwide (WHO 2014).

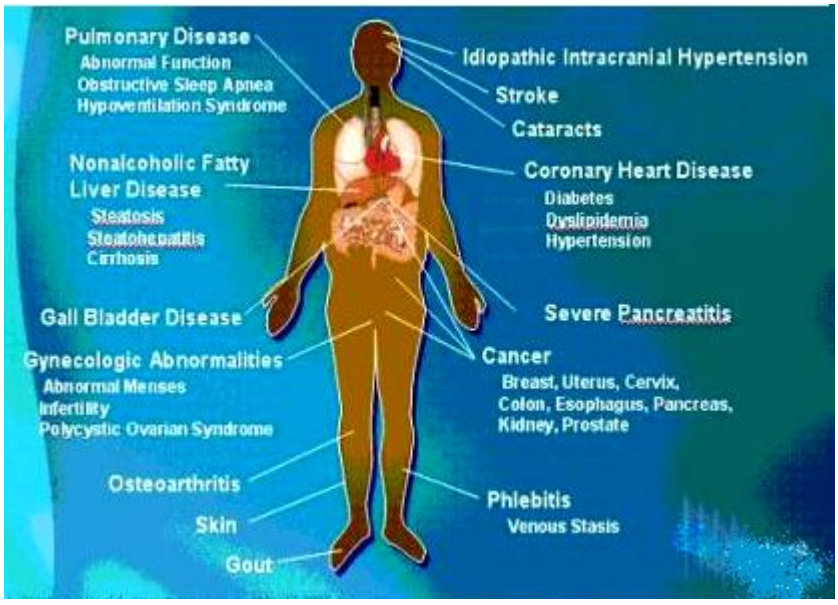


Figure 2: Obesity-related comorbidities.

1.2 The changing colour of fat

Adipose tissue is composed of three distinct forms, beige, brown and white, with fundamentally different functions. Beige cells express UCP1, that do not possess the complete molecular characteristics of that in brown adipocytes (Petrovic et al. 2010; Walden et al. 2012), Tbx 15, Shox2, Hoxc9 Zic1, unique marker of classical brown adipose tissue depots (Walden et al. 2012). It has been proposed that the formation of beige cells is stimulated by irisin, a hormone produced by skeletal muscle in response to exercise The stimulation of lipolysis and the activation and regulation of thermogenesis during chronic exposure to cold are regulated through the release of noradrenaline, which interacts with a β3-AR on the plasma membrane of adipocytes (Arch 2008).

BAT is specialized for the oxidation of FA, through the presence of UCP1, a specific mitochondrial protein which dissipates heat generation by non shivering mechanisms, dispersing the proton gradient across the inner mitochondrial membrane (Ricquier and Bouillaud 2000; Cannon and Nedergaard 2004). Heat generation by BAT may be evoked for thermoregulation, to dissipate calories excess as a mechanism for the maintenance of energy balance, or body weight (Cannon and Nedergaard 2004; Cannon and Nedergaard 2009), and for the arousal from hibernation (Cannon and Nedergaard 2004). It has been proposed that the termogenic gene expression in brown fat may involve the macrophages activation which in turn stimulate catecholamines realese (Nguyen et al. 2011).

WAT is specialized on fuel storage rather than for the oxidation of stored fatty acids, providing substrate to other tissues such as muscle during fasting and periods of high energy demand. Its function and regulations are largely discussed in the next sections.

1.3 White adipose tissue

*1.3.1 An o*verview

White adipose tissue (WAT) forms a layer called the panniculus adiposus or hypodermis in the connective tissue under the skin that provides a significant thermal insulation against cold by reducing the rate of heat loss. It is richly supplied with blood vessels, and surrounded by reticular fibers (type III collagen), which are secreted by the adipocytes. Special stains also reveal the presence of unmyelinated nerve fibers and numerous mast cells (Ross and Wojciech) (Table 2).

The two most abundant depots are visceral and subcutaneous adipose tissues, which produce unique profiles of adipokines (Fried, Bunkin, and Greenberg 1998; Samaras et al. 2010), but

also takes place throughout the body in association with multiple organs, including heart, kidneys, bone marrow, lungs and the adventitia of major blood vessels (Fig. 3).

Features	White Adipose Tissue
Location	Subcutaneous layer, mammary gland, greater omentum, mesenteries, retroperitoneal space, visceral pericardium, orbits (eye sockets), bone marrow cavity
Function	Metabolic energy storage, insulation, cushioning, hormone production, source of metabolic water
Adipocyte morphology	Unilocular, spherical, flatten nucleus, rim of cytoplasm
	Large diameter (15–150 μm)
Transcription factors "master switch" in differentiation	PPAR-γ/RXR
UCP-1 genes expression	No
Mitochondria	Few, poorly developed
Innervation	Few sympathetic nerve fibers
Vascularization	Few blood vessels
Response to environmental stress (cold exposure)	Decreased lipogenesis. Increased lipoprotein lipase activity
Growth and differentiation	Throughout entire life from stromal-vascular cells

Table 2: A summary of white adipose tissue features. Adapted from Ross and Wojeciech (Ross and Wojciech, 2010).

It has been shown that high-calorie diets can promote the development of a pro-inflammatory state in these depots in a similar manner to that observed in subcutaneous and visceral adipose tissue (Chatterjee et al. 2009). Although the functional importance of many of these individual adipose depots is generally not known, evidence suggests that diet-induced changes in their adipokine secretion can influence the function of the associated tissue (Takaoka et al. 2009).

The amount of an individual's adipose tissue is determined by two physiological systems: one associated with short-term weight regulation and the other one with long-term weight regulation. The first system controls appetite and metabolism on a daily basis through two small peptide hormones produced in the gastrointestinal tract, ghrelin, an appetite stimulant, and PYY, an appetite suppressant (Ross and Wojciech, 2010). Ghrelin is a small 28-amino-acid polypeptide produced by gastric epithelial cells, which in addition to its appetite stimulatory role, acts on the anterior lobe of the pituitary gland to release growth hormone. In humans, ghrelin increases the sense of hunger through receptors located in the hypothalamus.

PYY is a small 36-amino-acid gastrointestinal hormone produced by the small intestine that as well to its appetite suppressor role through receptors in the hypothalamus, promotes and maintains weight loss by inducing a greater sense of fullness soon after a meal and desire to

stop eating. In experimental clinical studies, the infusion of PYY into humans has been shown to reduce food intake by 33% over a period of 24 hours (Ross and Wojciech, 2010).

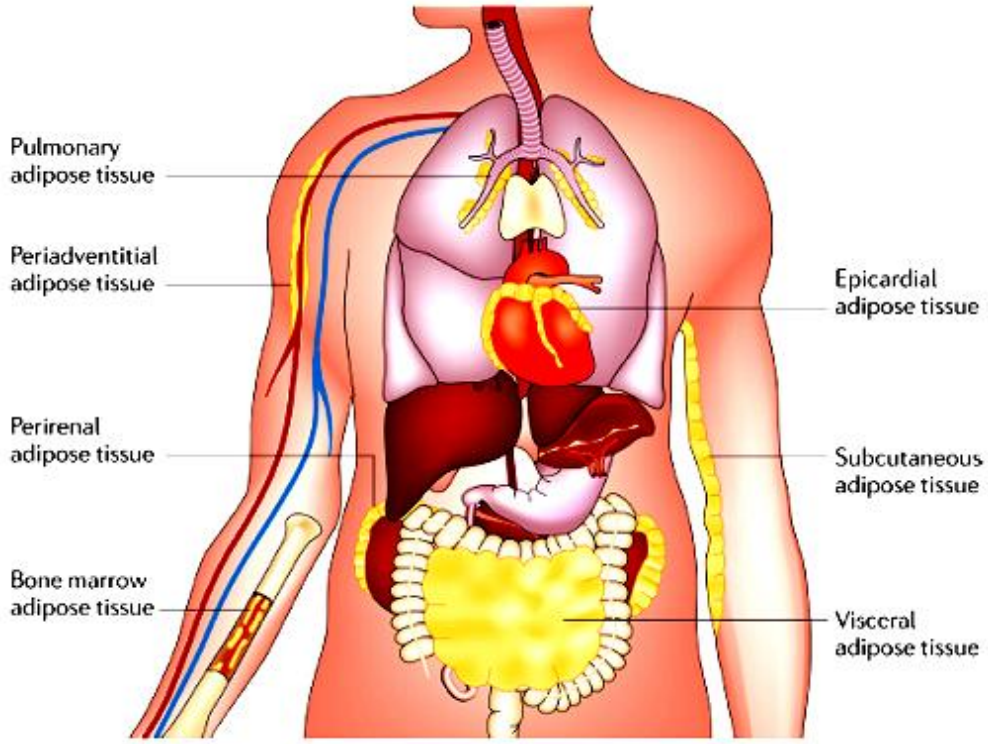


Figure 3: Adipose tissue depots. Under conditions of obesity, adipose tissue expands in these and other depots throughout the body. Common sites of adipose tissue accumulation include the heart, the kidneys and the adventitia of blood vessels. From Noriyuki Ouchi et al. (Noriyuki Ouchi et al. 2011).

The second system controls appetite and metabolism on a continual basis (over months and years) and it is influenced by leptin and insulin, along with other hormones, including thyroid hormone, glucocorticoids, and hormones of the pituitary gland.

Leptin, identified in *ob/ob* mice by positional cloning, regulates the amount of fat stored in the body. In experimental animal models, the addition of recombinant leptin to obese, leptin-deficient *ob/ob* mice causes them to reduce their food intake and lose about 30% of their total body weight after 2 weeks of treatment. Unlike mutant mice, in most obese humans, levels of leptin mRNA in adipose tissue as well as serum levels of leptin are elevated.

Insulin is a pancreatic hormone that regulates blood glucose levels and enhances the conversion of glucose into triglycerides by the adipocyte. Like leptin, insulin regulates weight by acting on brain centers in the hypothalamus, but in contrast to leptin, insulin is required for the accumulation of adipose tissue.

The interconnected hormonal and neural signals emanating from the adipose tissue,

alimentary tract, and central nervous system form the brain–gut–adipose axis that regulates appetite, hunger, satiety, and energy homeostasis (Fig. 4). In fact, WAT is innervated by sympathetic nerve endings, being the sympathetic system the principal physiological mediator of lipolysis (Hales, Luzio, and Siddle 1978) with marked activation occurring in situations where there is net lipolysis (Garofalo et al. 1996; Migliorini, Garofalo, and Kettelhut 1997).

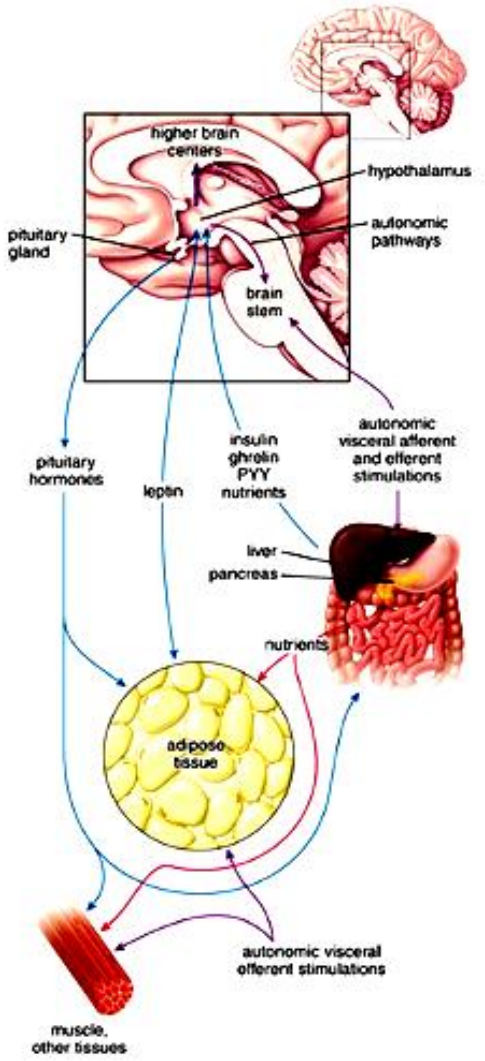


Figure 4: Regulation of energy homeostasis. This schematic diagram shows the relationship between adipose tissue, digestive processes and functions of the central nervous system. From (Ross and Wojciech, 2010).

1.3.2 Cellular complexity

WAT is mainly comprised of adipocytes, although other cell types contribute to its growth and function, including fibroblasts, vascular cells and immune cells (Fig. 5). Adipocytes are derived from undifferentiated mesenchymal stem cells associated with the adventitia of small venules (Fig. 6). Current evidence suggests that PPARγ in complex with the RXR play a

critical role in adipocyte differentiation and initiation of lipid metabolism. It induces the maturation of early lipoblasts (adipoblasts) or preadipocytes into fat cells. Most of the PPARγ target genes in adipose tissue influence lipogenic pathways and initiate the storage of triglycerides. Therefore, PPARγ/RXR is regarded as the "master switch" regulator in the white adipocytes' differentiation (Ross and Wojciech, 2010).

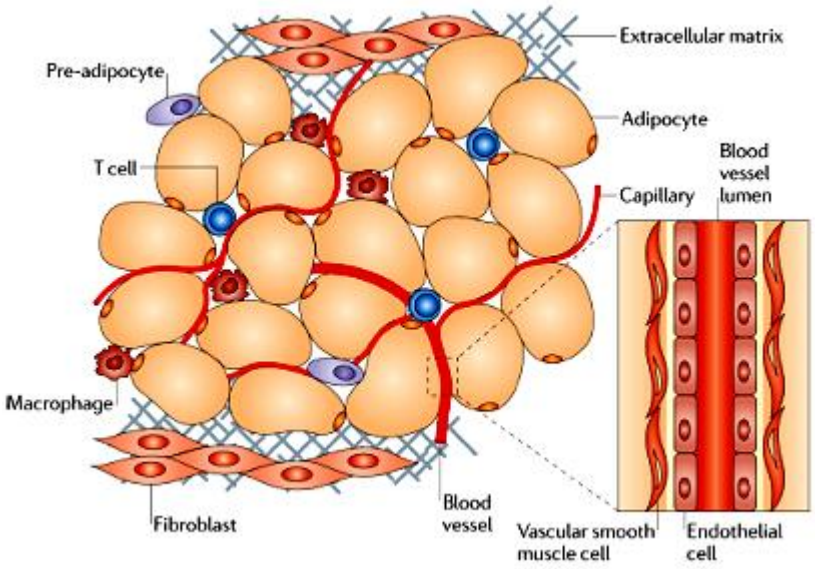


Figure 5: Components of adipose tissue. Adipocytes are the main cellular component of adipose tissue, and they are crucial for both energy storage and endocrine activity. The other cell types that are present are precursor cells (including pre-adipocytes), fibroblasts, vascular cells (endothelial cells and vascular smooth muscle cells) which are associated with the major blood vessels and immune cells (macrophages and T cells). These last type of cells constitute the stroma vascular fraction of adipose tissue. From Noriyuki Ouchi et al. (Noriyuki Ouchi et al. 2011).

When isolated, white adipocytes are spherical, but they may appear polyhedral or oval when crowded together in adipose tissue. Their large size is due to the accumulated lipids in the cell that are deposited in a single unilocular droplet occupying the central portion of the cytoplasm. The nucleus is flattened and displaced to one side of the lipid mass; the cytoplasm forms a thin rim around the lipid (Ross and Wojciech) (Fig. 7). The perinuclear cytoplasm contains a small Golgi apparatus, free ribosomes, short profiles of RER, microfilaments, and intermediate filaments.Filamentous mitochondria and multiple profiles of SER are also found in the thin rim of cytoplasm surrounding the lipid droplet (Ross and Wojciech, 2010).

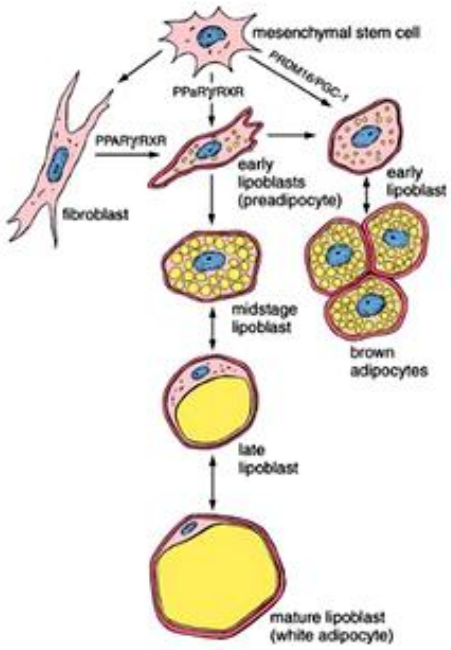


Figure 6: Development of adipose tissue. Like all connective tissue cells, adipocytes are derived from undifferentiated mesenchymal stem cells. From Ross and Wojciech (Ross and Wojciech, 2010).

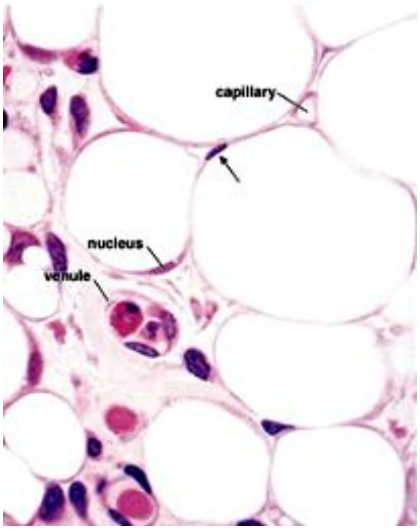


Figure 7: WAT. Photomicrograph of WAT showing its meshwork profiles. From Ross and Wojciech Ross and Wojciech, 2010).

Interest has been paid to the status of the vasculature in adipose tissue, showing that obesity can lead to capillary rarefaction in adipose tissue leading to localized hypoxia (J. Ye et al. 2007; Pasarica et al. 2009). Theoretically, a restriction of blood flow adipose tissue could contribute to an inflammatory state, possibly as a result of ischaemia-induced adipocyte necrosis and the subsequent recruitment of macrophages. Furthermore, obesity leads to the

downregulation of anti-inflammatory factors and the upregulation of pro-inflammatory factors that activate endothelial cells and promote a dysfunctional phenotype (Takaoka et al. 2009). In turn, the activated vascular endothelium expresses adhesion molecules and chemotactic factors that accelerate and localize inflammatory processes.

Fibroblasts produce extracellular matrix components. It has been shown that metabolically dysfunctional adipose tissue produces excess matrix components that may interfere with adipose mass expansion and contribute to metabolic dysregulation (T. Khan et al. 2009). The intercellular communication within adipose tissue is required for normal metabolic function. Examples of this, include the counter-regulation between adiponectin and sFRP5, adipocyte derived anti-inflammatory factors, and the TNF and WNT5a, which are macrophage-derived pro-inflammatory factors. Under conditions of obesity, TNF and WNT5a are upregulated, whereas adiponectin and sFRP5 are downregulated (Noriyuki Ouchi et al. 2003; Berg and Scherer 2005; Noriyuki Ouchi et al. 2010) (Fig. 8).

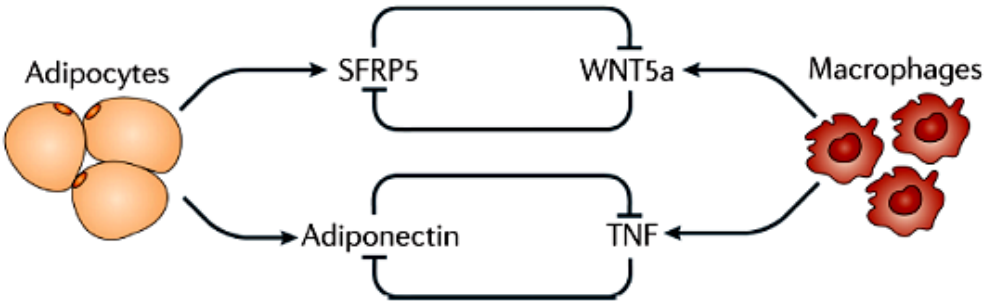


Figure 8: Examples of intercellular communication between different adipose tissue cell types include the counter-regulation between adiponectin and TNF, and between SFRP5 and WNT5a. Under conditions of obesity the pro-inflammatory factors (TNF and WNT5a) predominate. From Noriyuki Ouchi et al. (Noriyuki Ouchi et al. 2011).

Macrophages are particularly important in the inflammatory response that occurs in the tissue as obesity develops, and the obese state is associated with a major recruitment of these cells (Xu et al. 2003; Weisberg et al. 2003; Cancello et al. 2005). Because a key function of macrophages is to remove apoptotic cells in an immunologically silent manner to prevent the release of noxious substances, their presence as crown-like structures in adipose tissue may reflect a pro-inflammatory state that is due, in part, to an impairment of the macrophage-mediated phagocytic process (Fig. 9). According with this notion, is the finding that the induction of adipocyte apoptosis in an inducible mouse model of lipoatrophy leads to macrophage accumulation in adipose tissues (Pajvani et al. 2005). However, the process may

be more complex as a recent paper has reported that adipocyte death is not increased by obesity in humans (Spalding et al. 2008).

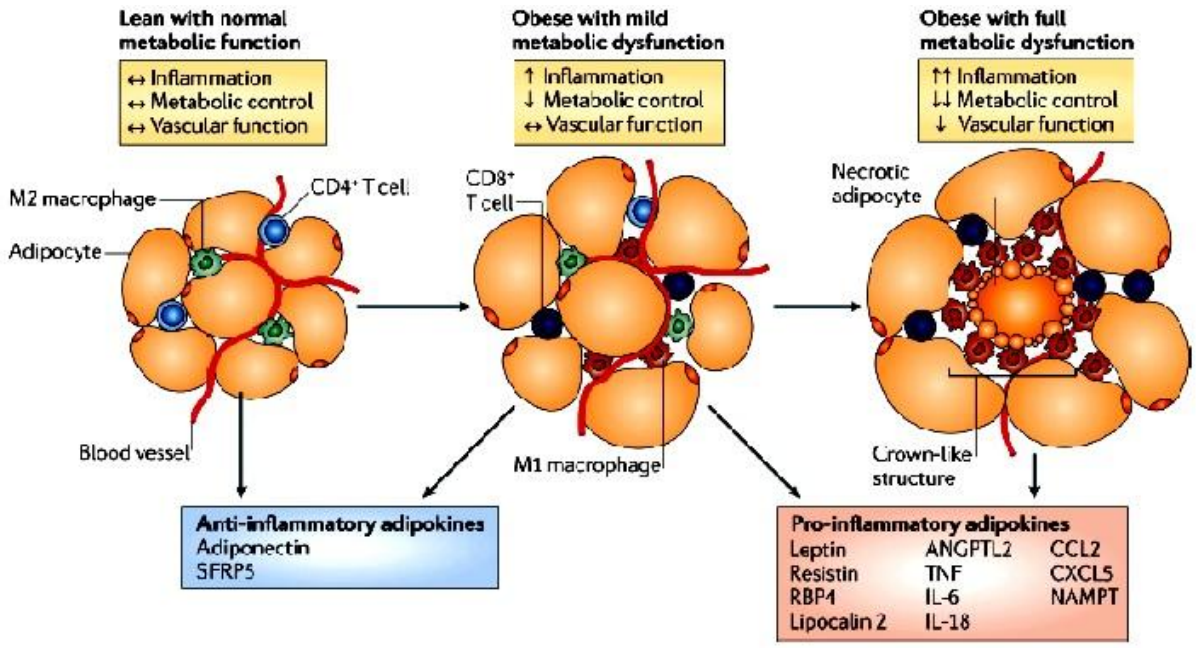


Figure 9: Phenotypic modulation of adipose tissue. As obesity develops, adipocytes undergo hypertrophy promoting the transition to a metabolically dysfunctional phenotype. This generates large amounts of pro-inflammatory factors, including leptin, resistin, RBP4, lipocalin 2, ANGPTL2, TNF, IL-6, IL-18, CCL2, CXCL5 and NAMPT. Metabolically dysfunctional adipose tissue can be associated with higher levels of adipocyte necrosis, and M1 macrophages are arranged around these dead cells in crown-like structures. From (Noriyuki Ouchi et al. (Noriyuki Ouchi et al. 2011).

1.3.3 Secretory role of WAT: adipokines

WAT metabolic function is mediated by the secretion of numerous proteins that are collectively referred to as adipokines, which by exerting their biological roles in autocrine, paracrine, or systemic manner, influence several physiological processes concerning energy, glucose metabolism and immunity (Waki et al. 2003) (Fig. 10).

Adipose tissue from lean individuals preferentially secretes anti-inflammatory adipokines such as adiponectin, TGFβ, IL-10, IL-4, IL-13, IL-1Ra and apelin, while obese adipose tissue mainly releases proinflammatory cytokines which are TNF-α, IL-6, leptin, visfatin, resistin, angiotensin II, and PAI-1 (Noriyuki Ouchi et al. 2011).

Adipokines enlisted in regulation of insulin resistance as well as in immunity and inflammation are: adiponectin, leptin, resistin, visfatin, chemerin, TNF- α, IL-1, IL-6, IL-8, IL-10, PAI-1, MCP-1 and RBP-4 (Table 3).

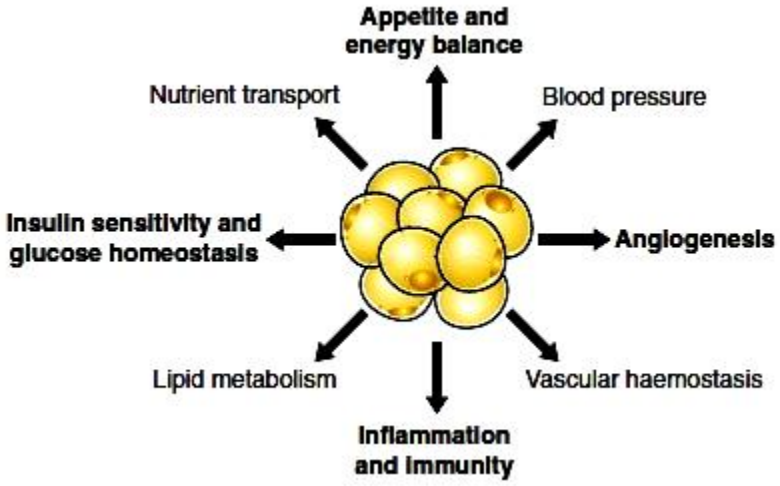


Figure 10: Illustration of the major physiological and metabolic processes with which adipose tissue is involved through the secretion of various adipokines from adipocytes. The interactions may be autocrine, paracrine, or endocrine. From Trayhurn (Trayhurn 2013).

Adipokine	Distribution	Function	Situation in obesity
Adiponectin	Only secreted by adipose tissue. Lower production in men	Insulin sensitizing effect. Improves insulin resistance and glucose metabolism	Decreased in mouse models and in human and correlated negatively with BMI. Increased after weight loss
Chemerin	In rodents and humans, expressed in placenta and WAT	Regulates adipocyte development and metabolic function	Circulating levels in T2D patients and correlated with body fat, glucose, and lipid metabolism
IL-1	Secreted mainly by adipocytes and macrophages	Role in macrophages chemotaxis and thermogenesis	In mice and human. Predictive of T2D
IL-6	Secreted predominantly by adipocytes for one-third of total circulating levels, in macrophages, skeletal muscle, endothelial cells, and fibroblasts	Controversial role in the development of insulin resistance. Affects glucose metabolism	In human and correlated with adiposity and reduced with weight loss. In plasma of T2D patients
IL-8	Secreted by adipocytes (visceral WAT > subcutaneousWAT) and Macrophages	Neutrophil chemotaxis	In subjects and related to fat mass and TNF-α levels
IL-10	Secreted by monocytes, macrophages, dendritic cells, and B and T cells	Improves insulin sensitivity and glucose transport	Attenuated in T2D patients and increased with weight loss
Leptin	Predominantly WAT and to a lesser degree in hypothalamus, gastric epithelium, placenta, and gonads	Regulates energy intake, expenditure, feeding behavior, storage of fat and insulin signaling	Mouse and human models and correlated with BMI and decreased with

			weight loss
MCP-1	Secreted by adipose tissue	Affects insulin sensitivity and increases macrophage recruitment in adipose tissue and inflammation	Increased in mouse models of obesity. Increased in T2D subjects
PAI-1	Expressed by WAT	Potent inhibitor of fibrinolytic pathway	In human and T2D subjects
Resistin	In rodents, secreted by adipocytes and in humans predominantly by circulating macrophages and monocytes and to a lesser degree by WAT	Regulation of neoglucogenesis and insulin resistance in rodents. Proinflammatory role in humans	In mouse models and in human correlated with insulin resistance in diabetic patients
RBP4	Secreted by adipocytes, macrophages, and hepatocytes	Affects insulin sensitivity, hepatic glucose output, and muscle insulin signaling	Circulating levels in subjects and correlated with BMI and insulin resistance
TNF-α	Expressed by macrophages and adipocytes (visceralWAT > subcutaneousWAT)	Provokes insulin resistance and stimulates lipolysis	Mouse models and in human correlated with BMI
Visfatin	Expressed in liver, muscle,WAT, bone marrow, and lymphocytes	Role in insulin sensitivity, insulin secretion and inflammatory properties	Correlates with visceral adiposity in humans

Table 3: Adipokines increased in obesity and/or diabetes. Modified from Makki et al. (Makki, Froguel, and Wolowczuk 2013).

Among all the adipokines, adiponectin (also known as ACRP30 and ADIPOQ) was identified as an adipocyte-specific adipokine (Scherer et al. 1995; Hu, Liang, and Spiegelman 1996). It exists in a wide range of multimer complexes in plasma and combines via its collagen domain to create three major oligomeric forms: a LMW trimer, a MMW hexamer, and HMW 12- to 18-mer adiponectin (Pajvani et al. 2003; Waki et al. 2003) (Fig. 11). HMW is the most biologically active form and best reflective of the reduction in total adiponectin levels associated with obesity (Ryo et al. 2004). This association is related to clinical observations that plasma adiponectin levels are negatively correlate with visceral fat accumulation (Ryo et al. 2004) and decreased in patients with T2D (Noriyuki Ouchi et al. 2003). With this regard, it has been proposed that reduced adiponectin levels caused by interactions between genetic factors, such as SNP 276G>T in the adiponectin gene itself, and environmental factors causing obesity, i.e. such as a HFD and a sedentary lifestyle, may play a crucial role in the development of insulin resistance, type 2 diabetes, and the metabolic syndrome (Kadowaki et al. 2006) (Fig. 12).

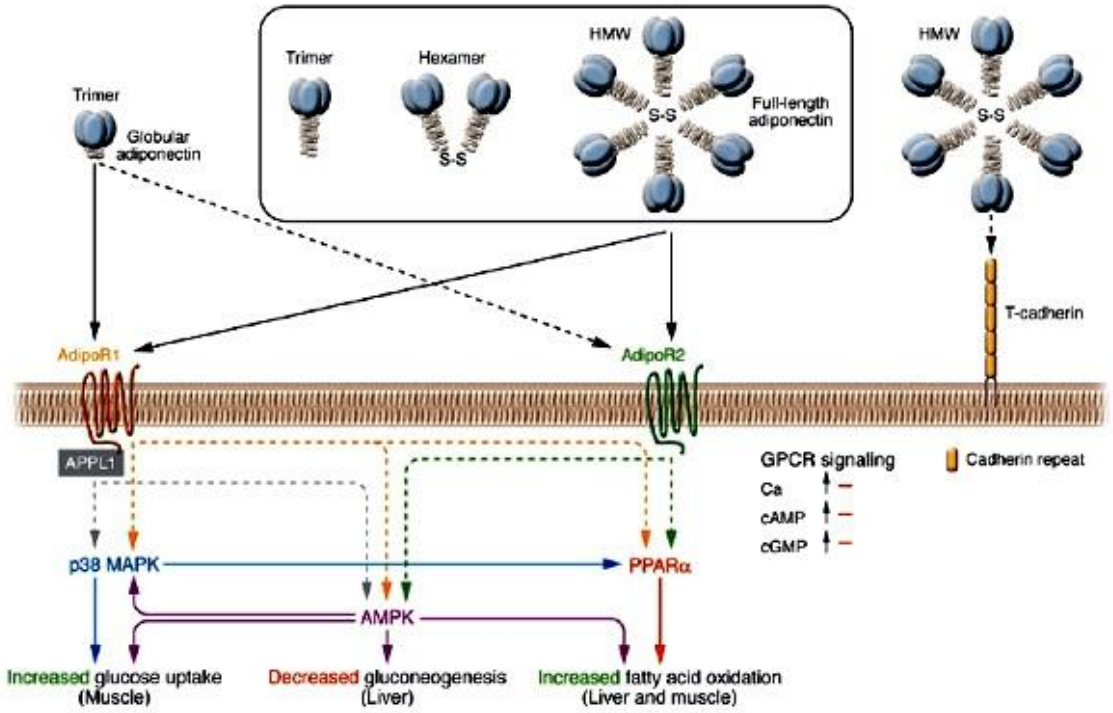


Figure 11: Signal transduction by adiponectin receptors. Globular adiponectin exists as a trimer, whereas full-length adiponectin exists as at least 3 species of multimers: an LMW trimer, an MMW hexamer, and an HMW multimer. AdipoR1 and AdipoR2 serve as receptors for globular and full-length adiponectin and mediate increased AMPK, PPARα ligand activities, p38MAPK, and adiponectin-induced biological functions. T-cadherin is capable of binding adiponectin but is thought to have no effect on adiponectin cellular signaling, since T-cadherin lacks an intracellular domain (Hug et al. 2004). Interaction of APPL1 with AdipoR1 appears to play important roles in adiponectin signaling and adiponectin-mediated downstream events such as lipid oxidation and glucose uptake (Mao et al. 2006) S-S, disulfide bond. From Kadowaki (Kadowaki et al. 2006).

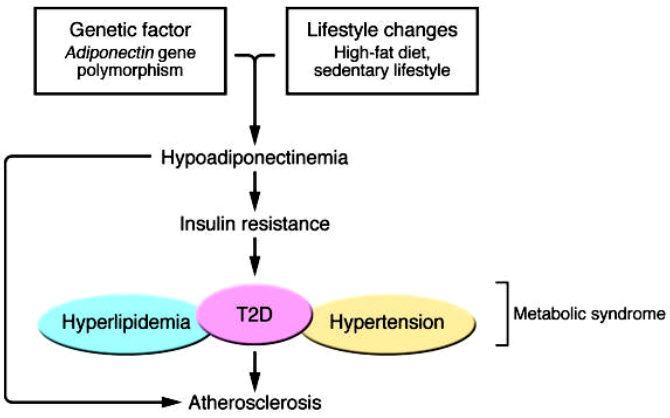


Figure 12: Adiponectin hypothesis for insulin resistance, metabolic syndrome and atherosclerosis. Interactions of genetic and environmental factors reduce adiponectin levels, appearing to play an important causal role in the development of insulin resistance, T2D and metabolic disease, thereby indirectly causing atherosclerosis. From Kadowaki et al.(Kadowaki et al. 2006).

Consistent with these, adiponectin-deficient mice develop hypertension and impaired endothelial cell-dependent vasodilation when fed an atherogenic diet (Li et al. 2007), and its disruption also leads to the enhancement of salt induced hypertension resulting in reduction of eNOS expression (Ohashi et al. 2006) and increased T cell accumulation in atheratoma (Okamoto et al. 2008). The formation of atherosclerotic lesions is inhibited by decreasing the expression of SR-A, TNF and VCAM1 (Okamoto et al. 2002; Toshimasa Yamauchi et al. 2003). In acordance with these, it has been reported that the homeostasis of the vascular function is promoted through the activation of AMPK–eNOS signalling pathway and of PGI2 (Ohashi et al. 2009). However, a recent study did not find an association between atherosclerosis and levels of circulating adiponectin, in the LDLR-deficient mouse model (Nawrocki et al. 2010).

Administration of adiponectin to diabetic mice has been shown to reduce hyperglycaemia by enhancing insulin activity (Berg and Scherer 2005), while given to obese mice it increases fatty acid oxidation in muscle tissue, reduces plasma levels of glucose, free fatty acids and triglycerides (Fruebis et al. 2001). In line with these observations, adiponectin deficient mice develop exacerbated diet-induced insulin resistance (Maeda et al. 2002; Nawrocki et al. 2006), whereas transgene-mediated overexpression of adiponectin in *ob/ob* mice improves glucose metabolism independently of weight loss (J.-Y. Kim et al. 2007). These beneficial effects on insulin sensitivity seem to be mediated in part by its ability to activate AMPK in skeletal muscle, increasing fatty acid oxidation and glucose uptake, and inhibiting gluconeogenesis in liver (Tomas et al. 2002; T. Yamauchi et al. 2002) through interactions with its cell surface receptors, AdipoR1 and AdipoR2 (Toshimasa Yamauchi et al. 2003) (Fig. 11). AdipoR1 is ubiquitously expressed, including abundant expression in skeletal muscle, whereas AdipoR2 is most abundantly expressed in the mouse liver. These receptors appear to be integral membrane proteins, with the N-terminus internal and the C-terminus external, opposite to the topology of all other reported GPCRs (Toshimasa Yamauchi et al. 2003) (Fig. 13).

The roles of these receptors in mediating the metabolic actions of adiponectin *in vivo* are controversial and incompletely understood. AdipoR1 deficiency results in reduced adiponectin-induced AMPK activation, increased glucose production and impaired insulin resistance, whereas AdipoR2 deficiency causes decreased activity of PPARα signalling pathways, attenuates high-fat diet-induced insulin resistance, but exacerbates glucose intolerance after long-term exposure to a high-fat diet, presumably owing to the dysfunction

of pancreatic β-cells (Bjursell et al. 2007; Toshimasa Yamauchi et al. 2007; Y. Liu et al. 2007). The deletion of both receptors abolishes adiponectin binding and actions leading to exacerbation of glucose intolerance.

Several studies have investigated the ability of adiponectin to suppress pro-inflammatory cytokine production. Adiponectin levels are negatively correlated with CRP levels in obese or diabetic patients, conversely in non-diabetic or healthy subjects (Noriyuki Ouchi et al. 2003). Additionally, adiponectin deficient mice have higher levels of TNF mRNA in adipose tissue and TNF protein in the blood (Maeda et al. 2002) and these parameters were restored to normal levels on administration of adiponectin. It is also known its role in controlling inflammation by mediating modulation of macrophage function and phenotype. It inhibits the transformation of macrophages into foam cells, abrogates LPS-stimulated TNF production (Yokota et al. 2000), TLR-mediated NF-κB activation and reduces intracellular cholesteryl ester content in human macrophages by suppressing the expression of class SR-A (N. Ouchi et al. 2001). Evidences suggest that adiponectin mediates modulation of macrophage function and phenotype, exerting its role in controlling inflammation, although the receptor-underlye pathways in these cells are poorly understood. Peritoneal macrophages and adipose tissue stromal vascular fraction cells of adiponectin-deficient mice show increased expression of pro-inflammatory M1-type markers and decreased expression of anti-inflammatory M2-type markers (Ohashi et al. 2010). Conversely, the systemic delivery of adiponectin to mice stimulates arginase 1 expression by these cells, and stimulation of cultured macrophages with recombinant adiponectin results in an increase in the levels of M2-type markers and a reduction in ROS generation (Ohashi et al. 2010). Because M2-like phenotype in macrophages is induced by the phagocytosis of early apoptotic cells (Savill et al. 2002), it has been suggested that adiponectin can function as a collectin protein. With this regard, similar to collectin protein like C1q and surfactant proteins A and D, adiponectin can binds to calreticulin and CD91 expressed on the apoptotic cells surface, facilitating their uptake by macrophages (Takemura et al. 2007). Despite its actions, the adiponectin receptor-mediated signalling in macrophages is poorly understood.

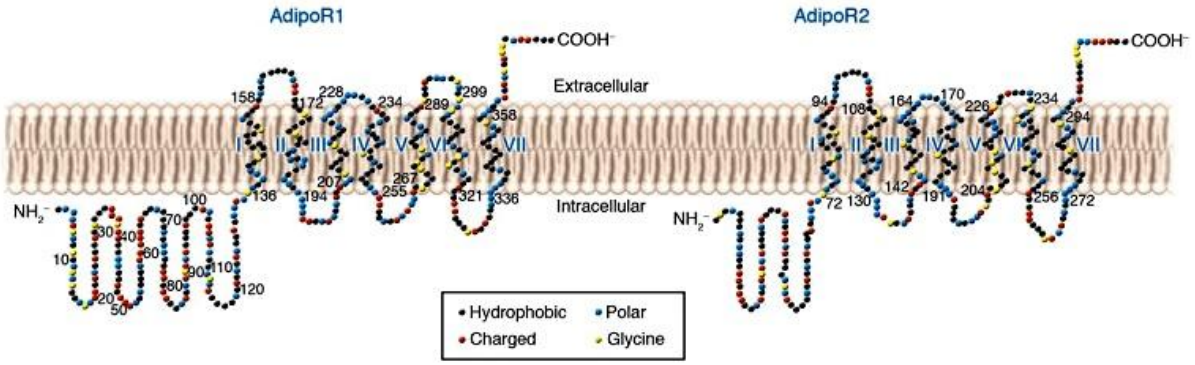


Figure 13: Structure of ADIPORs. AdipoR1 and AdipoR2 are predicted to contain 7 transmembrane domains but are structurally and topologically distinct from GPCRs. Modified from (Kadowaki et al. 2006).

1.4 Substrate and general metabolism

1.4.1 Glucose metabolism

WAT is a net exporter of lactate, particularly in obesity (Hosogai et al. 2007; Hodson et al. 2013) where the percent of glucose converted to lactate - correlated directly with fat cell size- is as much as 70% (Marin et al. 1987).

As adipose tissue mass increases, hypoxia develops in adipose tissue, leading to a switch into anaerobic metabolism, so more glucose is catabolized to lactate rather than oxidized through the citric acid cycle (Trayhurn 2014). The rise in lactate production in hypoxic adipocytes involves the recruitment of MCTs (Halestrap and Meredith 2004; Perez de Heredia, Wood, and Trayhurn 2010; Halestrap and Wilson 2012) for the remove of monocarboxylate, preventing cellular toxicity by the metabolites. Human adipocytes have been shown to express three family members called MCT1, MCT2, and MCT4. It has been suggested that both their transcription and expression levels are regulated through HIF-1, leading to a decreasing of MCT2 and a stimulation of MCT1 and MCT4 expression, but only MCT1 protein levels are increased (Perez de Heredia, Wood, and Trayhurn 2010).

Recent evidence suggests that lactate run as a signaling factor (Rooney and Trayhurn 2011), inducing insulin resistance in skeletal muscle (Choi et al. 2002), stimulation of inflammation in macrophages (Samuvel et al. 2009) and the inhibition of lipolysis in adipocytes (C. Liu et al. 2009). This findings raise the possibility of lactate shuttling between adipocytes and other cells within adipose tissue (Doherty and Cleveland 2013).

The increase in glucose uptake in adipocytes exposed to hypoxia implies increased hexose transport (Trayhurn 2013). Among the several members of GLUTs, GLUT1 gene expression and protein levels are markedly increased for several days in murine and human adipocytes in

response to hypoxia, accompanied with a substantial fall in GLUT4 gene expression by 48 h (Trayhurn 2014). It seems that glucose uptake in hypoxic adipose tissue is modulated by HIF-1 system (Trayhurn 2014). Transgenic mice with a selective knockdown of HIF-1β in adipose tissue exhibit a decrease in 2-deoxy-glucose uptake accompanied by a reduction in GLUT1 and GLUT4 gene expression, while in 3T3-L1 adipocytes it has been shown to result in a fall in GLUT1 and GLUT4 protein (Lee et al. 2011).

Although the induction of GLUT-1 expression is frequently utilized as a marker for the cellular response to low PO_2, and GLUT4-mediated glucose uptake may be compromised by chronic exposure to hypoxia, several other GLUTs are expressed. With this regard, authors have suggested GLUT12 as a potential therapeutic target linked to insuline resistance (Pujol-Gimenez et al. 2013), although it has been reported that this transporter is expressed in all the major human and rats depots, but not in 3T3-L1 cells (Wood, Hunter, and Trayhurn 2003). The interest about this facilitative transporter is due to a direct relation with the glycolytic metabolism of normal human skeletal muscle, where in response to insulin GLUT12 is translocated to the plasma membrane together with GLUT4 (Stuart et al. 2009). Conversely, basing on a recent study, seem that the expression of GLUT12 is increased in the diabetic myocardium, potentially as a compensatory mechanism for the observed downregulation of active GLUT4, and may probably be functioning as a basal non-insulin dependent GLUT located primarily at the cell surface (Waller et al. 2013). Even though informations about GLUT12 have been released, still little is known about its functional characteristics and its physiological role.

1.4.2 Insulin Sensitivity

Insulin stimulated glucose uptake is one of the fundamental mechanisms responsible for the maintenance of glucose homeostasis in the body. Impaired insulin action or insulin resistance which occurs in obesity leads to TD2 (Hotamisligil 2000; Kopelman 2000) whose risk increases by more than tenfold for those with a BMI of 30 (Trayhurn 2014).

Insulin-sensitizing action is linked to an impaired state between adiponectin secretion, that decreases under conditions of low PO_2, and the release of IL-6 that raises, conversely (Trayhurn 2014).

It seems that the antilipolytic action of insulin in WAT is dysregulated by hypoxia (T. Yamauchi et al. 2001; Berg and Scherer 2005; Wang, Wood, and Trayhurn 2007) and that this effect is driven by both HIF-1 and HIF-2, since their overexpression mimics the effects of low

PO_2 (Regazzetti et al. 2009), while their inhibition attenuates the hypoxia-induced downregulation of the insulin signaling pathway. This is a consequence of a reticence in the insulin-induced phosphorylation of IR-β and IRS-1.

In the insulin signaling cascade, PI3K and PTEN play a central role (Taniguchi et al. 2006). For the first time, authors reported that PIP3 increases GLUT4 surface expression and glucose uptake as well as glucose utilization at the cellular level mediated by AKT/PKCζ/γ phosphorylation (P. Manna and Jain 2013). This raises the suggestion that defects in insulin stimulated glucose metabolism in T2D are due to the uncoupling of AKT/PKCζ/γ /GLUT4 signaling pathways, which may in turn be linked with the decreased production of PIP3 in the hyperglycemic environment (P. Manna and Jain 2013).

It is well known that after insulin binding to the receptor, IRS-1 is phoshorylated and PI3K activated. This last one catalyzes the addition of a phosphate group to the membrane lipid PIP2, converting it to PIP3 that can be transformed by PTEN back to PIP2. PIP3 binds Akt, that catalyzing the phosphorylation of key proteins, leads to an increase in glycogen synthase activity and recruitment of GLUT4 to the membrane (Hemmings and Restuccia 2012) (Fig. 14).

On the other hand, IRS-1 can activate Ras-MAPK pathway that lead to recruitment of GRB2 binds to the guanine nucleotide exchange factor SOS activating it (Zarich et al. 2006). Activated SOS then promotes the removal of GDP from a member of the Ras subfamily that can bind GTP and become active. Activated Ras make actives RAF kinase that in turns activates MEK1 and MEK2 and sequentially ERKs (Avruch et al. 2001). The series of kinases from RAF to MEK, and to MAPK, is an example of a protein kinase cascade. Such series of kinases provide opportunities for feedback regulation and signal amplification.

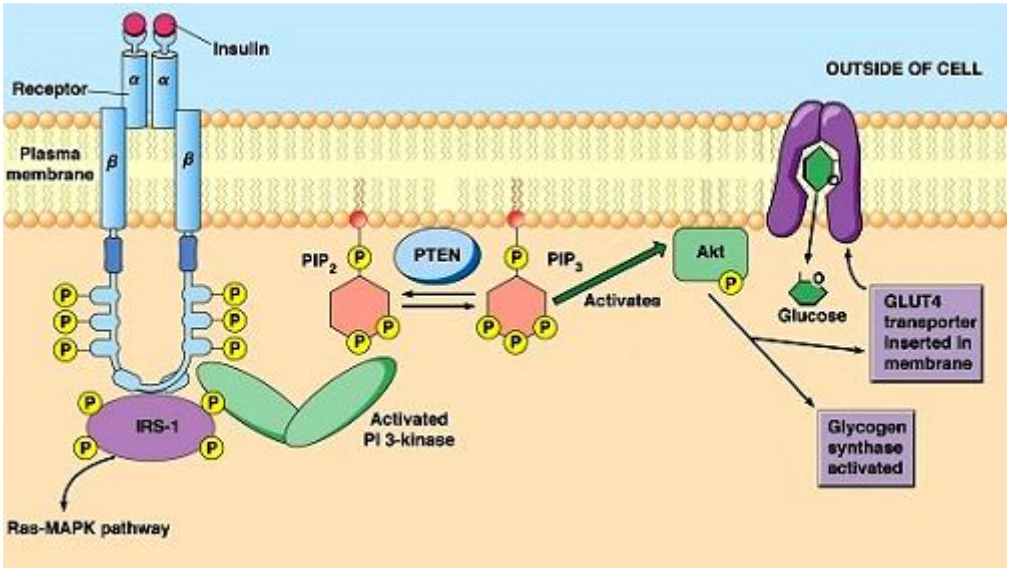


Figure 14: Insulin cascade signaling. When insulin receptor binds insulin, the active receptor phoshorylates the IRS-1 activating PI3-kinase. This one catalyzes the addition of a phosphate group to the membrane lipid PIP2, thereby converting it to PIP3 that can be converted by PTEN back to PIP2. PIP3 binds Akt, that catalyzes phosphorylation of key proteins, leading to an increase in glycogen synthase activity and recruitment of GLUT-4 to the membrane. Modified from Pearson Education, Inc 2012.

1.4.3 Lipid and Oxidative Metabolism

Adipocytes store extra energy in the form of TGs inside lipid droplets or LDL-C. When a continuous storing of TGs occurs, adipocytes must expand in size and become stressed to synthesize more proteins for LDL-C formation (Zha and Zhou 2012). So, they secrete multiple cytokines that activate resident and circulating macrophages forming the signature "crownlike structures" found in obese tissue (Surmi and Hasty 2008). During this cascade, amplified cytokines secretion can increase adipocyte lipolysis which in turns lead to a raise of circulating FFA that are deposited in muscle and liver ("lipid dumping") resulting in a decreased insulin sensitivity in these tissues (Mittra, Bansal, and Bhatnagar 2008). Particularly, FFA from visceral adipose tissue is directly deposited into the portal vein, growing the possibility of fatty liver disease, an high-risk factor for cardiovascular syndromes (Kabir et al. 2005; Yoshii et al. 2006). An increase in FFA release is also induced by cellular insulin insensitivity.

Lipid oxidation and lipolysis are the top pathways modulated in adipocytes by hypoxia (Geiger et al. 2011; Mazzatti et al. 2012). In response to low pO_2, basal lipolysis has been reported to be either unchanged (Lolmede et al. 2003) or increased (Yin et al. 2009; O'Rourke et al. 2013), whereas the uptake of fatty acids from the circulation is reduced (Yin et al. 2009)

by an attenuation of lipogenesis (O'Rourke et al. 2013) through suppression of adipogenic transcription factors PPAR-c and C/EBPβ (Yun et al. 2002; Swiersz, Giaccia, and Yun 2004; Gentil et al. 2006; J.-Y. Kim et al. 2007; Park and Park 2010). This underlies the elevation of plasma FFA levels in obesity (Frayn et al. 2003). A model for stimulatory lipolysis pathways is reported in figure 15.

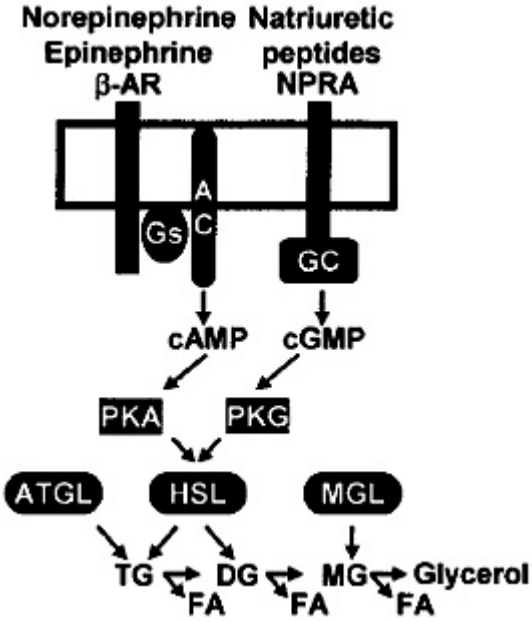


Figure 15: Model for stimulatory pathways in human adipose tissue lipolysis. Catecholamines and natriuretic peptides are the major hormones stimulating hydrolysis of TG and release of FFAs ( ). PKA- PKG activation induced by β2-ARs and NPRA stimulates the expression of HSL and ATGL. The MGL completes monoglycerides hydrolysis into glycerol and FA. From (Langin et al. 2005).

It has been suggested that the effect of hypoxia on lipolysis and lipogenesis is mediated through inhibition of HBS and that low pO_2 impairs the buffering capacity of adipocytes and contributes to lipotoxicity (O'Rourke et al. 2013). Specifically, HBS induces LG and LP in VAT but not in SAT and promotes FAO in the same manner in these tissues. Hypoxia also inhibits HBS, as well as LG, and induce LP. This shifts lipid metabolism towards LP, inhibiting adipocyte lipid storage and buffering capacity, increasing FFA release, and thus promoting systemic lipotoxicity (Fig. 16).

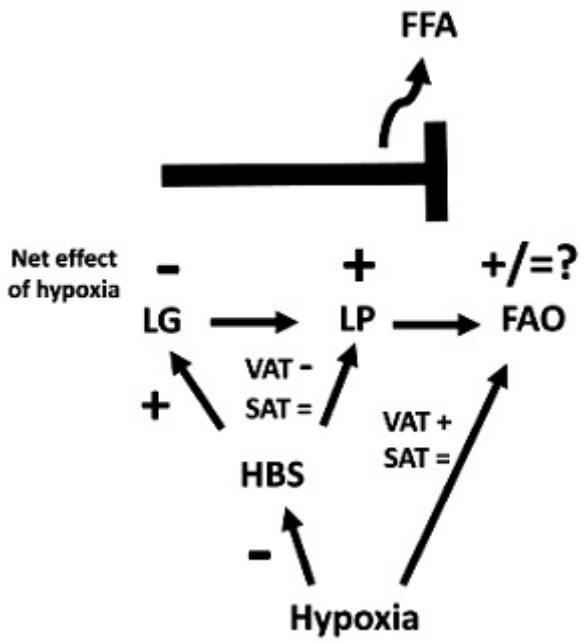


Figure 16: A model for hypoxia/HBS-mediated adipocyte overflow. HBS induces LG and LP in VAT but not in SAT and promotes FAO at the same way in these tissues. Hypoxia also inhibits HBS, so LG, and induce LP. This shifts lipid metabolism towards LP, inhibiting adipocyte lipid storage and buffering capacity, increasing FFA release, and thus promoting systemic lipotoxicity. From O'Rourke et al. (O'Rourke et al. 2013).

CHAPTER 2
PHARMACOLOGICAL STRATEGIES AND DIETARY SUPPLEMENTATION

2.1 Therapeutic intervention

Reducing body weight requires manipulation of the energy balance equation to produce energy deficits that should be accomplished through diet and exercise, pharmacological interventions, or surgical means. However, each of these methods comes with disadvantages. For instance, many diet and exercise lifestyle interventions are not usually long-term lasting (Barte et al. 2010). Furthermore, pharmacological and/or surgical means to reduce body weight are typically expensive and are sometimes accompanied by potentially unpleasant and/or dangerous side effects (Kernan et al. 2000; McEwen et al. 2010). Currently, there are few options for obesity pharmacotherapy. One of the approved drug for weight management in Europe is Orlistat (Xenical®) (Yumuk et al. 2014) that acts by inhibiting pancreatic lipase activity reducing the triglyceride digestion and absorption by 30% (Yumuk et al. 2014), but have several common adverse effects (Filippatos et al. 2008; de la Garza et al. 2011).

In addition, there are other categories that act by suppressing appetite, increasing energy expenditure or by redistributing adipose tissue (Chatzigeorgiou et al. 2014) (Table 4), but the European organizations still seem skeptical due to these medications' unwanted effects (Chatzigeorgiou et al. 2014). The only ones approved by the FDA are phentermine, an appetite-suppressant amphetamine, and topiramate, an anantiepileptic-controlled release drug that decrease lipogenesis and modifies the food taste via inhibition of carbonic anhydrase isoenzymes.

As such, recent evidences suggest that the consumption of natural ingredients and/or dietary supplements may provide a safe and effective means to induce weight loss and improve overall health (Lopez et al. 2013; Outlaw et al. 2013). The beneficial effects of poly- and monounsaturated fats on the recommended indexes ω3 have been determined to result in decreased obesity and IR (Burrows, Collins, and Garg 2011), as well as high-fiber cereals and whole grains (E. Q. Ye et al. 2012). A diet rich in soluble fiber (20 g/1000 kcal) and low in polyunsaturated fats (20% of total calories) and a decreased consumption of food items with a

high GI, can decrease the prevalence of MetS by improving BP and IR (Cho et al. 2013; Cuenca-Garcia et al. 2014). This suggests an overall food pattern in line with the Med Diet.

Drug	Compound	Clinical trials	References
Thyroid hormone receptor subtype β-agonists	Eprotirome (KB 141)	Terminated at phase III	(Sharma et al. 2014)
Growth hormone analogues	AOD9604	Insufficient efficacy in clinical trials	(A. Khan et al. 2012)
11β-HSD1 inhibitors	BVT-3498	Terminated at phase III	(Wang 2006)
ADRβ3	L-796568	Not effective in clinical trial	(Larsen et al. 2002)
Diazoxide	Diazoxide choline	Completed several phase I and II clinical studies	(Alemzadeh et al. 2008)
Sirtuin 1 activators	SRT2104	In phase II	(Baksi et al. 2014)
Angiogenesis inhibitors	TNP-470	In phase II	(J.-Y. Kim et al. 2007)
Inhibitors of methionine aminopetidase 2	Belonarib	In phase II	(Hughes et al. 2013)

Table 4 : Anti-obesity drugs involved in increasing energy expenditure or adipose tissue redistribution. Modified from Chatzigeorgiou et al. (Chatzigeorgiou et al. 2014).

2.2 Mediterranean diet

The Med Diet was first described by Oldways, the Harvard School of Public Health and the European Office of the World Health Organization at a conference in Cambridge, MA, in the 1993 (Oldways 2009) . This dietary pattern, based from the observations on dietary habits of people living in different regions of the Mediterranean basin (Keys 1995), it is generally illustrated by a diet pyramid graphic (Fig. 17).

As a source of minimally processed foods, it gathers a proper ratio between macronutrients, low glycemic index meals and energy density, with a high nutritional value (Bach-Faig et al. 2011; Gargallo Fernandez et al. 2012).

Previous studies exploring the adherence to the Med Diet have been conducted in Italy (di Giuseppe et al. 2008), Greek (Panagiotakos et al. 2007) and Spain (Mar Bibiloni et al. 2011; Bibiloni et al. 2012). A stronger association between the Med Diet and lower likelihood of being obese, hypertensive and having diabetes has been reported (Esposito et al. 2004; Psaltopoulou et al. 2004; Nunez-Cordoba et al. 2009; Domenech et al. 2014; Grosso et al. 2014). The negative correlation between the adherence to MedDiet and the weight gain (Mendez et al. 2006; Field et al. 2007), is ascribed to the customary use of olive oil, high in MUFA and PUFA (Bach-Faig et al. 2011), probably more easily oxidated, conversely to a

SFA and TFA intake (Field et al. 2007). In 2004, the FDA of the USA allowed a claim on olive oil labels concerning "the benefits on the risk of coronary heart disease of eating about two tablespoons (23 g) of olive oil daily, due to the MUFAs in olive oil" (Covas 2007).

Stated as a health model, the UNESCO has also recognized it as an Intangible Cultural Heritage of Humanity.

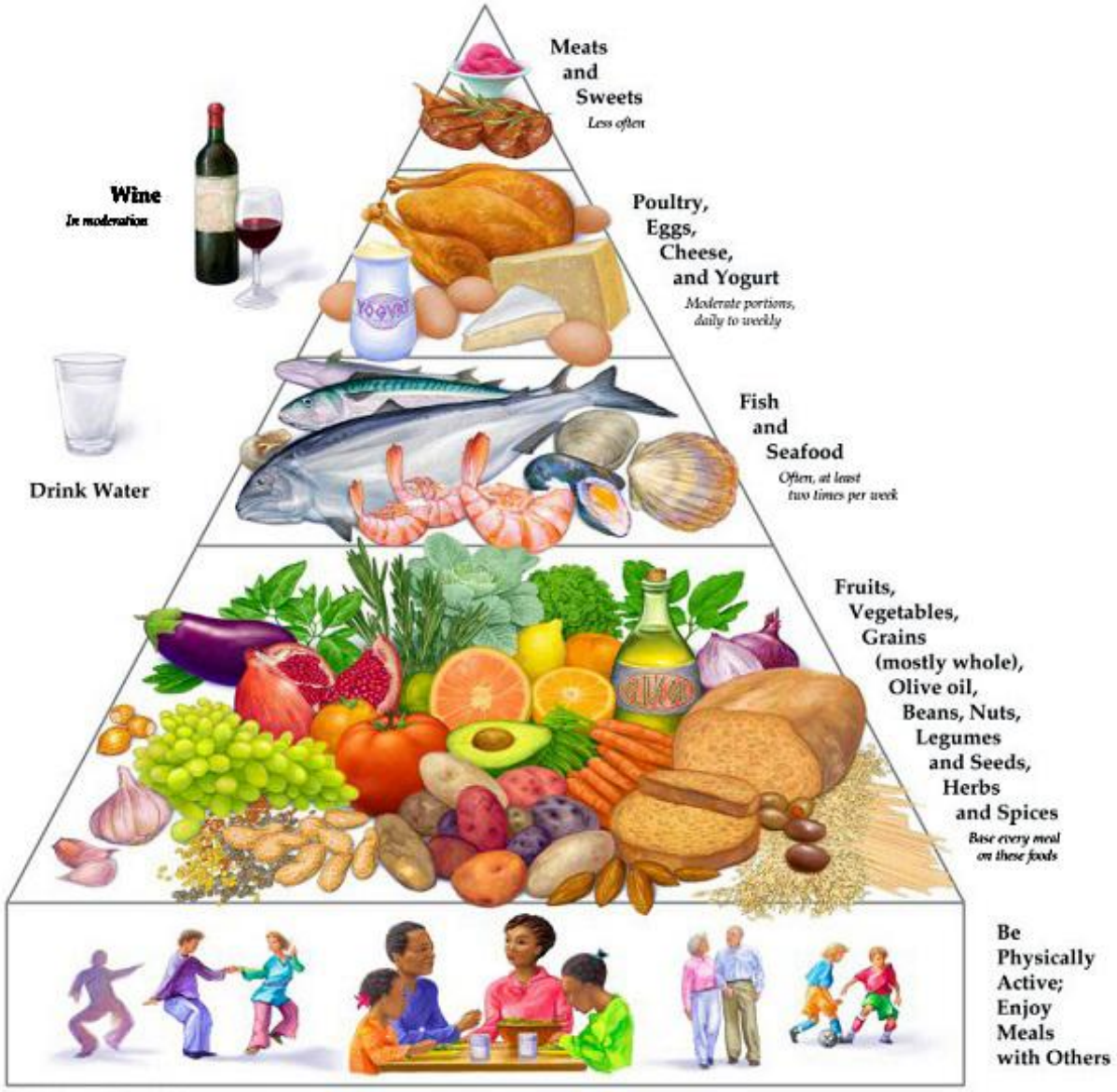


Figure 17: Graphic representation of MedDiet Pyramid graphic. This pyramid is a well-known guide to what is now universally recognized as the "gold standard" eating pattern that promotes lifelong good health. Modified from Oldways (Oldways 2009).

2.3 Olive oil

2.3.1 The plant

Olea europaea is an evergreen fruit plant that belongs to the family Oleaceae. It is native to the southern European countries and throughout the entire Mediterranean region, where represents the prime source of olive oil (Bouskou 1996).

The plant grows to approximately 6-9 meters in height, has rigid branches and a grayish bark. The leaves are opposite, lanceolate, or ovate-lanceolate, mucronate, shortpetioled, green above, and hoary on the underside. The flowers are small, in short, axillary, erect racemes, very much shorter than the leaves. The corolla is short, white, with 4 broad, ovate segments; the calyx short and 4-toothed (Y. Khan et al. 2007) (Fig. 18).

Figure 18: Illustration of Olea europeae L. Branch with ripe olive (A), flowering olive branches (B) and olive flowers (C). Modified from Y. Khan et al (Y. Khan et al. 2007).

The fruit is an oval-shaped drupe with a typical size of 2–3 cm (width and length) made up of 3 parts, epicarp or skin, mesocarp or pulp and endocarp or stone. The epicarp that is covered with wax, during the growth phase turns from light green to purple and brown or black. The mesocarp, with a soft pulpy flesh, accounts for 84–90% (of the total fruit mass). The endocarp containing the seed or kernel may differ from 13 to 30% of fruit weight. The seed contains 2–4 g oil /100 g. The fruit weight may range from 2–12 g, although some varieties may weigh as much as 20 g (Bouskou 1996; Niaounakis and Halvadakis 2006). Its growth and ripening is a long process which takes about 5 months in usual climatic conditions. It is composed by water (50%), protein (1.6%), oil (22%), carbohydrate (19.1%), cellulose (5.8%), inorganic substances (1.5%), phenolic compounds (1–3%), pectin, organic acids, and pigments (Bouskou 1996). However, this composition depends on a large amount of parameters including agronomical factors (*e.g.*, olive cultivar) (Romani et al. 1999), agroclimatic conditions (Vinha et al. 2005) and irrigation management (Patumi et al. 2002).

2.3.2 Cultivar

Although the tree is now cultivated in several parts of the world, the Mediterranean area serves as the major producer accounting for about 99% of the world's olive cultivation (Ghanbari et al. 2012). Among this area, the major olive producers countries are Spain, Italy and Greece which accounts for only the 12.1%, but has the largest per capita consumption of olive oil worldwide, over 26 liters per person per year. Spain produces 43.8% of olive oil world making, of whose the 75% comes from the region of Andalucía, particularly within Jaén province (FAOSTAT 2012). Although Italy is a net importer of olive oil, it still accounts for 21.5% of the world's production. Major Italian producers are known as "Oil Cities", including Lucca, Florence and Siena. However the largest production is harvested in Puglia and Calabria.

Italian and Spanish extra-virgin olive oils are the best-known and top-quality in North America, where are sold at high prices, often in prestige packaging. A large part of USA olive oil imports come from Italy, Spain, and Turkey (Sibbett and Ferguson 2005).

2.3.3 Extraction process

The mechanical and physical processes of olive oil production involve harvesting, washing, crushing, malaxation, decantation and filtration.

2.3.3.1 Collection

Olives are harvested in the autumn and winter, but the time varies in each country and with the season and the cultivar. Most of their today are harvested by shaking the boughs or the whole tree, but the found lying using on the ground can result in poor quality oil, due to damage. Another method involves standing on a ladder and "milking" the olives into a sack tied around the harvester's waist. This one produces high quality oil. A third method uses a device called an oli-net that wraps around the tree trunk and opens to form an umbrella-like catcher from which workers collect the fruit. Another method uses an electric tool, 'the oliviera', which has large tongs that spin around quickly, removing fruit from the tree. Olives harvested by this method are used for oil.

In some countries like Italy, Croatia and Greece, olives are harvested by hand because the terrain is too mountainous for machines. As a result, the fruit is not bruised and leads to a superior finished product.

2.3.3.2 Washing

The purpose of the washing section is to remove leaves, dirt, stones and other heavy extraneous objects before the olives enter the first part of the processing line.

Olives are placed in special washing machines where the washing water is decanted into a reservoir and circulated within the process. Olives passing accross different automatically conveyors are defoliatoring and washed. Leaves, dirt and stones are discharged through a suitable duct.

2.3.3.3 Crushing

This operation is designed to tear the fruit cells to release the droplets of oil from the inner cavity (vacuole). Like an important part of extracting olive oil, it affects its physical and chemical properties, therefore its quality.

Olives are grinded by a metal crusher-hammer or a toothed-disc. Using the hammer increases the oil extraction yield because the intercellular structure is destroyed by using the stone mill and consequently oil droplets may be retained inside the cells that are cut without destroying the intercellular structure (Veillet et al. 2009). Although the metal crusher may increase the yield of extraction from olives, but because of high speed it may create more emulsion than a stone crusher, the produced paste needs to stay longer in the malaxation process. In addition, the hammer crusher instead of a stone crusher cuts better the olives and increases the amount of total phenols components, so the stronger antioxidant power in the oil. This can be ascribed to the higher temperature which is caused by the speed of the hammer crusher as well as solubilisation phenomena by which more phenolic compounds pass into the oil (Veillet et al. 2009).

2.3.3.4 Malaxation

Malaxation process (also called beating or kneading) is essential for increasing extraction yields, to enhance the effect of crushing and to make the paste uniform. During this step the small droplets of the oil, by means of slow and continuous kneading of the paste produced by metallic crusher, merge into large drops that can be easily separated by the separating apparatus. The bioactive compounds in olive oil can be significantly improved by various factors including temperature, time (Servili et al. 2003), and the use of enzymes (Vierhuis et al. 2001). Addition of commercial enzyme preparations such as pectolytic, hemicellulolytic, and cellulolytic during the olive oil malaxation process resulted in degrading the cell wall of the fruit and reducing the complex of hydrophilic phenols with polysaccharides, increasing

the concentration of free phenols in the olive paste and their consequent release into the oil and wastewaters through processing (Aliakbarian et al. 2008).

2.3.3.5 Decantation

In this section the oil is separated from the paste and the vegetation water by using decanters. They are of two types named three-phases (10–30 L of added water per 100 kg of olive paste) and two-phases decanter that operates without adding any water. Actually, in three phase system in order to reduce the viscosity of pastes and to separate oil easily from the solid phase, sufficient water needs to be added before centrifugation (Montedoro et al. 1992). Disadvantages of this process include higher amounts of waste water (1.25 to 1.75 times more water than press extraction), loss of valuable components (e.g., natural antioxidants, phenolics) in the water phase, and problems of disposal of the oil mill waste water. Therefore, pressure system that does not need to add water to the olive paste offers a large amount of phenolic compounds in comparison to those obtained by the centrifugation system (Montedoro et al. 1992). The oil produced by a two-phase system has higher phenolic compounds content (Montedoro et al. 1992), stronger antioxidant activity (2-fold greater) and higher resistance to oxidation than that obtained by a three-phase system due to the higher amount of hydroxytyrosol, as an orthodiphenol compound (Galli and Visioli 1999; C. Manna et al. 1999).

2.3.3.6 Filtration

It is the final step in olive oil processing and can be carried out with fibrous materials and starch in combination with the filtration hardware to improve performance (Lozano-Sánchez, Segura-Carretero, and Fernández-Gutiérrez 2011). This process eliminates remaining solid particles that may reduce the shelf life and nutritive quality of olive, and removes humidity. The remaining paste (pomace) still contains a small quantity (about 5–10%) of oil that cannot be extracted by further pressing, but only with chemical solvents. This is done in specialized chemical plants, not in the oil mills. The resulting oil is not "virgin" but "pomace oil", as discussed following.

2.3.4 Classification

The grades of oil extracted from the fruit can be classified as:

- EVOO: Comes from virgin oil production only, and is of higher quality. It contains no more than 0.8% free acidity (see below), and is judged to have a superior taste, having some fruitiness and no defined sensory defects. EVOO accounts for less than 10% of oil in

many producing countries, with a higher percentage in the Mediterranean countries (Greece: 80%, Italy: 65%, Spain 30%);

- VOO: Comes from virgin oil production only, but is of slightly lower quality, with free acidity of up to 1.5%, and is judged to have a good taste;
- ROO: It is obtained by refining virgin olive oils with a high acidity level and/or organoleptic defects that are eliminated after refining, without alterations in the initial glyceridic structure. It has a free acidity, expressed as oleic acid, of not more than 0.3%. It is extracted by using charcoal and other chemical and physical filters. Oils labeled as pure olive oil or olive oil are primarily refined olive oil, with a small addition of virgin-production to give taste;
- POO: It is refined pomace olive oil often blended with some virgin oil. It has a more neutral flavor than pure or virgin olive oil and the same fat composition as regular olive oil, giving it the same health benefits. It also has a high smoke point, and thus is widely used in restaurants as well as home cooking in some countries.

The analytical parameters of olive oil merchandise classification according to 1989/03 UE Regulation are reported in table 5.

In Italy exists 40 types of olive oil whose 38 qualitatively branded as PDO and 2 as PGI. The production rules for the brands of PDO and PGI often involve the use of traditional techniques and restrictive rules with the aim of ensuring a higher quality products, and with particular reference to the traditional varieties used, which must be native.

	Extra Virgin Olive Oil	Virgin Olive Oil	Ordinary Virgin Olive Oil
Acidity (%)	≤ 0,8	≤ 2,0	>2
Peroxide Value (meq O2/Kg)	≤ 30	≤ 20	-
Waxes (mg/Kg)	≤ 250	≤ 250	≤ 300
Saturated fatty acids in position 2 of the triglyceride (%)	≤ 1,5	≤ 1,5	≤ 1,5
Stigmastadiene mg/kg	≤ 0,15	≤ 0,15	≤ 0,50
ECN42 HPLC and ECN42 Difference: theoretic calculation	≤ 0,2	≤ 0,2	≤ 0,3
K232	≤ 2,50	≤ 2,60	_
K270	≤ 0,22	≤ 0,25	_

ΔK	≤ 0,01	≤ 0,01	_
Organoleptic evaluation: median value of the defect (Md)	Md = 0	Md ≤ 2,5	Md > 2,5
Organoleptic evaluation: median value of the "fruity" (Mf)	Mf > 0	0 Mf > 0	_
Acidic composition (%)			
Miristic (%)	0,05	≤ 0,05	≤ 0,05
Linolenic (%)	≤ 1,0	≤ 1,0	≤ 1,0
Arachidic (%)	≤ 0,6	≤ 0,6	≤ 0,6
Eicosanoic (%)	≤ 0,4	≤ 0,4	≤ 0,4
Behenic (%)	≤ 0,2	≤ 0,2	≤ 0,2
Lignoceric (%)	≤ 0,2	≤ 0,2	≤ 0,2
Trans-linolenic isomers sum (%)	≤ 0,05	≤ 0,05	≤ 0,05
Sterols composition (%)	≤ 0,5	≤ 0,5	≤ 0,5
Cholesterol (%)	≤ 0,1	≤ 0,1	≤ 0,1
Brassicasterol (%)	≤ 4,0	≤ 4,0	≤ 4,0
Campesterol (%)	< camp _	< camp	-
Stigmasterol (%)	≥ 93,0	≥ 93,0	≥ 93,0
β-sitosterol (%)	≤ 0,5	≤ 0,5	≤ 0,5
Δ -7-stigmastenol (%)	≤ 0,5	≤ 0,5	≤ 0,5
Total sterols (%)	≥ 1000	≥ 1000	≥ 1000
Eritrodiol and uvaol (%)	≤ 4,5	≤ 4,5	≤ 4,5

Table 5: Analytical parameters of olive oil merchandise classification according to 1989/03 UE Regulation that modifies the 2568/91 UE Regulation. Modified from Servili et al. (Servili et al. 2010).

2.3.5 Chemical composition: the phenolic compounds

From a chemical point of view, olive oil can be divided into two fractions, saponifiable and unsaponifiable, depending on their behavior in presence of a strong alkaline solution (NaOH or KOH) and heating. Saponifiable (98% to 99% of total weight), able to form soaps in the mentioned conditions, include free fatty acids or esterified fatty acids with glycerol to form triglycerides, diglycerides and monoglycerides. It contains 75% to 85% of unsaturated fatty

acids (mainly oleic and linoleic acids) and 15% to 25% of saturated fatty acids (palmitic andstearic acids). The unsaponifiable (1-2%), formed by microcomponents that do not form soaps, is present in small quantities and contains mostly sterols, fat-soluble vitamins, waxes, aliphatic alcohols, aromatic compounds and polyphenols (Fig. 19). This fraction is very important from a nutritional and analytical point of view, to check the authenticity of the oil and its stability. For instance, the sterol profile is species-specific, so adulterations of the product with other oils are easily detectable ("Olive Oil Reference Book").

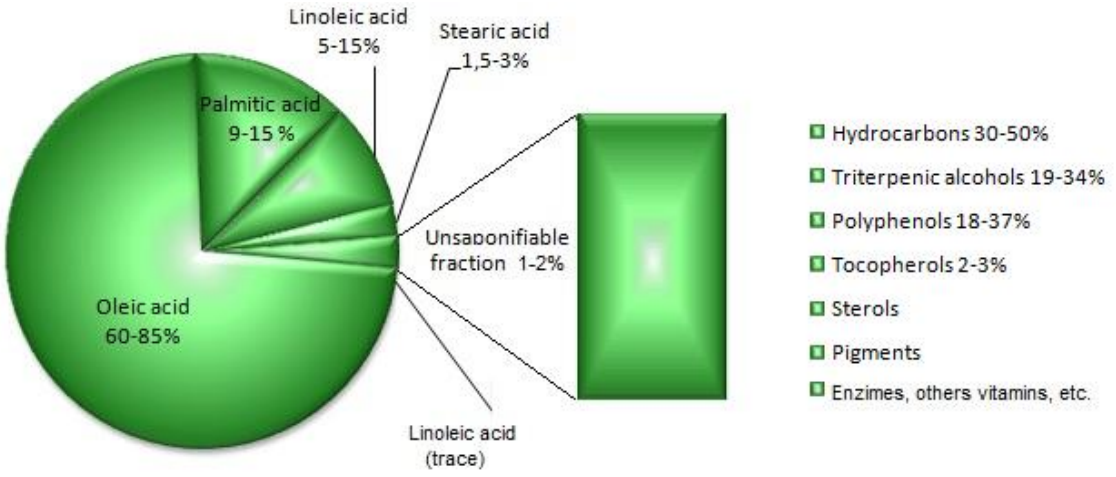


Figure 19: Chemical composition of unsaponifiable and saponifiable fractions of olive oil. Updated from ("Olive Oil Reference Book").

As mentioned previously, Spain and Italy are the major extra-virgin olive oils producer countries. The fatty acid composition of their oils is reported in table 6. Regarding Italy, the Calabria region produce and olive oil with a high content of palmitic acid (Table 7).

Origin area	Oleic acid (%)	Linoleic acid (%)	Palmitoleic acid (%)	Palmitic acid (%)	Stearic acid (%)
Italy	64.1 – 85.0	1.0 – 15.0	0.2 – 5.5	7.1 – 17.5	0.3 – 3.4
Spain	65.3 – 79. 6	5.1 – 19.8	-	-	-

Table 6: Fatty acid composition of olive oil from Italy and Spain. From Servili et al. (Servili et al. 2010).

Origin area	Oleic acid (%)	Linoleic acid (%)	Palmitic acid (%)	Stearic acid (%)
Apulia	79.6	7.3	9.3	2.3
Calabria	75.6	6.0	13.8	2.6
Liguria	80.6	5.2	10.0	2.6
Sardinia	74.6	9.2	12.5	2.0
Sicily	72.9	8.7	12.6	2.8
Tuscany	77.6	6.7	11.6	6.7
Umbria	77.7	5.9	10.9	5.9

Table 7: Fatty acid composition of olives from different Italian areas production. From Servili et al. (Servili et al. 2010).

Oleic acid, the principal fatty acid that determines olive oil free acidity, is claimed to increase the HDL-C and Apo A-I, decreasing the LDL-C cholesterol and Apo B-I (Kanter et al. 2012; Estevez-Gonzalez, Saavedra-Santana, and Betancor-Leon 1998), preventing cardiovascular diseases that are the major cause of mortality in industrialized countries. If these beneficial effects of olive oil can be attributed only to MUFAs content, any type of high oleic acid oils such as rapeseed oil, or any MUFAs-rich food would have shown the same health benefits. This indicates that olive oil's effects are due to more than MUFAs content. It has been suggested that the phenolic profile of olive oil is likely to have far greater benefits on blood lipids and oxidative damage than those shown by MUFAs (M.-I. Covas 2008). Based on this evidence, olive oil can be categorized as a functional food that besides having a high level of oleic acid, contains other medicinally important minor components with multiple biological activities (Covas 2007).

A linear relationship between the phenolic content and the oxidative stability of extra-virgin olive oil has been found. The phenolic content, that contribute to the resistance of olive oil to oxidative rancidity (Bouskou 1996), depends on a number of factors but mainly on the production and storage of the oil ("IOOC" 2014). Its content has been reported to be 232 ± 15 mg/kg in extra-virgin olive oil and 62 ± 12 mg/kg in refined olive oil (Owen et al. 2000), although there are several discrepancies, may due to inaccuracy of the two methods commonly used to determine the total phenol content. These methods are the Folin-Ciocalteau (FC) colorimetric assay, a simple and highly efficient procedure limited by a low specificity (Huang, Ou, and Prior 2005; Magalhaes et al. 2006), and analysis by HPLC, sensitive and specific but very time-consuming (Romani et al. 2001). Recently, also the correlation of polyphenolic content with the extinction coefficient at 225 nm (K 225) was studied, mainly to estimate the sensorial attribute of bitterness (Garcia-Mesa and Mateos 2007) or bitterness and pungent.

The phenolic compounds in EVOO and their chemical structure are reported in table 8 and figure 20, respectively.

Phenolic acids and derivatives	Phenolic alcohols	
Vanillic acid	3,4 DHPEA	
Syringic acid	p-HPEA	
p-Coumaric acid	3,4-dihydroxyphenyl ethanol glucoside	
o-Coumaric acid		
Gallic acid	Lignans	Flavones
Caffeic acid	(+)-1-Acetoxypinoresinol	Apigenin
Protocatechuic acid	(+)-Pinoresinol	Luteolin
p-Hydroxybenzoic acid		
Ferulic acid	Hydroxy-isocromans	
Cinnamic acid		
4-(acetoxyethil)-1,2-Dihydroxybenzene		
Benzoic acid		
Secoiridoids		
3,4 DHPEA-EDA		
p-HPEA-EDA		
Oleuropein aglycon 3,4 DHPEA-EA		
Ligstroside aglycon		
Oleuropein		
p-HPEA-derivative		
Dialdehydic form of oleuropein aglycon		
Dialdehydic form of ligstroside aglycon		

Table 8: Phenolic compounds in Extra Virgin Olive Oil (EVOO). From Servili et al. (Servili et al. 2014)

The efforts spent in recent years studying EVOO phenolic compounds can be ascribed to the fact that these substances show many healthy benefits, as depicted in figure 21.

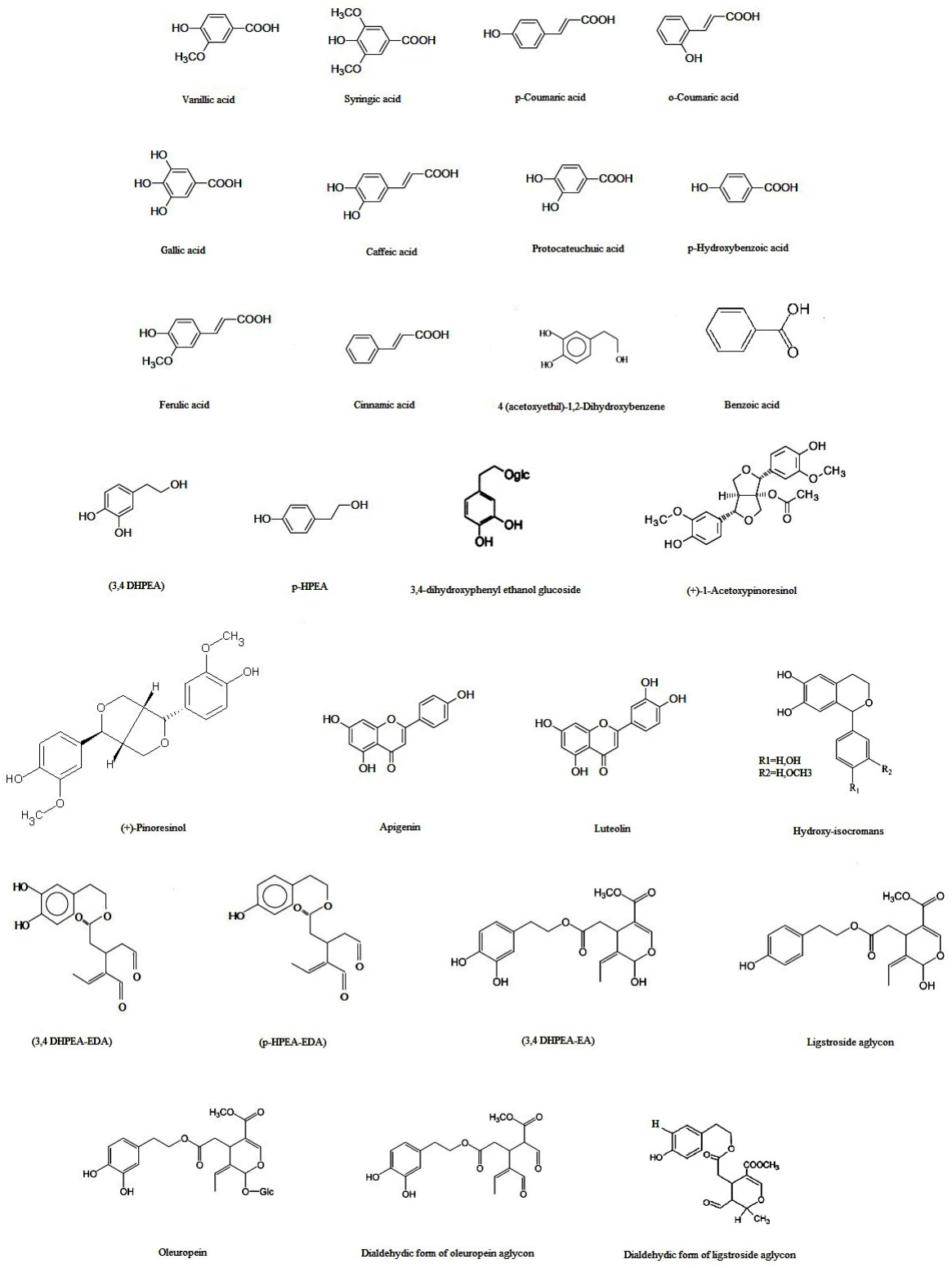


Figure 20: Chemical structures of the prevalent phenolic compounds in EVOO. Adapted from Reddy, Ghanbari and Servili et al. (Reddy and Caldarelli 2011; Ghanbari et al. 2012; Servili et al. 2014).

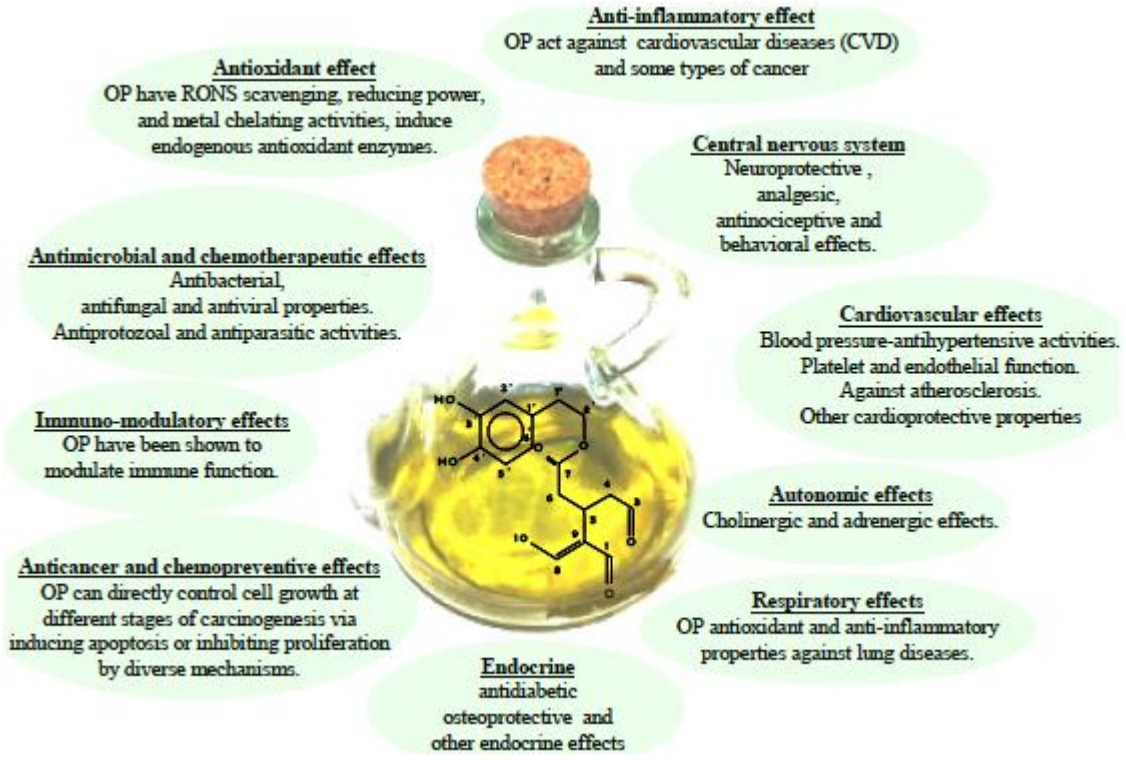


Figure 21: Schematic representation of the correlation among EVOO health properties and phenolic compounds, 3,4DHPEA-EDA in particular, was given. *From Servili et. al. (Servili et al. 2014).*

Polyphenols work inside cellular compartments as the first line of defense against free radicals, thanks to their antioxidant capacity, such as cellular redox status modulation by enzyme systems. The oxidation processes inside cells are due to the formation of ROS whose unbalanced levels drive the oxidation process of LDL in a large number of acute and chronic degenerative diseases. In particular, this oxidation process represents one of the first stages of the onset of atherosclerotic lesions (Carnevale et al. 2014). In this respect, a decrease in lipid oxidative damage in addition to an increase in the cholesterol level of HDL, strongly dependent on the phenolic content of EVOO has been observed (Gimeno et al. 2002; Castaner et al. 2011; Castaner et al. 2012). Because the increase in HDL cholesterol levels is one of the goals of current cardiovascular disease therapies (Covas et al. 2006; Helal et al. 2013; Martin-Pelaez et al. 2013), HDL functionality should be more important than its amount in the blood, might be due to the promotion of cholesterol efflux from macrophages in the so-called "reverse cholesterol transport" process (Rosenson et al. 2012). In this context polyphenols might counterbalance HDL oxidation (Shao 2012; Farras et al. 2013).

The larger fraction of hydrophilic phenols in EVOO are secoiridoids, among which, in *Olea europaea*, oleuropein, demethyl-oleuropein, ligstroside and oleoside represent the

predominant phenolic oleosides. Between the oleosides, oleuropein is the major constituent of the leaves, virgin olive oil and unprocessed olive (Syed Haris Omar 2010).

2.3.5.1 Oleuropein: chemistry and biological effects

Oleuropein structure was first recognized in 1958 from fresh leaves in amount from 1.5% to 2% (panizzi, Scarpati, and Oriente 1990). It occurs not only in the Olea genus but also in many other genera belonging to the Oleaceae family like Fraximus excelsior, angustifolia and chinensis, Syringa josikaea and vulgaris, Philyrea latifolia, Ligustrum ovalifolium and vulgare, and others (Soler-Rivas, Espin, and Wichers 2000). Its biosynthesis in Oleaceae proceeds via a branching in the mevalonic acid pathway from the secondary metabolism, resulting in the formation of oleosides (Fig. 22) (Damtoft, Franzyk, and Jensen 1993).

During fruit maturation and olive processing, chemical and enzyme reactions occur, which reduce the concentration of oleuropein raising that of degradation products. Oleuropein accumulation starts during the olive growth phase, declines throughout the green maturation phase concomitantly with chlorophyll and ligstroside levels, and continue to fall until the black maturation, when anthocyanins appear and verbascoside increase. As the oleuropein levels decline, elenolic acid glucoside and demethyloleuropein -which are glucosylated derivatives of oleuropein- tyrosol, hydroxytyrosol and tyrosol glucoside emerge. It has been suggested that elenolic acid glucoside and demethyloleuropein are formed from oleuropein by the action of esterases, that increase during the maturation phase (Capozzi, Piperno, and Uccella 2000), while the increase in tyrosol, hydroxytyrosol and tyrosol glucoside levels are consistently correlates with the activation of b-glucosidases during crushing and malaxation of the fruits (Obied et al. 2008) (Fig. 23). The elenolic acid glucoside and hydroxytyrosol contents are considered indicators for the maturation of olives (Syed Haris Omar 2010).

The concentration of hydroxytyrosol is dependent not only on the concentration of demethyloleuropein but also on that of oleuropein decomposed by methylesterase, since high concentrations of 3,4-DHPEA-EDA are found in cultivars characterized by low demethyloleuropein concentration.

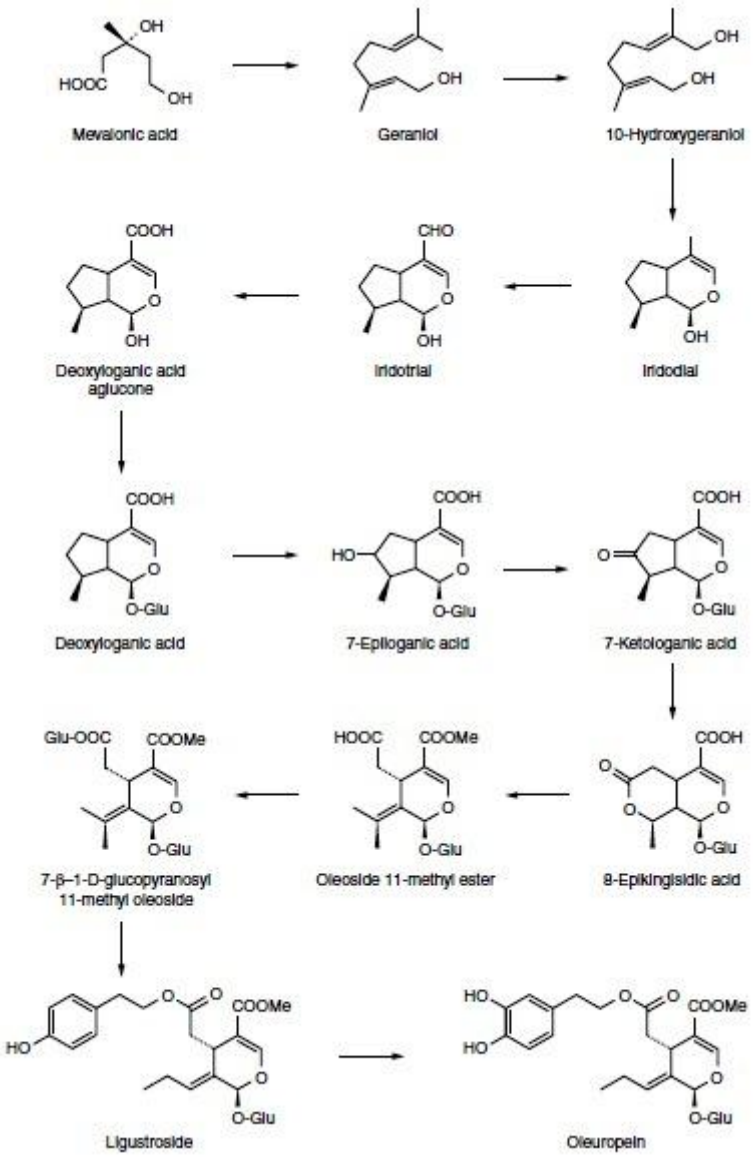


Figure 22: Proposed biosynthetic pathway for OL in Oleaceae. From Syed Haris Omar (Syed Haris Omar 2010).

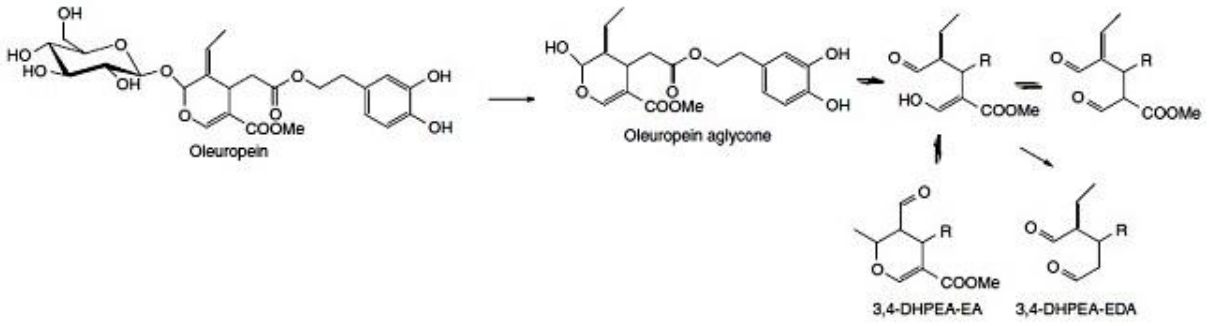


Figure 23: Degradation pathway of oleuropein. From Bulotta et. al. (Bulotta et al. 2013).

Rapidly absorbed after oral intake with a maximum of plasma concentration (55-60%) occurring around 2 h, oleuropein is secreted for the 15% in urine as tyrosol and hydroxytyrosol (Vissers et al. 2002). The mechanism underlying the absorption is different for the glucosylated and unglucosylated forms. After ingestion, oleuropein aglycone is partially hydrolyzed into free hydroxytyrosol and free tyrosol in the stomach, and some remain intact and enter the small intestine un-hydrolyzed (Giulia Corona et al. 2006). In this site, the quote of hydroxytyrosol and tyrosol are absorbed by passive diffusion (C. Manna et al. 2000) and metabolized primarily to O-glucuronidated conjugates but also O-methylated by the action of COMT (Giulia Corona et al. 2006). In contrast, the glucosylated form appears not to be subject to gastric hydrolysis (Vissers et al. 2002), and enter the small intestine unmodified (Fig. 24). The mechanism of absorption is unclear but may involve SGLT1 or paracellular movement facilitated by the opening of paracellular junctions in response to hypotonicity (Edgecombe, Stretch, and Hayball 2000). The hydrolysis reaction would be performed by lactic acid bacteria (Santos et al. 2012).

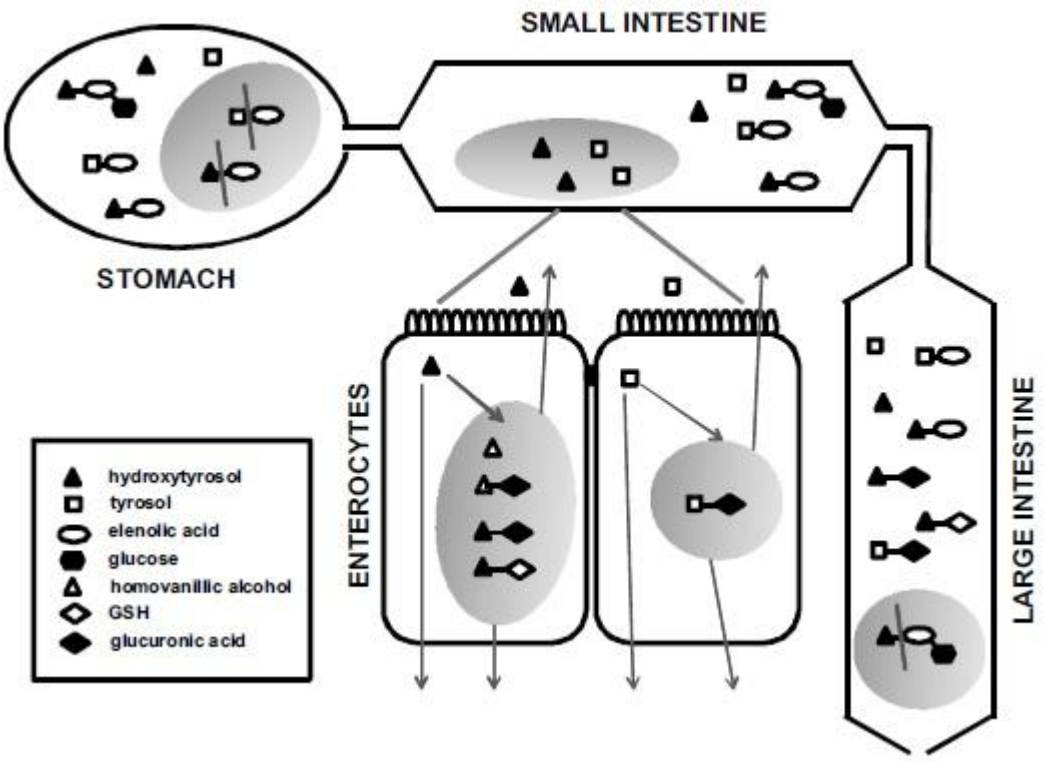


Figure 24: Schematic representation of gastrointestinal absorption and metabolism of olive oil phenolics. From G. Corona et al. (G. Corona, Spencer, and Dessi 2009).

Oleuropein and its derivates have demonstrated several biological and pharmacological properties (Fig. 25) (Bulotta et al. 2013) .

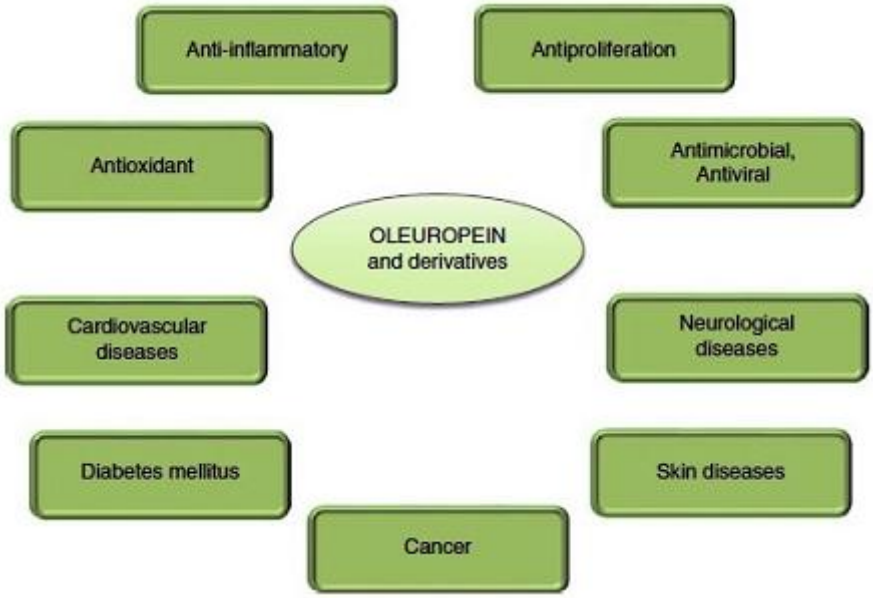


Figure 25: Biological and pharmacological effects of oleuropein and derivatives. From Bulotta et. al. (Bulotta et al. 2013).

A close relationship between anti-oxidant, hypoglycemic and hypolipidemic activity after supplementation of oleuropein and hydroxytyrosol has been observed. In animal experimental models of alloxan-induced diabetes, oleuropein and hydroxytyrosol rich extract decreased blood glucose levels concomitantly to the restoration of most of the enzymatic and nonenzymatic endogenous antioxidants (Hamden et al. 2009; Jemai, El Feki, and Sayadi 2009). This close relationship was also confirmed using a high-carbohydrate, high-fat diet-induced model of the metabolic syndrome in rats, by improving glucose tolerance and normalized abdominal fat deposition as well as plasma triglyceride and total cholesterol concentrations; These effects were accompanied by reduced plasma uric acid and malondialdehyde concentrations, therefore suggesting a decrease in ROS (Poudyal, Campbell, and Brown 2010).

Since ROS-induced alteration contribute to the pathogenesis of diabetes (Kaneto et al. 2007), these properties have been also investigated in insulin-secreting pancreatic β-cells. It has been found that oleuropein significantly suppressed cell death induced by cytokine- or H_2O_2-mediated apoptosis and/or necrosis, and was able to inhibit ROS generation and to preserve insulin secretion in INS-1 cells. Because these findings were accompanied by the maintenance

of intracellular glutathione content and the activity of antioxidant superoxide dismutase, an involvement of the preservation of redox homeostasis in the protective action was suggested (Cumaoglu et al. 2011; Cumaoglu et al. 2011). About the molecular mechanism, interestingly, oleuropein was able to down-regulate the hepatic mRNA levels of LCN2 (Y. Kim, Choi, and Park 2010) whose deficiency appears to protect mice from developing aging- and obesity associated insulin resistance and hyperglycemia (Law et al. 2010).

By using an in vitro model, the 3T3-L1 cells, it was proved that oleuropein (200 and 300 μmol/L) and hydroxytyrosol (100 and 150 μmol/L) are able to inhibit both the intracellular triglyceride accumulation down-regulating PPARγ, C/EBPα and SREBP-1c transcription factors and their downstream targets genes GLUT4, CD36 and FASN, and 3T3-L1 division during mitotic clonal expansion causing cell cycle delay (Drira, Chen, and Sakamoto 2011). It seems that this effect is also mediated by Hydroxytyrosol acetate (Hd-Ac), a polyphenol approximately equal to that of Hd in some olive varieties (Brenes et al. 1999), at concentrations of 0–75 μmol/L, was able to stimulate, dose-dependently, lipolysis both phosphorylating perilipin and the HSL at Ser563 (Drira and Sakamoto 2013). Furthermore, by considering mitochondrial metabolism and ATP synthesis decline in concert with a reduction of key factors regulating mitochondrial biogenesis in patients with insulin resistance, type 2 diabetes and obesity (Patti et al. 2003; Drira, Chen, and Sakamoto 2011), a novel approach was proposed. It has been reported that hydroxytyrosol ranged at 0.1–10 μmol/L stimulated PPARGC1α and its downstream target factors Nrf1, Nrf2 and Tfam, which leads to an increase in mtDNA and in the number of mitochondria. Since the mitochondrial enhancement function also resulted in an increase Mitochondrial Complexes I, II, III and V expression and activity parallel to the oxygen consumption and a decrease in free fatty acid contents, hydroxytyrosol it has been suggested as an activator of 5′AMPK (Hao et al. 2010).

Although it was shown that oleuropein and hydroxytyrosol exert many anti-inflammatory effects (Table 9), nowadays it is reported in literature about their involvement into the impaired state of low-grade inflammation which occurs during obesity into an in vitro model.

Phenolic compound	Molecular mechanisms	References
Hydroxytyrosol	↑,↓ iNOS (↑,↓ NO)	(Giner et al. 2011)
	↓COX 1/2	(Procopio et al. 2009; Giner et al. 2011)
	↓Lipoxygenase	(de la Puerta, Ruiz Gutierrez, and Hoult 1999)
	↓MMP-9	(Giner et al. 2011)
	↓ iNOS (↓NO)	(Maiuri et al. 2005; Zhang, Cao, and Zhong 2009)
	↓COX 1/2	(Procopio et al. 2009; Zhang, Cao, and Zhong 2009; Scoditti et al. 2014)
	↓PGE-2	(Impellizzeri et al. 2011)
	↓Myeloperoxidase	(de la Puerta, Ruiz Gutierrez, and Hoult 1999)
	↓ IL-1β mRNA	(Gong et al. 2009)
	↓Thromboxane A2, B2	(Gonzalez-Correa et al. 2008)

Table 9: Molecular mechanisms of the anti-inflammatory action of oleuropein and its hydroxytyrosol. Modified from Bulotta et al. (Bulotta et al. 2013).

AIM

Nowadays, a team of chemical engineers in Department of Computer Science, Modeling and Systems Engineering (DIMES), University of Calabria, Cosenza, "have managed to structure lipid systems to check the consistency of the oil", preserving its quality.

In order to assess if this structured fat could be considered ‘‘healthy’, the aim of this study was to investigate the impact of spread bio oil intake on the risk of being obese, diabetic and hypertensive.

This work is placed in a PON project designed by the DIMES, and is result of a collaboration between the Department of Biology, Ecology and Earth Science (diBEST), University of Calabria and the supporting of the Department Of Nutrition, Food Science and Physiology (CAF), University of Navarra, Pamplona.

CHAPTER3
MATERIALS AND METHODS:AN IN *VIVO* STUDY

3.1 Animals

Sprague Dawley rats (n=45) (Harlan Laboratories, Udine, Italy), weighing 200–250 g, aged 8 weeks, were used. Rats were housed in a temperature controlled room (25 ± 1 ◦C), 12-h light:12-h dark cycle environment with ad libitum access to water and food for 16 weeks. Food and water measurements were taken to monitor the day to-day health of the rats. The rats were randomly divided into six groups based on their diet: the control (ND; n=15) fed a standard pelleted diet (44,2% of energy as protein, 18,6% as carbohydrate, 6,2% as lipid and 31% as fibers and vitamins), the overweight (HFD; n=15) a high-fat diet (17,3% of energy as protein, 48,5% as carbohydrate, 21,2% as lipid and 13% as fibers and vitamins) and the Spread Bio Oil (SBOD; n=15) a control diet surpluses with the 15% of Spread Bio Oil. After 16 treatment weeks, rats were anaesthetized by i.p. injection of ethyl carbamate (1 g/kg body weight) and euthanized. Descending aortas, SAT and liver were collected and frozen (−80°C) for further analysis. Animal care and its killing were in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health 1996).

3.2. Spread Bio Oil processing

SBO was structured by using extra virgin olive oil, a range between 60 and 70% (Gabro, Italy), distilled water, Cocoa Butter (ICAM, Italy), a vegetal fat rich in natural saturated components, monoglycerides and fatty acids in the range between 2 and 5% (MYVEROL, Kerry Group, Ireland) as organogelator. The organogel was produced at high temperature (above 60 °C) by mixing the ingredients of the oily phase. Quickly cooled with thermal ramp, it was brought at a lower temperature lower than the onset of the crystallization temperature (Tco), at which the system appears uniformly consistent. The emulsification with water was carried out at room temperature with a rotor-stator homogenizer (Ultra Turrax T50, Ika, Germany). The emulsion was rested at a controlled temperature (4 °C) for one week before being used.

3.3 Plasma biochemistry analysis

At the end of experimental period, rats were put in the fasting for 4h, blood collected from the tail vein into heparinized capillary tubes and centrifuged at 5,000 g for 15 minutes. Plasma samples were separated and stored at −80°C for analysis. TC, HDL-C and LDL-C were assayed using a kit (EHDL-100, EnzyChrom^{TM}, BioAssay Systems) following manufacturer's instructions. Basal glucose levels were determined by using an automated blood glucose analyzer.

3.4 Anthropometric assays

Animal weight and anthropometric assessment were performed during the experimental period once a week. A trans-abdominal incision was performed, chest opened with a bilaterally cut along the bottom edge, rib cage raised and descending aorta, SAT and liver removed, weighed and immediately frozen (−80°C) for further analysis. Organs weight, abdominal circumference and fat measurement were normalized relative to the length squared at the time of their removal. BMI was calculated as [body weight (g) /body length squared (cm^2)].

3.5 Western Blot analysis

Descending aortas and liver were sonicated in RIPA buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100, 10% glycerol, 1 mM PMSF, protease inhibitor cocktail) and tissue lysates cleared by centrifugation (13,000 rpm, 30 min at 4°C). Protein concentration was determined using the Bio-Rad DC protein assay®. The soluble lysates were electrophoresed through a reducing SDS-PAGE and electroblotted onto a nitrocellulose membrane (GE Healthcare). To confirm equal loading the transfer were stained with Red Ponceau (Sigma Aldrich®). Membranes were blocked and incubated with the primary polyclonal IgG for GLUT-12 (Bioss Inc.), monoclonals for p-AMPKα1/2 (Thr 172), AMPK α1/2, FAS, CPT1A, pAKT (Ser473), AKT all purchased from Cell Signaling Technology (Beverly, MA,USA), monoclonals for PKC, Ox-LDL R-1, COX-2, and polyclonals for GLUT-1, GLUT4, HIF-1α and NOS2 acquired from Santa Cruz Biotechnology Inc. The levels of proteins and phosphoproteins were detected with horseradish, goat and mouse peroxidase-linked secondary antibodies (Santa Cruz Biotechnology, Inc.) and revealed using the Enhanced Chemiluminescence system (GE Healthcare). Tubulin (Cell Signaling

Technology®) was used as a loading control. Immunoblots were quantified using Image J software (National Institutes of Health).

3.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (San Diego,CA, USA). Data were presented as mean ± SEM. Groups were analysed using one-way analysis of variance (ANOVA) following by Bonferroni's test. $P < 0.05$ was considered significant.

CHAPTER 4
RESULTS

5.1 SBO processing

The extra virgin olive oil composition is reported in appendix 1. Myverol, the commercial emulsion, was able to promote oil gelation, and cocoa butter (rich in stearic acid) to increase the saturated fat content. In this way, a stable network structure was obtained, also, useful to identify the optimal operating conditions (i.e. cooling and shear rate) necessary to enhance oil structuration. High cooling rates and low shear conditions, favoured the production of a large amount of small fat crystals, yielding a strong and extended crystalline network able to entrap and stabilize the water droplets during the emulsification (Lupi et al. 2011).

5.2 Plasma biochemistry analysis

Lipid and blood glucose levels are given in Figure 26. Results from lipid profile showed that HFD significantly increases LDL-C ($p<0.001$), HDL-C ($p<0.001$) and CHOL ($p<0.001$) compared with ND and SBOD, that conversely did not influence these parameters ($p<0.001$). SBO group showed a comparable profile to the ND ($p<0.001$), and interestingly decreased HDL-C ($p<0.001$). Blood glucose levels increased in HF group ($p<0.001$), while SBO intake did not alter glucose levels ($p<0.001$).

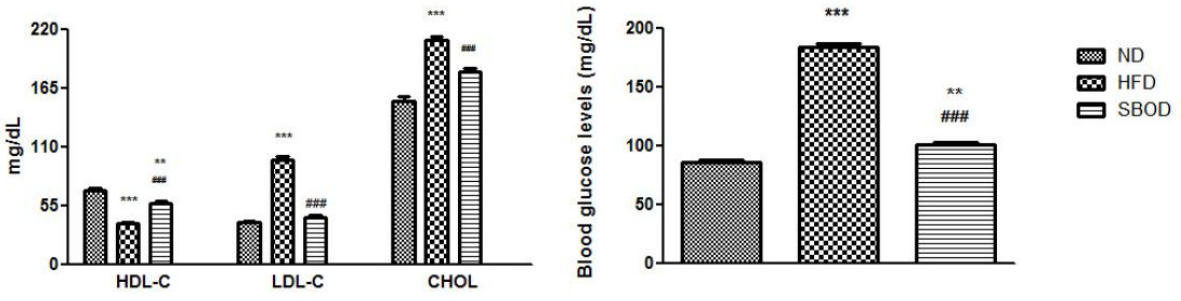


*Figure 26: Evaluation of biochemical profiles in ND, HFD and SBOD groups after diets. Data are shown as means ± SEM, n = 5 per group. **P<0.01; ***P<0.001, different from ND. ##P<0.01, ###P<0.00,1, different from HFD at 16 weeks.*

5.3 Anthropometric assays

Figure 27 shows anthropometric measurements, including animal weight, performed during the diet period. Obesity induced by the HFD was characterized by a significantly higher weight gain, BMI, abdominal fat and circumference ($p<0,001$) than the control and SBOD groups. It was also noticeable that although with an equal fat content percentage of HFD, SBO supplementation did not alter anthropometric assessment ($p<0.001$), and interestingly cause a reduction of BMI ($p<0,001$).

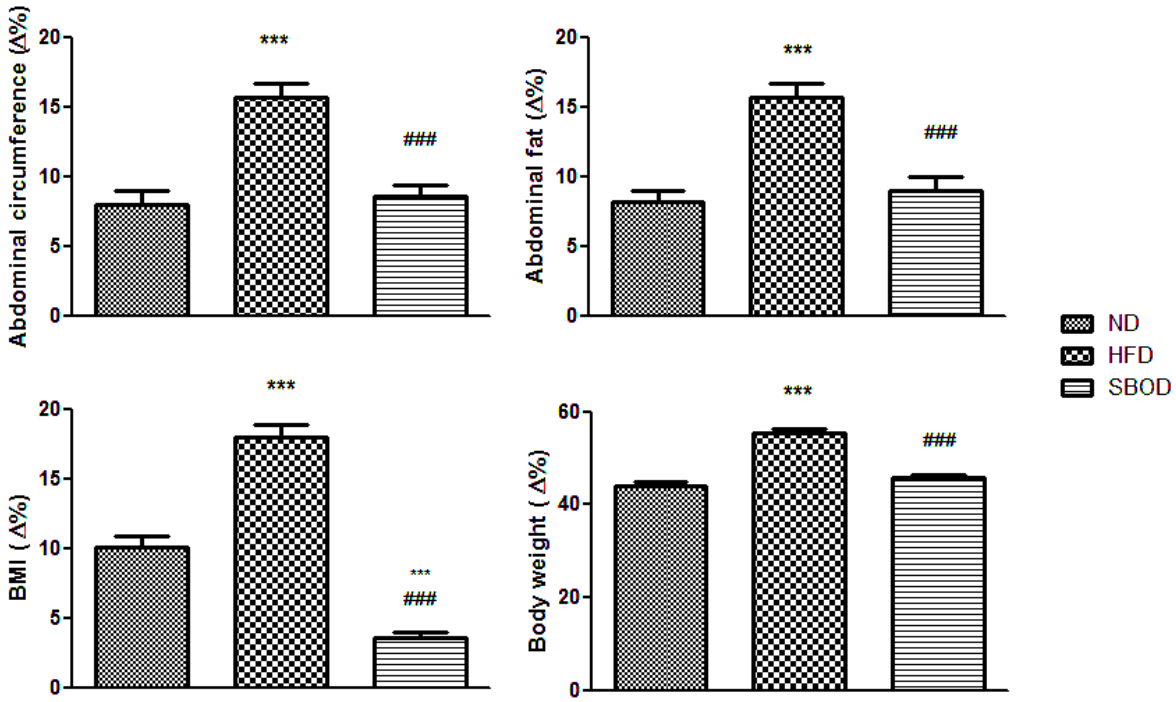
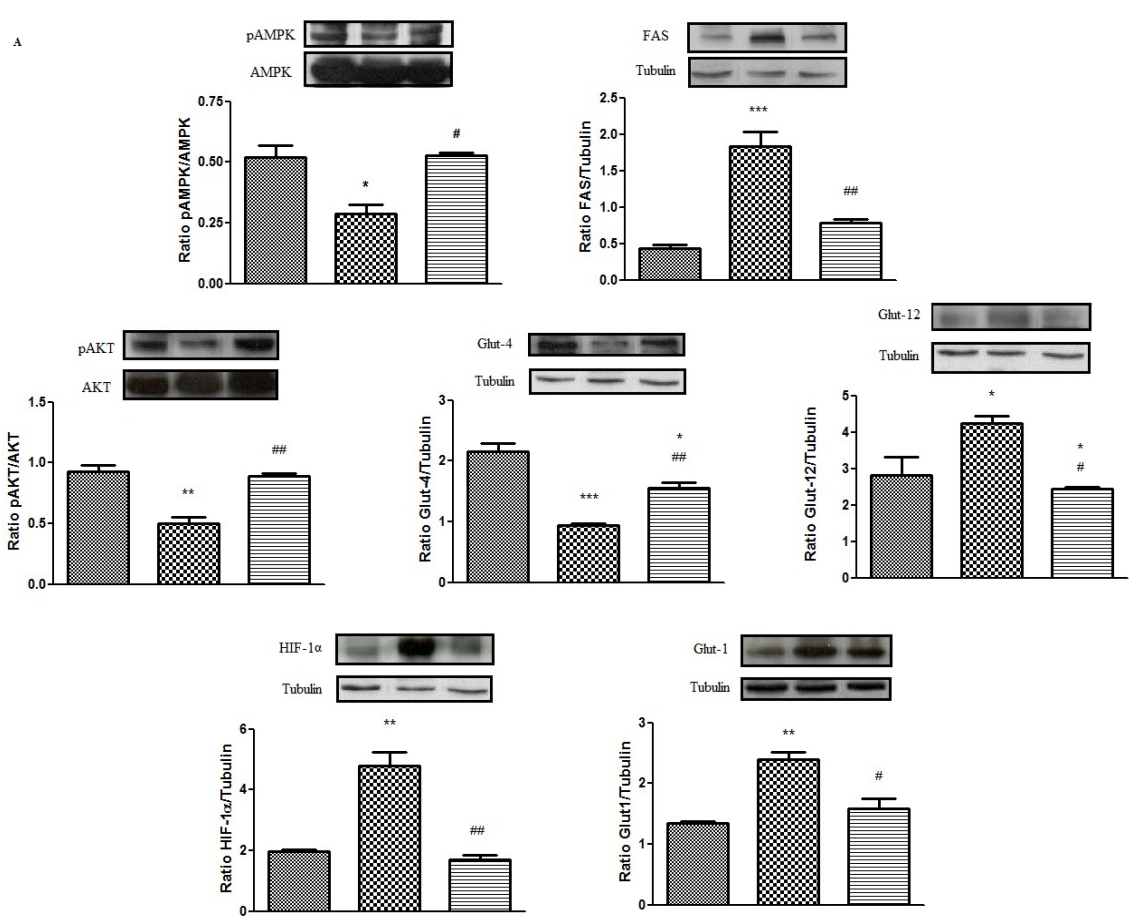


*Figure 27: Abdominal circumference and fat, BMI and weight after diets. Data are shown as means ± SEM, n = 5 per group. ***P< 0.001, different from ND ., ### P< 0.001, different from HFD at 16 wk.*

5.4 Western Blot analysis

Western blotting has been used to accurately assess the trasductional pathway activated in liver and aorta by SBO (Fig. 28). Conversely to the HF group, and with a comparable trend to the ND group, rats given SBO intake showed ability to stimulate AMPK phosphorylation ($p<0.01$) by reducing FAS ($p<0.01$). Concomitantly, it was able to stimulate glusose metabolism up-regulating pAKT ($p<0.01$), Glut-4 ($p<0.01$) and down-regulating Glut-1 ($p<0.05$) through the inhibition of HIF-1 α ($p<0.01$). Interstengly, a role in Glut-12 regulation ($p<0.05$). was observed, although the trend showed was disputable (Fig. 28A). Furthemore,

immunoblots on isolated rats orta, demonstrated that SBO supplementation also did not impair the vascular function, by attenuating NOS2 ($p<0.05$), COX-2 ($p<0.01$) and ox-LDL-R1($p<0.05$) expressions (Fig. 28B) .



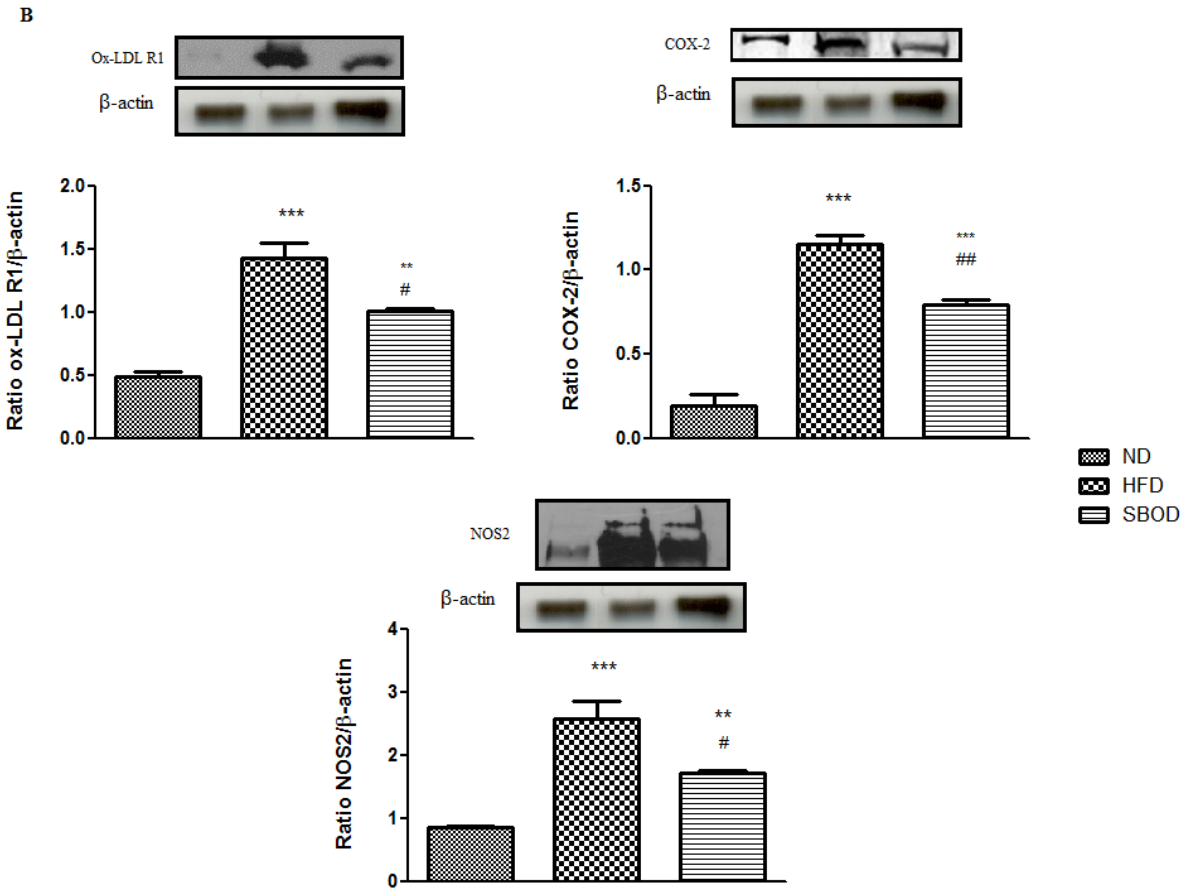


*Figure 28: Immunoblots and densitometry analysis of representative liver (A) and aorta (B) protein extracts. Tubulin and β-actin serves as a loading control. The signal was quantified using ImageJ program and normalized to loading controls. Data are shown as means ± SEM, n = 3 per group. *P<0.05, **P<0.01; ***P<0.001, different from ND. #P<0.05, ##P<0.01, different from HFD.*

CHAPTER 5
DISCUSSIONS

In this study, the formulation of an oil with a specific spreadability was used. SBO made by structuring lipid systems that was able to check its consistency, was utilized to assess its healthy effects in comparison with an experimental model able to induce metabolic syndrome. Structured emulsions are widely used in the food industry. In the case of water-in-oil emulsions, an oil phase structuration is achieved by the creation of a saturated fat crystalline network inside where water droplets are entrapped. Traditional technology based on the hydrogenation of vegetable oils, leads to the formation of saturated trans-fatty acids, considered unhealthy owing to their potential contribution to cardio-vascular diseases. As a consequence, nowadays the use of hydrogenated fatty acids has been reduced and the consumption of healthy oils has increased. Since oils need to be properly structured to be used as solid fat replacers, a W/O emulsion with a structured continuous oil phase has been made. In order to produce a structured fat that could be considered ‘‘healthy’, the main ingredients of these innovative emulsions were extra virgin olive oil, a typical element in the Mediterranean diet, cocoa butter, with its high stearic acid content, and a combination of mono- and di-glycerides of fatty acids, a common food emulsifier (Lupi et al. 2011).

(Merrill et al. 1997). In our experimental model, HFD diet produced raised blood glucose levels, conversely to SBO supplementation which maintained glucose levels similar to those found in the normal diet group. Concomitantly with a peripheral insulin resistance, the raising in glucose levels lead to TD2 (International Diabetes Federation 2013). Several drugs that have long been used for the treatment of diabetes, such as TZDs, exert some of their beneficial effects through the indirect activation of AMPK (Neil B. Ruderman et al. 2013). Like a metabolic sensor to the cellular energy status, AMPK acts regulating glucose homeostasis and lipid metabolism (Hardie 2004; Carling 2004; Kemp et al. 2003). Glucose homeostasis is maintained by a balance between hepatic glucose production and glucose uptake by peripheral tissues. To inhibit the fasting hyperglycemia in liver (Consoli 1992; Saltiel 2001), AMPK acts suppressing the transcription of PEPCK and G6Pase (Saltiel 2001; Lochhead et al. 2000) and restoring the energy balance inhibiting ACC to reduce malonyl-CoA concentration, thereby driving the entry of long-chain acyl-CoA into the mitochondria

for β-oxidation (Merrill et al. 1997). The rate-control step is regulated by CPT1, whose transfers long-chain acyl-CoA into the mitochondria, and this process is inhibited allosterically by malonyl-CoA (N. B. Ruderman et al. 1999), synthesized by ACC (Trumble, Smith, and Winder 1995). In our study, SBO was able to stimulate pAMPK expression, leading to the inhibition of FAS. This in turn enhanced insulin-sensitizing action through the coupling signaling pathway pAKT/GLUT-4 down-regulating GLUT-1 expression controlled by HIF-1α. Limited data are available on the effects of olive olive or its constituents on liver (Burgess et al. 2011). Since defects in insulin stimulated glucose metabolism in T2D are due to the uncoupling of AKT/PKCζ/γ /GLUT4 regulated by PI3K and PTEN (Taniguchi et al. 2006) signaling pathways, which may in turn be linked with the decreased production of PIP3 in the hyperglycemic environment(P. Manna and Jain 2013), we supposed that SBO is able to activate PI3K.

Regarding Glut-12, a trend was observed. Even though some articles about Glut-12 have been published, still little is known about its functional characteristics and its physiological role, and nowadays no data has reported about its expression in liver. Based on a recent study, where it seems that the expression of Glut-12 is increased in the diabetic myocardium, potentially as a compensatory mechanism for the observed downregulation of active Glut-4, and may probably be functioning as a basal non-insulin dependent Glut located primarily at the cell surface (Waller et al. 2013). This would be in accordance with what was reported previously by authors (Pujol-Gimenez et al. 2013). Supporting this hypothesis, in the vascular endothelium RGC-32, an angiogenic inhibitor acts down-regulating GLUT-12 expression to maintain the glucose homeostasis (Guo et al. 2014). In addition, it seems that Glut-12 expression is tissue-specific (Wood, Hunter, and Trayhurn 2003).

Diabetics also exhibit dyslipidemia, characterized by decreased levels of HDL-C and elevated levels of LDL-C and TG (Wilson, Kannel, and Anderson 1985; Barrett-Connor, Grundy, and Holdbrook 1982). According with this, in our experimental model, HFD diet produced changes in plasma lipid profile and anthropometric variables. Conversely, SBO supplemented in equal fat percentage of HFD, normalized abdominal fat deposition as well as plasma LDL-C, HDL-C and total cholesterol concentrations. Interestingly, it did not cause weight gain and reduced BMI index. It is well known that foods rich in fat are an example of palatable foods that inhibit the satiety signals and up-regulate hunger sensation (Erlanson-Albertsson 2005). Despite of that, it would seem that SBO with its fat content, provided a higher amount of energy supplying macronutrients able to reduce food intake or induce satiety, by suppressing

appetite neurotransmitters. This could explain the negative correlation with its supplementation and the body gain and BMI increase. In addition, as the fat deposition did not increase, we suppose that SBO may increase the basal metabolism, since it is closely associated to the percentage of lean mass that consume more energy (Suarez 2012). With this regard, since the basal metabolism decrease with the age (Suarez et al. 2012), we decided to use a young rat model (8 weeks).

When diabetes occurs, hypertension progress, as a result of an impaired NO-endothelium-dependent vasodilatation (DeSouza et al. 2005), that lead to a decrease of endothelial activation markers, and an increase of proinflammatory cytokine levels (Hadi, Carr, and Al Suwaidi 2005). One of the inflammatory protein secreted is the inducible form of COX, named COX-2, not expressed under physiological conditions (Davidge 2001). COX-2 is a rate-limiting enzyme that catalyzes the conversion of free arachidonic acid into prostaglandin H2. In this sense, it has been shown that the high expression of COX-2 in VSMC in a type 2 diabetic mouse model (Z. Guo et al. 2005) and in coronary arteries from diabetic patients (Szerafin et al. 2006), is closely related to increased expression of the antiapoptotic protein Bcl-2 (Redondo et al. 2011). This mediates an increased proliferation/apoptosis ratio in VSMC and a subsequent pathological vascular remodeling (Ruiz et al. 2006). In the scenario that leads the progression of hypertension to atherosclerosis, oxidation of LDL is one of the key steps (Fito et al. 2000). The oxidation of LDL-C induces endothelial cell death through the activation of NF-κB and AP-1 pathways increasing the $\dot{O}^{2-}$ formation and upregulating Ox-LDL R1 (Valente et al. 2014), throughout the evolving of macrophages into foam cells (Hansson 2005). The increased of $\dot{O}^{2-}$ production from NAD(P)H oxidase increases NOS2 expression (Alvarez et al. 2008). It has been shown that pretreatment with antioxidant significantly prevented ox-LDL-induced apoptosis (Chen et al. 2007) and with oleuropein and hydroxytyrosol the expression of NOS2 and COX-2 (Palmieri et al. 2012). According with this finding, SBO supplementation also did not impair the vascular function, by attenuating NOS2, COX-2 and ox-LDL-R1 expressions, suggesting its protection against the free radical-induced impairment of the NO·-mediated relaxation, thereby preserving the role of vascular endothelium in vasodilatation.

SUGGESTIONS

Is SBO able to reverse in experimental *in vivo* and *in vitro* models conditions of diabetes, obesity and hypertension?

Suggested as a healthy fat structured oil, are the beneficial effects of the emulsion due to the synergic effects of polyphenols, or to the amounts of other minor components?

Is Glut-12 a novel second insulin-sensitive Glut? It may probably function as a basal non-insulin dependent Glut primarily located at the cell surface? Are its expression and function tissue-specific?

Further analysis might clarify these aspects.

CONCLUSIONS

This study showed for the first time the correlation with the intake of SBO, an oil with a specific spread-ability, and lower likelihood of being obese, diabetic and hypertensive. SBO may have important clinical implications in the way that diabetics need to be treated to prevent cardiovascular complications originated by the inflammatory process.

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APPENDIX 1

® Centro Analisi Biochimiche Sas
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Laboratorio Operante secondo la norma UNI CEI EN ISO/IEC 17025:2005 Accreditato ACCREDIA al N. 0859
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Riconosciuto come laboratorio ufficiale per l'analisi degli oli alimentari dal Ministero delle Politiche Agricole e Forestali - GU n.46 del 25/02/2009 - DM 09/02/2009
LAB N° 0859

Rapporto di Prova N. 462/14

Rizziconi 16/01/2014

Committente: Oleificio Gabro Srl (174-08)
C.da Sisto - Str. Prov.le Lauropoli - Sibari Km 3. 87011 CASSANO allo JONIO (CS)

Numero campione:	462/14	**Data inizio prove:**	15/01/2014	**Data Di Campionamento:**	15/01/2014
Data ricevimento:	15/01/2014	**Data termine prove:**	16/01/2014	**Verb. di Campionamento N.**	/
Temperatura Arrivo in °C:	15.2	**Temperatura Conforme:**	Si		

Categoria Merceologica: 0000 - OLI ALIMENTARI
Prodotto dichiarato: 01. Olio d'oliva vergine extra
Descrizione Campione: Olio d'oliva vergine extra
Etichetta Campione:
Descrizione Sigillo: Nessuno
Quantita' Campione: 750 ml **Restituzione Campione:** No
Imballaggio: 1 Bottiglia in vetro con tappo a vite
Procedura Campionamento: (*) A cura del Committente

I risultati analitici si riferiscono al campione sottoposto a prova. Il presente Documento può essere riprodotto solo per intero. La riproduzione parziale deve essere autorizzata con approvazione scritta del ns. Laboratorio. Un controcampione viene conservato, in condizioni di temperatura idonea a preservarne l'integrità, per un periodo massimo di 30 gg, in rapporto alla sua deperibilità. Tutti i dati relativi all'analisi vengono archiviati per un periodo di 4 anni.
L'accreditamento del laboratorio non costituisce approvazione del prodotto da parte dell'organismo di accreditamento. Le eventuali valutazioni riportate non fanno parte della prova accreditata Accredia. I risultati delle prove non possono essere utilizzati a fini pubblicitari.
(*) - Prove non accreditate Accredia.
L'incertezza estesa è calcolata con un livello di probabilità del 95%, coefficiente di copertura k = 2
I risultati riportati nel presente rapporto di prova sono ottenuti su n. 1 prova.
Legenda: LOQ = Limite di Quantificazione (LOQ = Limiti Of Quantification) - NV = non valutabile in quanto < del LOQ (NV= Not Valuable because < LOQ)
FL = Annotazione che indica un valore oltre il limite regolamentare. U.M. = Unità di Misura.

Cod. Prova - Nome Prova - U.M.	Valore	Incertezza	Limite	
0046-00 - Acidi grassi liberi, metodo a freddo - in %	**0.59**	± 0.05	Max 0.8	(15)
Cod. Met. / Metodo: 0046-00 / Reg CEE 2568/1991 11/07/1991 GU CEE L248 05/09/1991 All II - Reg CE 702/2007 21/06/2007 GU CE L161 22/06/2007				
0050-00 - Numero di Perossidi - come meq. di O2/Kg. Olio	**10.0**	± 1.2	Max 20	(15)
Cod. Met. / Metodo: 0050-00 / Reg CEE 2568/1991 11/07/1991 GU CEE L248 05/09/1991 All III				
0052-00 - Analisi Spettrofotometrica nell'ultravioletto				
Cod. Met. / Metodo: 0052-00 / Reg CEE 2568/1991 11/07/1991 GU CEE L248 05/09/1991 All IX - Reg CEE 183/1993 29/01/1993 GU CEE L22 30/01/1993				
- K232	**2.01**	± 0.10	Max 2.5	(15)
- K266	**0.14**	± 0.02		
- K270	**0.13**	± 0.02	Max 0.22	(15)
- K274	**0.13**	± 0.02		
- Delta K	**0.00**	± 0.003	Max 0.01	(15)
0175-00 - Determ. del contenuto di Metil ed Etil Esteri degli Acidi Grassi (FAME e FAEE)				

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Riconosciuto come laboratorio ufficiale per l'analisi degli oli alimentari dal Ministero delle Politiche Agricole e Forestali - GU n.46 del 25/02/2009 - DM09/02/2009
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Rapporto di Prova N. 462/14

Committente: Oleificio Gabro Srl (174-08)

Cod. Prova - Nome Prova - U.M.	Valore	Incertezza	Limite	
Cod. Met. / Metodo: 0175-00 / Reg (UE) No 61/2011 24/01/2011 GU CEE L23/1 27/01/2011				
- Metil Palmitato - in mg/Kg	1.9			
- Etil Palmitato - in mg/Kg	1.9			
- Metil Linoleato - in mg/Kg	0.6			
- Metil Oleato - in mg/Kg	7.1			
- Metil Stearato - in mg/Kg	0.2			
- Etil Linoleato - in mg/Kg	0.7			
- Etil Oleato - in mg/Kg	6.6			
- Etil Stearato - in mg/Kg	0.5			
- Somma Esteri Metilici - (FAME C16+C18) - in mg/Kg	9.0			
- Somma Esteri Etilici - (FAEE C16+C18) - in mg/Kg	8.5			
- Somma FAME + FAEE - in mg/Kg	17.5		Max 75	
- FAME / FAEE	1.1			
- FAEE / FAME	0.9			
0047-00 - STEROLI				
Cod. Met. / Metodo: 0047-00 / Reg CEE 2568/1991 11/07/1991 GU CEE L248 05/09/1991 All V - Reg CEE 183/1993 29/01/1993 GU CEE L22 31/01/1993				
- Colesterolo in %	0.09	± 0.03	Max 0.5	(15)
- Brassicasterolo in %	< 0,01		Max 0.1	(15)
- 2,4-Metilencolesterolo in %	0.11			
- Campesterolo in %	2.14	± 0.10	Max 4	(15)
- Campestanolo in %	0.12			
- Stigmasterolo in %	1.37	± 0.08		
- Delta-7 Campesterolo in %	< 0,01			
- Delta-5,23 Stigmastadienolo in %	< 0,01			
- Clerosterolo in %	0.94			
- Betasitosterolo in %	81.44			
- Sitostanolo in %	0.77			
- Delta-5 Avenasterolo in %	11.32			
- Delta-5,24 Stigmastadienolo in %	0.93			
- Delta-7 Stigmastenolo in %	0.14	± 0.05	Max 0.5	(15)
- Delta-7 Avenasterolo in %	0.64			
- STEROLI TOTALI, mg/Kg	1455	± 80.0	Min 1000	(15)
- BETASITOSTEROLO TOTALE, %	95.40	± 0.30	Min 93	(15)
- ERITRODIOLO + UVAOLO in %	1.74	± 0.30	Max 4.5	(15)
- ERITRODIOLO + UVAOLO in ppm	25.8			(15)
0049-00 - Esteri Metilici degli Acidi Grassi - in %				
Cod. Met. / Metodo: 0049-00 / Reg CE 796/2002 06/05/2002 GU CE L128 15/05/2002 All XB + Reg CEE 2568/1991 11/07/1991 GU CEE L248 05/09/1991 All XA - Reg CEE 1429/1992 26/05/1992 GU CEE L150 02/06/1992				
- C12:0 - Acido Laurico	0.00			
- C14:0 - Acido Miristico	0.01	± 0.01	Max 0.05	(15)
- C16:0 - Acido Palmitico	15.07	± 0.42	[7.5; 20]	(15)
- C16:1 - Acido Palmitoleico	1.73	± 0.05	[0.3; 3.5]	(15)
- C17:0 - Acido Eptadecanoico	0.17	± 0.03		
- C18:0 - Acido Stearico	2.61	± 0.15	[0.5; 5]	(15)
- C18:1 - Acido Oleico	71.53	± 0.60	[55; 83]	(15)
- C18:2 - Acido Linoleico	6.95	± 0.10	[3.5; 21]	(15)

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Rapporto di Prova N. 462/14

Committente: Oleificio Gabro Srl (174-08)

Cod. Prova - Nome Prova - U.M.	Valore	Incertezza	Limite	
- C20:0 - Acido Arachico	0.46	± 0.05	Max 0.6	(15)
- C18:3 - Acido Linolenico	0.56	± 0.07	Max 1	(15)
- C20:1 - Acido Eicosenoico	0.30	± 0.05	Max 0.4	(15)
- C22:0 - Acido Beenico	0.14	± 0.03	Max 0.2	(15)
- C22:1 - Acido Erucico	0.00			
- C24:0 - Acido Lignocerico	0.08	± 0.01	Max 0.2	(15)
0049-01 - Analisi G.C. Isomeri Trans degli Acidi Grassi - in %				
Cod. Met. / Metodo: 0049-00 / Reg CE 796/2002 06/05/2002 GU CE L128 15/05/2002 All XB + Reg CEE 2568/1991 11/07/1991 GU CEE L248 05/09/1991 All XA - Reg CEE 1429/1992 26/05/1992 GU CEE L150 02/06/1992				
- Trans C18:1 - (Elaidinico)	0.01		Max 0.05	(15)
- Trans C18:2 + C18:3	0.02		Max 0.05	(15)
0053-00 - Stigmastadieni				
Cod. Met. / Metodo: 0053-00 / Reg CEE 2568/1991 11/07/1991 GU CEE L248 05/09/1991 All XVII - Reg CE 656/1995 28/03/1995 GU CE L069 29/03/1995				
- Stigmasta 3,5 Diene in mg/Kg	0.02	± 0.02	Max 0.1	(15)
0178-00(*) - POLIFENOLI - in ppm				
Cod. Met. / Metodo: 0178-00 / Met. HPLC (Tirosolo/St. Int. Ac. Siringico)				
- (*) - Polifenoli Totali	256.5	± 2.3		
- (*) - Idrossitirosolo	7.5	± 0.123		
- (*) - Tirosolo	5.0	± 0.142		
0190-00(*) - SAGGIO KREISS	NEGATIVO			
Cod. Met. / Metodo: 0000-00 / Metodo Interno di Prova				
0000-00(*) - in mg/Kg o ppm - LOQ = 0,01				
Cod. Met. / Metodo: 0001-00 / UNI EN 1528-1:1997 + UNI EN 1528-2:1997 + UNI EN 1528-3:1997 + UNI EN 1528-4:1997				
- (*) 2,4 D	< 0,01			
- 2,4 DDD - (o Mitofane) o-p	< 0,01			
- 4,4 DDD - p-p	< 0,01			
- 2,4 DDE - p-p	< 0,01			
- 4,4 DDE p-p	< 0,01			
- 2,4 DDT - o-p	< 0,01			
- 4,4 DDT - p-p	< 0,01			
- 3,5-Dichloroaniline	< 0,01			
- 3-Idrossicarbofuran	< 0,01			
- 2-Phenylphenol	< 0,01		Max 0.25	(170
- Abamectin	< 0,01			
- Acephate	< 0,01			
- Acetamiprid	< 0,01			
- Acibenzolar S Methyl	< 0,01			
- Aclonifen	< 0,01			
- Acrinathrin	< 0,01			
- Alachlor	< 0,01			
- Aldicarb	< 0,01			
- Aldicarb Sulfone	< 0,01			
- Aldicarb Sulfoxide	< 0,01			

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Cod. Prova - Nome Prova - U.M.	Valore	Incertezza	Limite	
- Aldicarb (sum of aldicarb, its sulfoxide and its sulfone, expressed as aldicarb)	< 0,01		Max 0.1	(175
- Aldoxicarb	< 0,01			
- Aldrin	< 0,01			
- (*) Ametryn	< 0,01			
- (*) Aminocarb	< 0,01			
- Amitraz	< 0,01			
- (*) Atraton	< 0,01			
- Atrazine o Desethylatrazine	< 0,01			
- Azinphos Ethyl	< 0,01			
- Azinphos Methyl	< 0,01			
- (*) Azobenzene	< 0,01			
- Azocyclotin	< 0,01			
- Azoxystrobin	< 0,01			
- Benalaxyl	< 0,01			
- Bendiocarb	< 0,01			
- Benfuracarb	< 0,01			
- Benfluralin	< 0,01			
- Benomyl	< 0,01			
- Benoxacor	< 0,01			
- Bentazone	< 0,01			
- Benthiavalicarb-isopropyl	< 0,01			
- Benzoximate	< 0,01			
- Beta BHC	< 0,01			
- (*) Bifenox	< 0,01			
- Bifenthrin	< 0,01			
- Biphenyl	< 0,01			
- bitertanol	< 0,01			
- Boscalid	< 0,01			
- Bromacil	< 0,01			
- Bromophos Ethyl	< 0,01			
- Bromophos Methyl	< 0,01			
- (*) Bromopropylate	< 0,01			
- Bromoxynil Octanoato	< 0,01			
- (*) Bromuconazole	< 0,01			
- Bupirimate (o Pirimidinol)	< 0,01			
- Buprofezin	< 0,01			
- Butafenacil	< 0,01			
- Butocarboxim	< 0,01			
- Cadusafos	< 0,01			
- Captafol	< 0,01			
- Captan	< 0,01			
- Carbaryl	< 0,01		Max 0.1	(176
- Carbendazim	< 0,01			
- Carbofuran	< 0,01			
- Carbophenothion	< 0,01			
- Carbosulfan	< 0,01			
- (*) Chinomethionate	< 0,01			
- (*) Chlomazone	< 0,01			

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Cod. Prova - Nome Prova - U.M.	Valore	Incertezza	Limite	
- Chlorantraniliprole	< 0,01			
- Chlorbromuron	< 0,01			
- Chlordane (cis + trans)	< 0,01			
- (*) Chlorfenson	< 0,01			
- Chlorfentezine	< 0,01			
- Chlorfenvinphos	< 0,01			
- (*) Chlorfiurenol	< 0,01			
- Chlormephos	< 0,01			
- (*) Chlorobenzilate	< 0,01			
- Chlorofenapyr	< 0,01			
- Chlorofluazuron	< 0,01			
- Chlorpropham	< 0,01			
- Chlorpyrifos Ethyl	< 0,01		Max 0.25	(149
- Chlorpyrifos Methyl	< 0,01			
- Chlorsulfuron	< 0,01			
- Chlorthal Dimethyl	< 0,01			
- Chlorthalonil	< 0,01			
- Chlorthiophos	< 0,01			
- Chlortoluron o Chlorotoluron	< 0,01			
- Chlozolinanate	< 0,01			
- Clothianidin	< 0,01			
- Coumaphos	< 0,01			
- Cyanophos	< 0,01			
- Cyazofamid	< 0,01			
- Cyanazina	< 0,01			
- Cyfluthrins	< 0,01			
- Cyhalotrin (Lambda)	< 0,01		Max 5	(169
- Cyhexatin	< 0,01			
- Cymoxanil	< 0,01			
- Cypermethrins	< 0,01		Max 0.25	(156
- Cyproconazole	< 0,01			
- Cyprodinil	< 0,01			
- Delta BHC	< 0,01			
- Deltamethrin	< 0,01		Max 5	(160
- Desmethryn	< 0,01			
- Demeton-S-Methylsulfone	< 0,01			
- Diafenthiuron	< 0,01			
- Dialifor o Dialifos	< 0,01			
- Diazinon	< 0,01			
- Dicamba	< 0,01			
- Dichlobenil	< 0,01			
- Dichlofenthion	< 0,01			
- Dichlofluanid	< 0,01			
- (*) Diclofop Methyl	< 0,01			
- Dichlorvos	< 0,01			
- Diclobutrazol	< 0,01			
- Dicloran	< 0,01			
- Dicofol	< 0,01			
- Dicrotophos	< 0,01			

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Laboratorio Operante secondo la norma UNI CEI EN ISO/IEC 17025:2005 Accreditato ACCREDIA al N. 0859
Iscritto al N. 07 dell'elenco ufficiale dei laboratori di analisi per l'autocontrollo degli alimenti, con Delibera Regione Calabria Decreto Dir. Gen. N. 12582 del 06/10/2006 per quanto concerne le analisi chimiche e microbiologiche.
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ACCREDIA L'ENTE ITALIANO DI ACCREDITAMENTO
LAB N° 0859

Rapporto di Prova N. 462/14

Committente: Oleificio Gabro Srl (174-08)

Cod. Prova - Nome Prova - U.M.	Valore	Incertezza	Limite	
- Dieldrin	< 0,01			
- Diethofencarb	< 0,01			
- Difenoconazole	< 0,01			
- (*) Diflubenzuron	< 0,01			
- Dimethoate	< 0,01		Max 10	(165
- Dimetomorph (cis + trans)	< 0,01			
- Diniconazole	< 0,01			
- Dinocap	< 0,01			
- (*) Dinotefuran	< 0,01			
- (*) Dioxathion	< 0,01			
- Diphenylamina	< 0,01			
- Disulfoton	< 0,01			
- Disulfoton Sulfone	< 0,01			
- (*) Disulfoton Sulfoxide	< 0,01			
- Ditalimphos	< 0,01			
- Dithianon	< 0,01			
- Diuron	< 0,01			
- Dodine	< 0,01			
- Emamectin	< 0,01			
- Endosulfan	< 0,01		Max 0.25	(175
- Endosulfan (cis + trans)	< 0,01			
- Endosulfan Solfato	< 0,01			
- Endrin	< 0,01			
- Endrin aldeide	< 0,01			
- Epoxiconazol	< 0,01			
- (*) EPTC	< 0,01			
- Esaclorobenzene HCB	< 0,01			
- Esfenvalerate	< 0,01			
- Etaconazole	< 0,01			
- (*) Ethephon	< 0,01			
- Ethiofencarb	< 0,01			
- (*) Ethofumesate	< 0,01			
- Ethoprophos	< 0,01			
- Ethoxyquin	< 0,01			
- Ethyl p-nitrophenyl phenylphosphorothioate (EPN)	< 0,01			
- Etofenprox	< 0,01			
- Etoxazol	< 0,01			
- Etridiazol	< 0,01			
- Etrimfos	< 0,01			
- Famoxadone	< 0,01			
- (*) Famphur	< 0,01			
- Fenamidone	< 0,01			
- Fenamiphos	< 0,01			
- Fenarimol	< 0,01			
- Fenazaquin	< 0,01			
- Fenbuconazole	< 0,01			
- Fenbutatin oxide	< 0,01			
- Fenhexamid	< 0,01			
- Fenitrothion	< 0,01			

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Cod. Prova - Nome Prova - U.M.	Valore	Incertezza	Limite	
- (*) Fenobucarb	< 0,01			
- Fenothiocarb	< 0,01			
- Fenoxaprop P Ethyl	< 0,01			
- Fenoxycarb	< 0,01			
- Fenpropathrin	< 0,01			
- Fenpropidin	< 0,01			
- Fenpropimorph	< 0,01			
- Fenpyroximate	< 0,01			
- (*) Fenson	< 0,01			
- Fensulfothion	< 0,01			
- Fenthion	< 0,01			
- (*) Fenthion-Oxon-Sulfoxide	< 0,01			
- (*) Fenthion-Sulfone	< 0,01			
- (*) Fenthion-Sulfoxide	< 0,01			
- (*) Fenthion (fenthion and its oxigen analogue, their sulfoxides and sulfone expr. as parent)	< 0,01		Max 0.05	(175
- Fenvalerate	< 0,01			
- (*) Ferclorfenuron	< 0,01			
- Fipronil	< 0,01			
- (*) Flonicamid	< 0,01			
- (*) Fluazifop Butyl	< 0,01			
- Fluazifop P Butyl	< 0,01			
- Fluazinam	< 0,01			
- Flucythrinate	< 0,01			
- Fludioxonil	< 0,01			
- Flufenacet	< 0,01			
- (*) Flufenoxuron	< 0,01			
- Fluopicolid	< 0,01			
- Fluoxastrobin	< 0,01			
- Fluquinconazole	< 0,01			
- Flusilazole	< 0,01			
- Flutriafol	< 0,01			
- Fluvalinate Tau	< 0,01			
- Folpet	< 0,01		Max 0.1	(184
- Fonofos	< 0,01			
- Forchlorfenuron	< 0,01			
- Formetanat	< 0,01			
- (*) Fosthiazat	< 0,01			
- (*) Furalaxyl	< 0,01			
- Furathiocarb	< 0,01			
- (*) Haloxyfop Ethoxy Ethyl	< 0,01			
- (*) Haloxyfop Methyl	< 0,01			
- Heptachlor	< 0,01			
- Heptachlor Epoxide	< 0,01			
- Heptachlor (sum of heptachlor and heptachlor epoxide expressed as heptachlor)	< 0,01		Max 0.05	(161
- Heptenophos	< 0,01			
- Hexaconazole	< 0,01			
- (*) Hexaflumuron	< 0,01			

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Cod. Prova - Nome Prova - U.M.	Valore	Incertezza	Limite	
- Hexythiazox	< 0,01			
- Imazamox	< 0,01			
- Imazalil	< 0,01			
- Imidacloprid	< 0,01			
- Indoxacarb	< 0,01		Max 0,1	(178
- Iodofenfos	< 0,01			
- Iprodione	< 0,01			
- Iprovalicarb	< 0,01			
- Isofenphos	< 0,01			
- (*) Isofenphos-methyl	< 0,01			
- (*) Isopropalin	< 0,01			
- Isoproturon	< 0,01			
- (*) Isoxaben	< 0,01			
- (*) Isoxaflutole	< 0,01			
- Kresoxim Methyl	< 0,01			
- Lenacil	< 0,01			
- (*) Leptophos	< 0,01			
- Lindan	< 0,01			
- Linuron	< 0,01			
- Lufenuron	< 0,01			
- (*) Malaoxon	< 0,01			
- Malathion	< 0,01			
- Mecarbam	< 0,01			
- Mecoprop-P	< 0,01			
- Mepanipyrim	< 0,01			
- Mepronil	< 0,01			
- Metalaxyl	< 0,01			
- Metamitron	< 0,01			
- Metazachlor	< 0,01			
- Methabenzthiazuron	< 0,01			
- (*) Methacrifos	< 0,01			
- Methamidophos	< 0,01			
- Methidathion	< 0,01			
- Methiocarb	< 0,01			
- Methiocarb Sulfoxide	< 0,01			
- (*) Methiocarb Sulfone	< 0,01			
- (*) Methiocarb (sum of methiocarb and methiocarb sulfoxide and sulfone, expressed as methiocarb)	< 0,01		Max 1	(155
- Methomyl	< 0,01			
- methoxychlor	< 0,01			
- Metobromuron	< 0,01			
- Metolachlor	< 0,01			
- Metrafenon	< 0,01			
- Metribuzin	< 0,01			
- Mevinphos	< 0,01			
- Mirex	< 0,01			
- Monocrotophos	< 0,01			
- (*) Molinate	< 0,01			
- Myclobutanil	< 0,01			

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Cod. Prova - Nome Prova - U.M.	Valore	Incertezza	Limite	
- Naled	< 0,01			
- Naptalam	< 0,01			
- (*) Napropamide	< 0,01			
- Nitepyram	< 0,01			
- (*) Nitrofen	< 0,01			
- (*) Nitrothal Isopropyl	< 0,01			
- Nuarimol	< 0,01			
- (*) Ofurace	< 0,01			
- Omethoate	< 0,01			
- Oxadiazon	< 0,01			
- Oxadixyl	< 0,01			
- Oxamyl	< 0,01			
- (*) Oxyfluorfen	< 0,01		Max 5	(161
- Paclobrutazolo	< 0,01			
- Paraoxon Ethyl	< 0,01			
- Paraoxon Methyl	< 0,01			
- Parathion Ethyl	< 0,01			
- Parathion Methyl	< 0,01			
- Parathion-methyl (sum of Parathion-methyl and paraoxon-methyl expressed as Parathion-methyl)	< 0,01		Max 0.1	(176
- Pencicuron	< 0,01			
- Penconazole	< 0,01			
- Pendimethalin	< 0,01			
- Permethrin cis	< 0,01			
- Permethrin trans	< 0,01			
- Permethrin (sum of isomers)	< 0,01		Max 0.25	(155
- Phenmedipham	< 0,01			
- Phenthoate	< 0,01			
- (*) Perthan	< 0,01			
- Phorate - Thimet	< 0,01			
- (*) Phorate Oxon	< 0,01			
- (*) Phorate Oxon Sulfone	< 0,01			
- (*) Phorate Sulfone	< 0,01			
- (*) Phorate (sum of phorate, its oxygen analogue and their sulfones expressed as phorate)	< 0,01		Max 0.1	(176
- Phosalone	< 0,01			
- Phosmet	< 0,01		Max 15	(163
- (*) Phosphamidon	< 0,01			
- Phoxim	< 0,01			
- Picoxystrobin	< 0,01			
- Piperonyl Butoxide	< 0,01			
- Piridaben	< 0,01			
- Pirimicarb	< 0,01			
- Pirimiphos Ethyl	< 0,01			
- Pirimiphos Methyl	< 0,01			
- Prochloraz	< 0,01		Max 0.25	(156
- Procymidone	< 0,01			
- Profenofos	< 0,01			
- Profluralin	< 0,01			

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Cod. Prova - Nome Prova - U.M.	Valore	Incertezza	Limite
- Promecarb	< 0,01		
- Prometryn	< 0,01		
- Propachlor	< 0,01		
- Propamocarb	< 0,01		
- Propanil	< 0,01		
- (*) Propaquizafop	< 0,01		
- Propargite	< 0,01		
- (*) Propazine	< 0,01		
- (*) Propham	< 0,01		
- Propiconazole	< 0,01		
- Propoxur	< 0,01		
- Propyzamide	< 0,01		
- (*) Proquinazid	< 0,01		
- Prosulfocarb	< 0,01		
- Prosulfuron	< 0,01		
- Prothioconazole	< 0,01		
- Prothiofos	< 0,01		
- Pymetrozine	< 0,01		
- Pyraclostrobin	< 0,01		
- Pyrazophos	< 0,01		
- (*) Pyrethrins	< 0,01		
- Pyridaben	< 0,01		
- Pyridaphenthion	< 0,01		
- Pyridate	< 0,01		
- Pyrifenox	< 0,01		
- Pyrimethanil	< 0,01		
- (*) Pirimicarb-Desmethyl	< 0,01		
- Pyriproxifen	< 0,01		
- Quinalphos	< 0,01		
- Quinoxyfen	< 0,01		
- Quintozene	< 0,01		
- (*) Quizalofop Ethyl	< 0,01		
- Resmetrin	< 0,01		
- (*) Rimsulfuron	< 0,01		
- Rotenone	< 0,01		
- S-Ethyl dipropylthiocarbamate (EPTC)	< 0,01		
- Simazine	< 0,01		
- (*) Simetryn	< 0,01		
- Spinosad	< 0,01		
- Spirodiclofen	< 0,01		
- Spiromesifen	< 0,01		
- Spirotetramat	< 0,01		
- Spiroxamine	< 0,01		
- Sulfotep	< 0,01		
- (*) SWEP	< 0,01		
- TDCP	< 0,01		
- Tebuconazole	< 0,01		
- Tebufenozide	< 0,01		
- Tebufenpyrad	< 0,01		

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Cod. Prova - Nome Prova - U.M.	Valore	Incertezza	Limite	
- Tecnazene	< 0,01			
- Teflubenzuron	< 0,01			
- Tefluthrin	< 0,01			
- Temefos	< 0,01			
- TEPP	< 0,01			
- Terbufos	< 0,01			
- Terbumeton	< 0,01			
- Terbuthylazina	< 0,01		Max 0.25	(126
- Terbutryn	< 0,01			
- Tetrachlorvinphos	< 0,01			
- (*) Tetraconazole	< 0,01			
- Tetradifon	< 0,01			
- Tetramethrin	< 0,01			
- TFM Tre-trifluorometil-4-nitrofenolo	< 0,01			
- Thiabendazole	< 0,01			
- Thiacloprid	< 0,01			
- Thiametoxam	< 0,01			
- Thiodicarb	< 0,01			
- Thiometon	< 0,01			
- Thionazin	< 0,01			
- (*) Thiophanate Methyl	< 0,01			
- (*) Thribenuron methyl	< 0,01			
- Tolclofos Methyl	< 0,01			
- Tolyfluanid	< 0,01			
- Transfluthrin	< 0,01			
- Triadimefon	< 0,01			
- Triadimenol	< 0,01			
- (*) Triamiphos	< 0,01			
- (*) Triasulfuron	< 0,01			
- Triazophos	< 0,01			
- (*) Tribenuron methyl	< 0,01			
- Trichlorfon	< 0,01			
- Tricyclazole	< 0,01			
- Triexapac Ethil	< 0,01			
- Trifenilmetano	< 0,01			
- Trifloxystrobin	< 0,01			
- Triflumizole	< 0,01			
- Triflumuron	< 0,01			
- Trifluralin	< 0,01			
- Thrisulfuron methyl	< 0,01			
- Triticonazol	< 0,01			
- Vamidothion	< 0,01			
- Vinclozolin	< 0,01			
- Zoxamide	< 0,01			

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(15) Reg. C.E.E. n. 2568/91, All. I; e succ. modifiche e integrazioni
(126 Reg. C.E.E. n. 149/2008
(149 Reg (CE) N. 839/2008 della Comm del 31 luglio 2008
(155 Reg. (EC) No 839/2008
(156 Reg. (EU) No 520/2011
(160 Reg. (EU) No 441/2012
(161 Reg. (EC) No 149/2008
(163 Reg. (EU) No 592/2012
(165 Reg. (EC) No 1097/2009
(169 Reg. (EC) No 459/2010
(170 Reg. (EU) No 834/2013
(175 Reg. (EU) No 310/2011
(176 Reg. (EU) No 899/2012
(178 Reg. (EU) No 35/2013
(184 Reg. (EU) No 251/2013

GIUDIZIO - Non Oggetto di accreditamento Accredia

[0000-010] - Limitatamente ai parametri richiesti, il campione analizzato risulta CONFORME al regolamento CEE 2568/91 e successive modifiche, per la categoria dichiarata.

ORDINE NAZIONALE DEI BIOLOGI - ALBO PROFESSIONALE - Dott. CARMINE DOMENICO VENTRE Iscrizione N. 42830

Il Responsabile del laboratorio
P.I. Chimico - Biologo

Dr. Carmine Ventre
Documento emesso con firma digitale

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