UNIVERSITÀ DEGLI STUDI DI MILANO

PhD course in Environmental Sciences

XXXI Cycle

Anthocyanins rescue Macrophage infiltration in a *Drosophila* model of obesity

PhD Thesis

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Academic Year: 2018-2019

Contents

Abstract

For years, scientific research has availed itself of the opportunity of using animal models to understand the cellular and molecular mechanisms that regulate several human diseases. In this thesis, I showed the great importance to employ *Drosophila melanogaster* as model organism for the study of chronic diseases. *Drosophila* is considered an excellent and innovative model thanks to its peculiar characteristics such as low cost, small genome size, short generation time.

About 75% of human disease-causing genes have functional homologs in *Drosophila*; furthermore, the fruit fly shows extreme genetic flexibility and a number of genetic systems has been developed to answer specific biological questions, such as the UAS-GAL4 binary system that allows the expression of target genes in a tissue-specific manner.

For all these reasons, *Drosophila* can be used for the study of different metabolic diseases among which obesity. In particular, in our laboratory we created a new *Drosophila* model of obesity that shows similar characteristics as those present in obese people, promoting an Adipose Tissue Macrophages infiltration (ATM) phenotype.

Using this new model of obesity, I tried to understand the role of important compounds like Flavonoids and Anthocyanins, both considered anti-inflammatory and antioxidant agents, which regular consumption reduces the risk to develop metabolic disorders.

Anthocyanins represent the major red, purple, and blu pigment in many plants and fruits. I have discovered that Anthocyanin are able to rescue the ATM phenotype by reducing the *Drosophila* hemocytes (similar to human macrophages) migration in the larval fat body, which carries out human adipose tissue and liver functions.

Anthocyanins also show potential health benefits by reducing ROS levels, thus acting as antioxidant agents. This antioxidant activity may be due to the capacity to modulate the expression of some redox regulators like Glutathione-S-Transferase (GST) and the Nuclear factor erythroid 2-Related Factor 2 (NRF2).

Riassunto

La ricerca scientifica, da molti anni, si è avvalsa dell'opportunità di utilizzare diversi modelli animali per comprendere i meccanismi cellulari e molecolari che regolano le malattie umane. In questa tesi, ho dimostrato come *Drosophila melanogaster*, un eccellente organismo modello, svolge un ruolo fondamentale nello studio delle malattie croniche.
Drosophila melanogaster è considerato un perfetto e innovativo modello animale poiché possiede alcune peculiari caratteristiche: bassi costi di mantenimento, dimensioni del genoma abbastanza piccole e un tempo di generazione breve; inoltre circa il 75% dei geni umani associati a malattie possiede un omologo funzionale in *Drosophila*.
Il moscerino della frutta è infine un modello genetico estremamente manipolabile, e l'utilizzo del sistema binario *UAS-GAL4* consente l'espressione di geni bersaglio in modo tessuto-specifico.
Per tali motivi, *Drosophila* è ampiamente utilizzata nella ricerca biologica per lo studio di diverse malattie metaboliche tra cui l'obesità. In particolar modo, nel nostro laboratorio abbiamo utilizzato animali transgenici che rappresentano un modello innovativo di obesità, mostrando caratteristiche simili a quelle presenti in persone obese, in cui è stata stimolata l'infiltrazione di macrofagi nel tessuto adiposo per determinare il fenotipo ATM. Utilizzando questo innovativo modello di obesità, ho cercato di comprendere il ruolo svolto da importanti composti, i Flavonidi e le Antocianine, entrambi considerati agenti antinfiammatori e antiossidanti; sembra che il loro regolare consumo riduca il rischio di sviluppare malattie metaboliche.
Le Antocianine rappresentano il principale pigmento rosso, viola e blu presente in molte piante e frutti. Gli esperimenti condotti con *Drosophila melanogaster*, con l'utilizzo delle Antocianine, hanno messo in evidenza il loro eccezionale potere antinfiammatorio, essendo in grado di ridurre la migrazione degli emociti (simili ai macrofagi umani) nei corpi grassi, tessuti che svolgono le stesse funzioni del fegato e del tessuto adiposo umano. Le Antocianine, in qualità di agenti antiossidanti, possiedono anche potenziali effetti benefici sulla salute riducendo i livelli di ROS; la loro probabile attività antiossidante potrebbe essere determinata dalla capacità di modulare l'espressione di alcuni geni codificanti per enzimi coinvolti in meccanismi di ossido-riduzione, come la Glutatione-S-Trasferasi (GST) e il Fattore di Trascrizione Nucleare Eritroide-2 (NRF2).

Chapter 1

1. General introduction

1.1 *Drosophila melanogaster*

Drosophila melanogaster, commonly known as “fruit fly” is considered a powerful model organism for the study of fundamental biological processes.
In 2000, *Drosophila* genome has been completely sequenced and has been found that, about 75% of human genes are conserved in *Drosophila* (1).
Furthermore, *Drosophila* is an excellent model thanks to the small genome size, high fecundity, low cost, short generation time and a flexible genetic background. For all these advantages, the fruit fly can be used for studying of many human diseases as metabolic disorders, but also neurodegenerative diseases such as Alzheimer, Parkinson and Huntington.

1.1.1 *Drosophila* life cycle

Probably, the best advantage of this model system is the short life cycle. This is temperature-dependent and requires about 10 days at 25° C from the egg to the adult fly. The life cycle consists of four distinct stages: egg, larva, pupa, and adult (**Fig.1.1**). Virgin females and males are placed in vials, within a few hours can lay many eggs, after 24 hours laying the eggs hatch into 1st instar larvae. The larval stage of this insect consists of three instars (5 days), during which the larva loses its spiracles, mouth, and hooks. At the fifth day after egg laying (d AEL), the larva encapsulates itself inside in a protective colored puparium and begins the pupariation process, where it happens metamorphosis, giving rise to the adult fly. During the pupal stage, the steroid hormone Ecdysone determines degeneration of all organs, a process called histolysis, to restructure them into the adult shapes. At 10 days from fertilization the eclosion process takes place, where the adult flies emerge through the operculum. Finally, sexual maturity is acquired in about 8–12 hr (2).

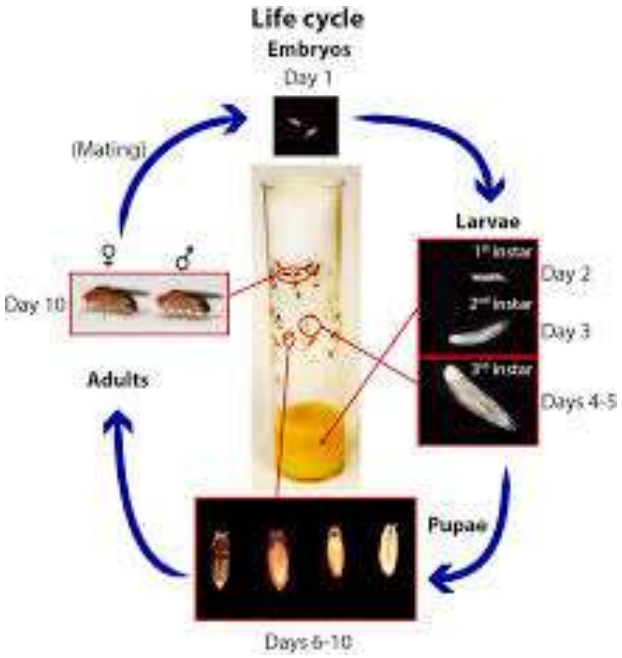

Fig. 1.1 Life cycle of *Drosophila melanogaster*. *Drosophila* life cycle consists of four distinct stages: egg, larva, pupa, and adult. This process is temperature-dependent; the flies are cultured in vials and are kept at 25°C to obtain adults after 10 days (*Hales et al., 2015*).

1.1.2 Regulation of growth by ecdysone

Ecdysone is a steroid hormone produced by the prothoracic gland (PG) **Fig 1.2** (A) and released into the hemolymph.

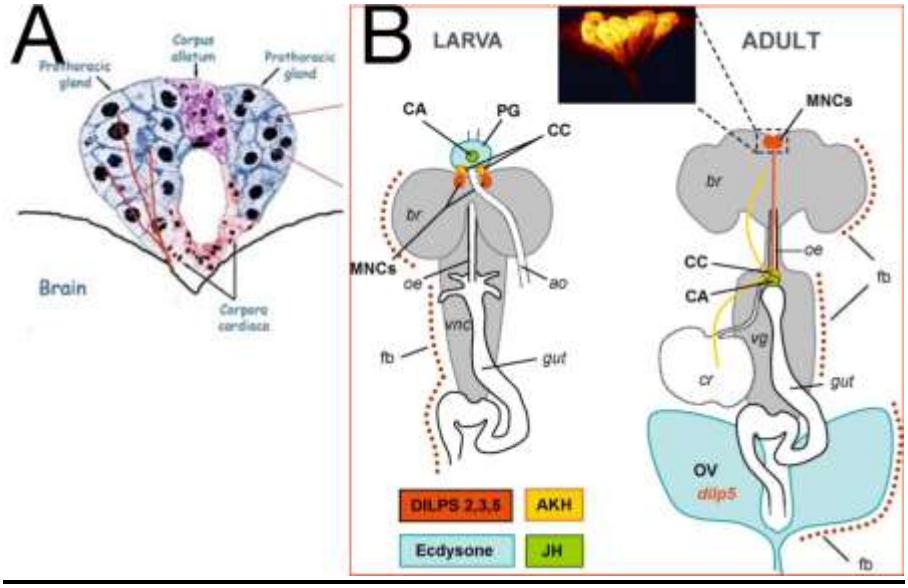

Fig. 1.2 A. prothoracic gland B. endocrine system in the larvae and adult.

Ecdysone controls animal growth and maturation since it is released from the PG right before the change of each instar by a complex mechanism that involves the coordination of secreted factors from the fat body and the presence of nutrients in the food (**Fig.1.3**).

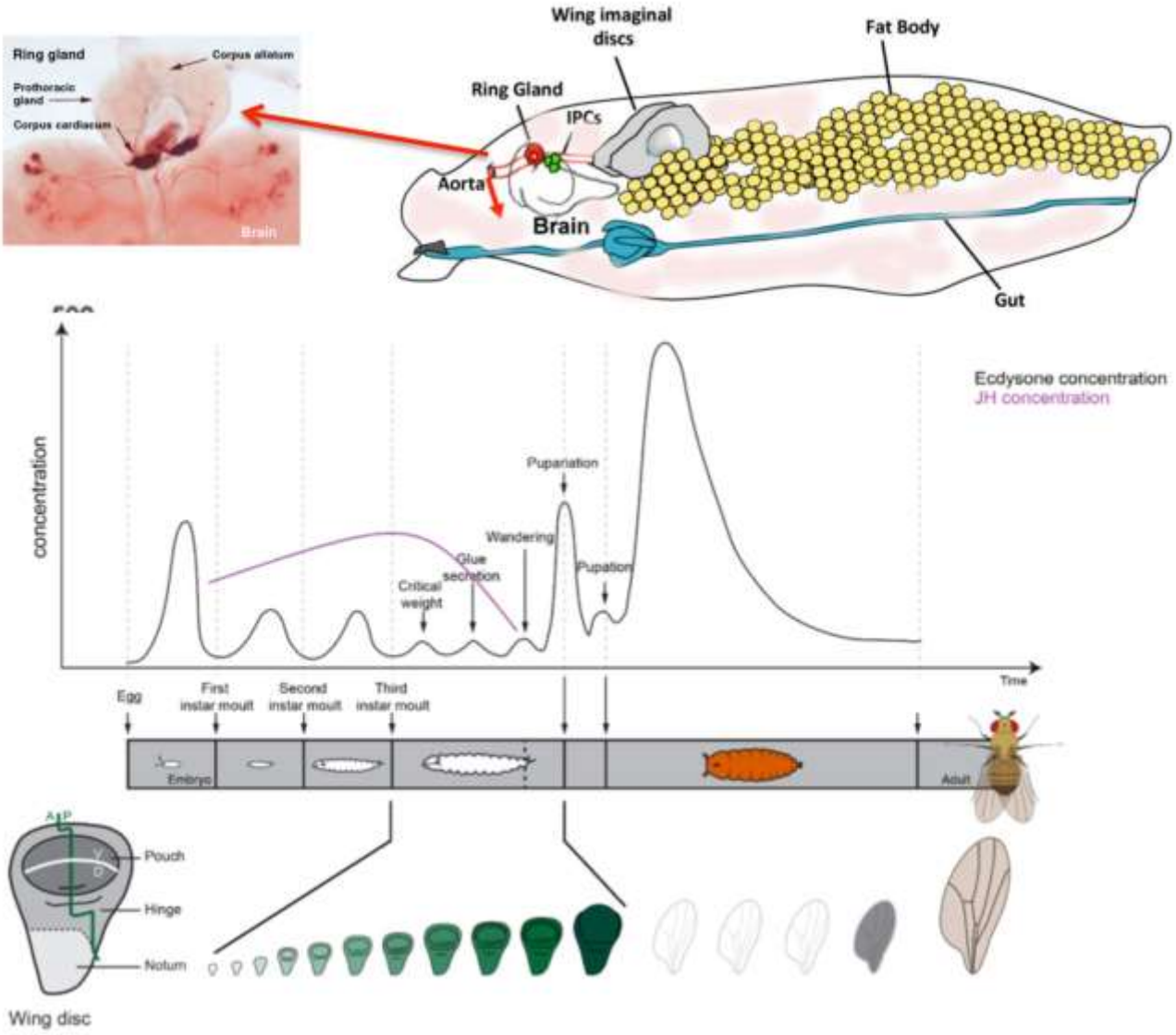

Figure 1. 3 Coordination between ecdysone, fat body, nutrients and molting during larval growth and maturation to pupa (modify from T. M. Lab, «The regulation of body and organ size).

1.1.2 UAS-GAL4 system

The UAS-GAL4 binary system is a method to induce gene expression, primarily used in *Drosophila*, although it has also been applied to mice and zebrafish. This powerful genetic technique has been developed by Andrea Brand and Norbert Perrimon in 1993. The UAS-GAL4 is a binary system that allows the ectopic expression of a transgene in a specific tissue and it consists of two components: the first is a construct that presents a "driver" that induces the expression of the transcription factor GAL4, normally expressed in yeast; the second is a construct where the transgene of interest is placed downstream of a promoter sequence called UAS (Upstream Activation Sequence), that consists of GAL4-binding domains **(Fig 1.4)** (3).

The great advantage to use *Drosophila* as a model to regulate the expression of specific genes is that the flies does not express an endogenous GAL4; in this way, the off-target effects are minimal. Furthermore, the transgene is only expressed when the two constructs are co-present in the F1 generation (2, 4).

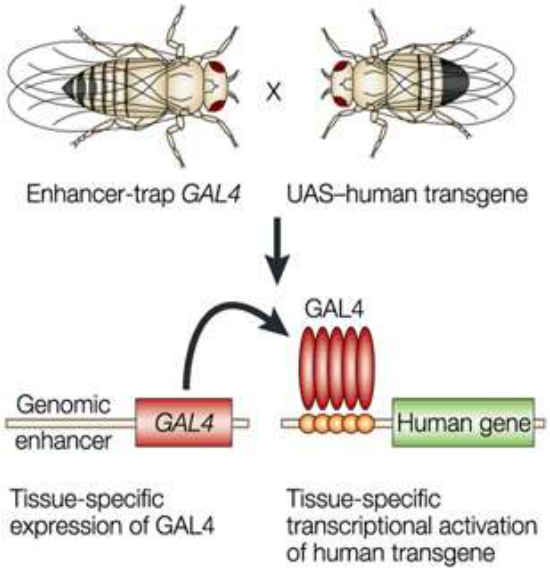

Fig. 1.4 UAS-GAL4 system. A tissue-specific promoter drives the expression of the transcription factor GAL4 that it binds the UAS sequence and allows the expression of the gene of interest. *(from* Daniel St Johnston *Nature Reviews Genetics* (2002) volume3, pages176–188).

Chapter 2

2.1 General introduction on Obesity in vertebrates

2.1.1 Obesity

Obesity is defined a metabolic syndrome widely spread worldwide, often associated with other chronic diseases such as cardiovascular disorders, type II diabetes and cancer (5).

Obesity is a public health concern, and the World Health Organization estimates that 1.9 billion adults are overweight (BMI > 25 kg/m2), among which 600 million are obese (BMI > 30 kg/m2) (**Fig. 2.1**). Based on data reported by the World Health Organization (WHO), the incidence of obesity continues dramatically to grow in specific countries, in particular in developed and developing countries like USA, Europe and United Arab Emirates. The onset of obesity is the result of multifactorial elements, including genetic predisposition to obesity and subsequently, the impact of several environmental factors like sedentary lifestyle and diet rich in fat and sugar and poor in phytonutrients (6, 7).

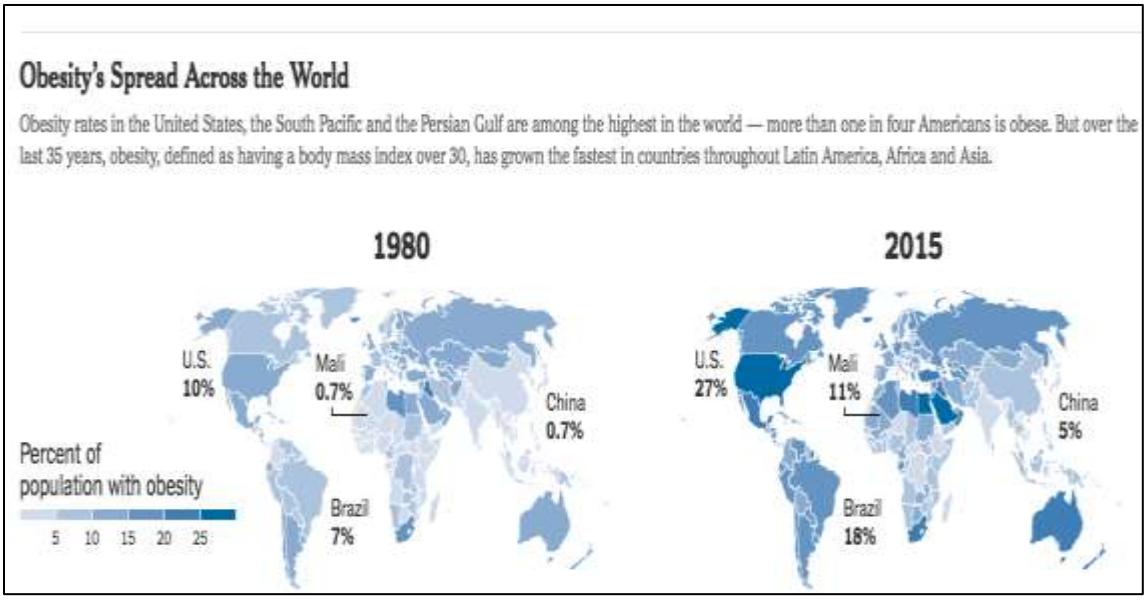

Fig. 2.1 Percent of population with obesity. Obesity rate spread in worldwide from 1980 to 2015. (*ANDREW JACOBS and MATT RICHTEL for The New York Times SEPT. 16, 2017*).

2.1.2 Causes that influence the insurgency of Obesity

Although obesity has been associated mostly with an excess of food intake many other factors are known to influence its insurgency like:
- **genetics** like for rare monogenic forms in many components of the leptin-melanocortin axis (8) (**Fig. 2.2**);
- **bad habits,** like lack of day-night circadian sleep;
- **environmental factors** like Bisphenol A that affected fatty acids synthesis;
- **health disparities** in USA is higher among Afro-Americans and Native Americans;
- **epigenetics changes**, one of the most relevant evidence that supports this hypothesis has been reported in the bio-sociological analysis performed on the F1 generation from women who were pregnant during 1944 famine in the Netherlands, known as the Dutch Hunger Winter. Those women had children and grandchildren who were unusually small or prone to diabetes and obesity (9).

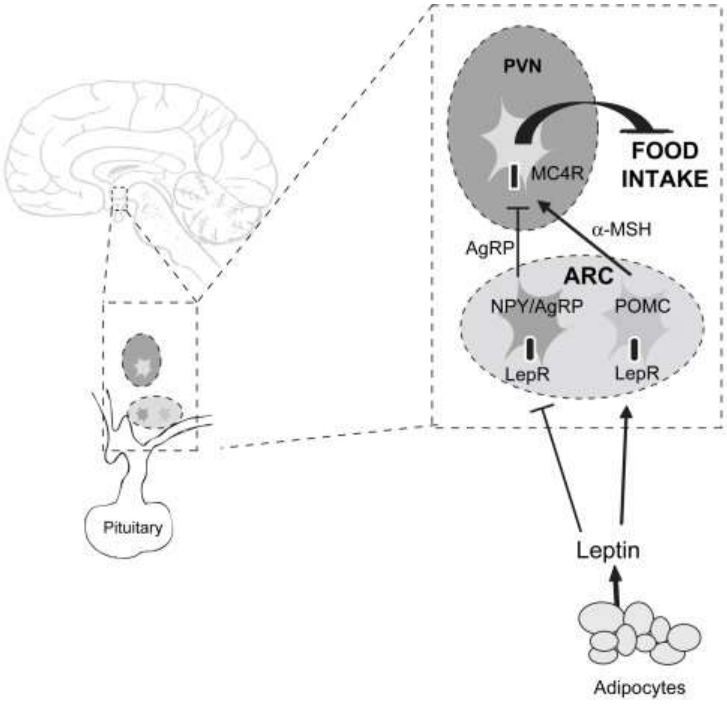

Fig. 2.2 Leptin-Melanocortin system of energy balance *(Ranadive, 2008).*

2.1.3 Obesity and inflammation

The "obesity state" triggers a low-grade chronic inflammation in metabolically active sites such as liver and adipose tissue (10, 11).

The inflammation is a defense process, which comprises a series of cellular and humoral reactions with the aim to protect the organism from various insults among which infection and tissue damage leading to the rescue of the affected tissue (12, 13). In normal conditions, the adipose tissue regulates the storing energy reserves in the form of triglycerides, however it also has an important functions as an endocrine organ, producing a variety of pro-inflammatory cytokines such as interleukin (IL-1, IL-6, IL-8), *interferon γ (*IFNγ) and tumor necrosis factor α (TNFα) (14, 15). In the adipose tissue of obese people, the production of these molecules by adipocytes, is abnormal and induces a low level of chronic inflammation that influences other systems by altering their functions (16) (Fig. 2.3). Furthermore, the obese adipose tissue diminishes its capability to store fats, leading to an increase of circulating free fatty acids (FFAs) that is thaught to promote insulin resistance and damage to the mitochondrial membrane enhancing oxidative stress caused by ROS release (17-19).

The role of the inflammatory response is to combat infection and tissue injury through the activation of the innate immune system with recruitment of the immune cells, the mammalian macrophages. The principal role of macrophages is host defense against pathogens through their phagocytic activity, but they show also other important functions such as homeostasis, inflammation and repair processes (20).

Once active, the macrophages infiltrate the adipose tissue and release further molecules such as NO, TNF-α, IL-6, IL-1 by increasing the serum FFA and triglyceride levels (21).

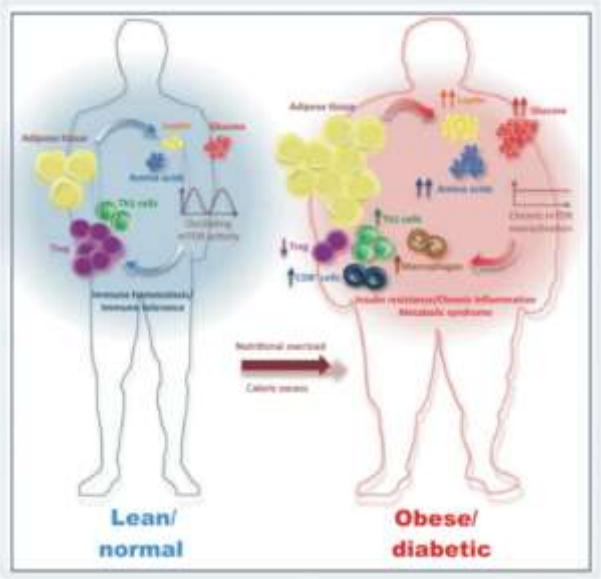

Fig. 2.3 Different mTOR levels in normal and obesity conditions. In physiological condition, the leptin and several stimuli activate the mTOR pathway contributing to the maintenance of the metabolic homeostasis, while in obese condition mTOR is overexpressed facilitating the accumulation of inflammatory cells (*Matarese, Procaccini, & De Rosa, 2012*).

2.1.4 Obesity and oxidative stress

In obese people, lipid accumulation and chronic inflammation increase oxidative stress with the production of high levels of reactive oxygen species (ROS) (22, 23). ROS are produced during mitochondrial electron transport, where the reduction of oxygen through the addition of electrons leads to the formation of different ROS species including free radicals such as superoxide anions (O_2^-) hydroxyl (HO), perhydroxy (HO_2^-) and alkoxy (RO) and non-radicals like hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (24).

In mammals, elevated ROS alterate physiological function of cellular processes throught damage of DNA, proteins and lipids.

ROS can interact with DNA causing modification of the nucleotide structure, triggering mutations and genomic instability, they can also oxidize proteins, this modifications result in changes in structure compromising biological functions, finally they can react with the polyunsaturated fatty acids of the membranes, causing cell death (24-26).

All this events can lead to many human pathologies including cancer, neurodegenerative disorders, cardiovascular disease, diabetes and aging. Despite their well-known toxic effects, it seems that at low concentrations they are involved in cellular signaling and defense against environmental insults (24).

In order to prevent excess ROS, the mammalian cells are equipped with defence mechanisms that comprise antioxidative compounds such as enzyme, phenolic compounds, vitamins and drugs, they are reducing agents able to counteract oxidative species formation. The enzymatic part regards the endogenous antioxidant activity and includes Catalase, Superoxide dismutase, Glutathione-S-Transferase (GST), while exogenous antioxidative compounds are vitamins A, C, E and phenolic compounds like Anthocyanins (27, 28).

2.1.5 Signaling pathways relevant in obesity

JNK pathway

The excessive production of ROS determinates the activation of the c-Jun-NH2-terminal kinase (JNK), member of a mitogen-activated protein kinases (MAPKs) [15].

MAPK cascade consists of four major component: c-Jun N-terminal kinases (JNK), p38 kinase (p38), the extracellular signal-related kinases (Erk1/2) and kinase Erk5 (29).

These kinases are evolutionarily conserved in eukaryotes and show a key role in cellular processes in response to a wide variety of signals among which growth factors, hormones, cytokines and reactive species. In particular, JNK signaling pathway is involved in few biological processes such as inflammation, cell proliferation, differentiation and apoptosis (24).

Activation of JNK can be determined by stress, inflammatory cytokines and growth factors and requires a cascade of phosphorylation events starting with the activation of the JNKKK kinases, such as ASK1, TAK1, that activate the JNKK as MKK4 and MKK7 (30).

In mammals, three JNK genes have been identified (JNK 1, 2, 3) with at least 10 different splicing isoforms. Regarding JNK 1 and 2, their expression occurs in every cell and tissue type, whereas JNK 3 is selectively expressed in the brain, heart and testis (31, 32).

GST (Glutathione-S-Transferase)

GST is one of the most efficient detoxifying enzymes, found mainly in the cytosol. The main biological roles of GST include detoxification and protection against oxidative stress by catalyzing the conjugation of electrophilic substrates to

glutathione (GSH), obtaining products no longer reactive and more soluble that can be eliminated from the organism. Over to detoxifying activity, GST presents other important functions: it can inhibit the activity of JNK by causing the block of apoptosis and promoting cell proliferation and tumor growth (33).
GSTs existing in mammals, are generally divided into three categories: cytosolic, microsomal, and mitochondrial. Up to now, seven classes of mammalian cytosolic GSTs have been identified: Alpha, Mu, Pi, Theta, Omega, Sigma and Zeta (34, 35).

NRF2

The nuclear factor erythroid 2-related factor 2 (NRF2) is considered one of the main regulators of the intracellular antioxidant response both in vertebrates and invertrebrates. NRF2 is a member of cap'n'collar (CNC) leucine zipper (bZIP) family of transcription factors and it is expressed in all cell types (36).
NFR2 is a master of redox response, but recent studies have identified other numerous functions, for example, NRF2 regulates lifespan extension by caloric restriction and it seems to prevent cancer in animal models, although in humans a costitutive NFR2 activation is linked to greater incidence of cancer (37-39).
]. Studies performed in mice and human cultured cells have shown that upon exposure to oxidative stress and electrophilic chemical insults, Nrf2 activity increases. Experiments in animal models have demonstrated that, once activated, NRF2 translocates to the nucleus and binds the Musculo-Aponeurotic Fibrosarcoma oncoprotein (Maf). The NRF2/Maf dimer regulates the expression of over 200 genes, containing antioxidant response elements (AREs), by promoting up-regulation of few redox regulators among which Glutathione S-transferase, Thioredoxin, Peroxyredoxins, NAD(P)H quinone oxidase 1 (NQO1) and Heme oxygenase 1(HO1) (40, 41).
In this way, NRF2 regulates many cellular responses such as homeostasis, proliferation, autophagy, DNA repair, and mitochondrial physiology. Under non-stressed conditions, NRF2 protein levels are kept low through its proteasomal degradation. Three E3 ubiquitin ligase complexes control the ubiquitylation and proteasomal degradation of NRF2: Kelch-like erythroid CNC homologue (ECH)-associated protein 1 (KEAP1), SKP1 and HRD1 (42).
In particular, the inhibition of NRF2 is orchestrated by its cytoplasmatic negative regulator, Keap1, associated with Cul3-based ubiquitinligase system (43, 44).

The inhibitory effect of Keap1 involves the supression of NRF2 activity in the cytoplasm becoming target for proteosomal degradation. Furthermore, Keap 1 can function as a redox sensor because its cysteine residues, indeed in a stress condition, the oxidants bind these residues determining the disassociation between Keap 1and NRF2 and preventing Nrf2 ubiquitination and proteolysis by Keap1(38).

2.1.6 Obesity and cancer

Recent data have expanded the consistent evidence that connects obesity to an increased risk of some cancers (45, 46).

Despite the limitations, many possible mechanisms exist that explain the obesity-cancer link by regarding studies performed on insulin, insulin-like growth factors (IGFs), sex hormones, and adipokines, but other candidate process are inflammation and oxidative stress (47).

In vitro and *in vivo* studies have shown that insulin and insulin-like growth factor 1 (IGF-1) are connected to obesity. It has been hypothesized that hyperinsulinemia causes the activation of insulin and IGFs pathway, these events determine a block of the apoptotic process and promote tumor growth in the colon, kidney, prostate, and endometrium (48-50).

Another evidence of a possible relationship between obesity and cancer has been found with sex hormones; in fact, they trigger cell division by promoting tumor progression. In particular, breast, uterine and prostate cancers are caused by sex steroid hormones activities (49, 51).

Reaserches performed on Adipokines, in particular on leptin, have suggested as this hormone is able to have a negative role in the development of different cancers, such as endometrial, breast, colon, and prostate cancer (52, 53).

Numerous cohort studies have reported that people who are overweight or obese are more likely to develop some cancers than normal-weight people including endometrial, breast, tyroid, liver, kidney and ovarian cancer (54-59).

2.1.7 Animal models to study obesity and related pathologies

As already mentioned before, obesity is an epidemic problem, and it is associated with several health problems, including diabetes, cardiovascular disease and cancer. The precise molecular mechanisms that link obesity to these health problems are not yet clear. To better understand the etiology of metabolic disorders, specific animal models have been obtained that present all the complications linked to the human disease. Below are reported some animal models associated with obesity-related pathology (60).

Most used mouse obese genetic models:

The *agouti* mutation was first reported among the several obese mice used in research and it has become the first obesity gene characterized at the molecular level (61).

Agouti is a gene that controls the distribution of the red/yellow pigment melanin in mammal hairs, in particular the lethal yellow mutant mouse (*Ay)* is a dominat mutation in the *agouti locus* and it has been considered a good obesity model. (*Ay)* mutation exhibits a typic obese phenotype associated with hyperinsulinemia, insulin resistance, hyperglycemia and hyperleptinemia; furthermore, *agouti* overexpression in the adipose tissue induces fat increase without alteration of food intake (62, 63).

In 1994, Zhang et al. have discovered that mutation in leptin gene caused mutation in the mouse gene obese (ob) (**Fig. 2.4**) (64). Leptin is a hormone secreted by adipocytes, in normal condition leptin induces a reduction in body weight, food intake and serum insulin, instead the mutation exhibits obesity, type 2 diabetes and insuline resistance, therefore leptin is considered a marker of obesity (65).

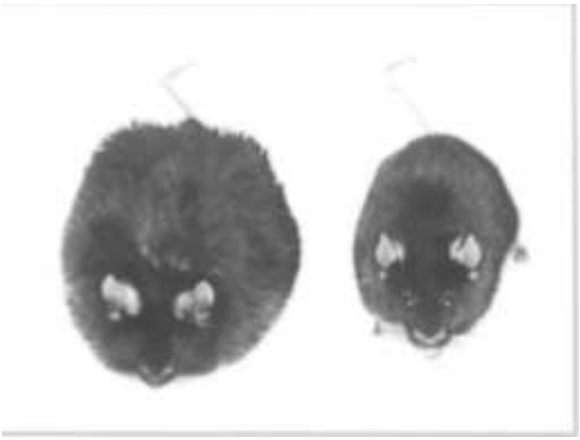

Figure 2.4 The ob/ob mouse (right) and wt mouse (from Zhang Nature 1994 vol 372 1 Dec 425-32)

Another type of obese model is LepRdb/db mouse; this time the mutation regards leptin-receptor gene, the metabolic profile leads to hyperphagia and obesity, with consequent hyperleptinemia and insulin resistance (66, 67).

Also a high-fat diet (HFD) model is used for studying the obese phenotype. Different mouse strains respond to HFD-induced obesity; in particular, the C57BL/6J strain shows similar characteristics to obese people when fed with HFD, which corresponds to a typical diet containing 45% of calories from lipids, the same diet used in developed countries (68).

2.2 General introduction on obesity in Drosophila

2.2.1 Obesity and inflammation in *Drosophila*

Obesity is defined a metabolic syndrome that affects all age and each kind of social class, the last data published by WHO reveal that obesity is one of today's most public health problems.
Obesity is determinated by excessive fat accumulation in adipose tissue that presents a risk to health. That pathologic condition stimulates the activation and release of inflammatory mediators such as TNFα and IL-6, promoting an inflammatory process and subsequently oxidative stress (69-72).
The inflammatory state induces activation of the innate immune system with recruitment of the immune cells, the mammalian macrophages, while in *Drosophila* it is orchestrated by hemocytes, circulating cells in the hemolymph, present at all stages of the life cycle and representing the fly's innate immune system.
The *Drosophila* hemocytes infiltrate the fat body, considered equivalent to the mammalian liver and adipose tissue, and in turn release more inflammatory mediators among which the TNF-α ortholog Eiger, to promote the ATM phenotype (71, 73).

The Hemocytes

In *Drosophila* melanogaster, the hemocytes, motile and phagocytic cells are produced in the limph gland, an hematopoietic organ and subsequently stored in specific sites called Hematopoietic Pockets located between epidermis and muscle layers (74).
In *Drosophila* are present three different cell types of hemocytes are present with slightly different functions:
-**Plasmatocytes** represent the majority of circulating hemocytes during the larval stage (~95%), they show phagocytic activity like mammalian macrophages, indeed these cells are able to migrate to sites of infection through of the protusions of actin-rich filopodia and lamellipodia (75).
-**Lamellocytes** represent about 2,5% of total hemocytes, they are rarely present in larva, however these particular cells are required in case of invasion by endoparasites in which they are involved in pathogen encapsulation (76).

-Crystal cells represent the remaining 2,5% of total hemocytes, they are released during pupariation phase, they are characterized by the presence of cytoplasmatic crystal-like inclusions; they secreted specif enzymes required for the melanization cascade (77).
It seems that, in response to injury or infection, the sessile hemocytes start to migrate to the damaged site and promote the phagocytic activity, but the specific signal that triggers the switch from sessile to mobile cells is still not known.
A recent work has hypothesized that hemocytes migration is linked to ecdysone activity (75).

The Fat body

The human adipose tissue regulates the homeostatic metabolism, it provides an energy storage in form of TAG, deposited in specific cells called adipocytes. During starvation, the lipases break TAGs down into free fatty acids (FFAs) that are released in the bloodstream to produce energy **(Fig. 2.5)** (78, 79).
Furthermore, the adipose tissue is also an endocrine organ, it produces several adipokines like leptin and adiponectin. The insect fat body is an organ analogue to vertebrate adipose tissue and liver, it is considered a multifunctional organ, indeed it regulates nutrient storage and energy metabolism, it coordinates insect growth with metamorphosis, it also carries out an exocrine fuction by producing several antimicrobial peptides (80).

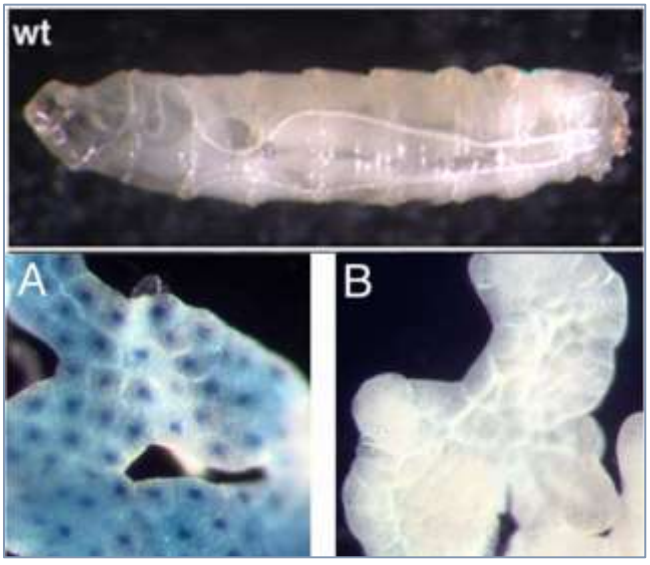

Figure 2.5 Photographs of larval fat body from wt animals A nuclei are stained with blue

Regarding the storage function, the fat body is fundamental for the life of insects, the energy in form of TAG is stored in adipocyte cells, characterized by the presence of numerous organelles called lipid droplets with metabolic activity.
The lipolytic activity includes two lipases: Brummer and Triglyceride lipase. The metabolic functions conducted by the fat body are regulated by hormonal signals such as insulin and ecdysone. During insect metamorphosis, the fat body undergoes to a "remodeling" process that consists in a destruction of larval tissues and concurrently the determination of adult tissues (81).
All this information indicates that *Drosophila* shares with mammals the same mechanisms of storage and mobilization of energy, so this insect becomes an excellent model to study lipid homeostasis (82).

2.2.2 Obesity and oxidative stress in *Drosophila*

In humans, the relationship between obesity and ROS production is still very controversial because many problems occur while using genetic models for this metabolic disease. However, it is very important to remember that the signals that trigger the increase of ROS level are highly conserved in mammals and flies, and this allows using *Drosophila* as a genetic model to analyse and find novel pathways involved in obesity and in other metabolic disorders. ROS production can be induced by different signals such as proinflammatory cytokines, UV light, radiation and environmental toxins (83).
Also in *Drosophila*, ROS perform an essential role in maintaining homeostasis by triggering cellular signaling pathways and host defense mechanisms (24).
Nevertheless, an excessive production of ROS causes an intracellular imbalance and determinates irreversible oxidative damage to DNA, proteins and lipids (84).
This cell dysfunction lead to severe complications and increases the risk to develop diseases such as diabetes, neurodegeneration, cancer and aging (85).
Fleming and colleagues have demonstrated that oxidative damage regulate the lifespan in *Drosophila* both in normal and stress conditions (86).
Another work showed that unrepaired damages caused by free radicals provoke aging and death in *Drosophila* (87).
As mentioned previously, in humans but also in *Drosophila*, cellular defense mechanisms against oxidative stress include enzymatic components like Glutathione

reductase (GSR), Glutathione peroxidase (GPx) and GST, and non-enzymatic components as vitamins and polyphenolic compounds (83).
Further data have shown that dietary antioxidants like vitamins and polyphenols ameliorate the effects of oxidative stress induced by paraquat. In *Drosophila* lifespan, in particular, it has been observed a sexual dimorphism, with females exhibiting an increase in lifespan compared to males (88-90).

2.2.3 Signaling pathways relevant in obesity conserved in *Drosophila*

The TNF-α orthologue Eiger

TNF-α belongs to a superfamily of TNF/TNFR, the name "Tumor Necrosis Factor" derives from its identification in hemorragic necrosis of human tumors caused by endotoxins (91).
In mammals TNF-α is considered an inflammatory mediator, it promotes the production of prostaglandins and other cytokines.
Furthermore, it was also reported that TNF-α is able to promote macrophages differentiation, proliferation and to induce programmed cell death (92, 93).
In *Drosophila*, TNF-α ortholog Eiger shows similar functions, it is a regulator of the death signal, indeed it can induce cell death by activating the JNK pathway, furthermore Eiger is required for innate immune response against extracellular pathogens (94, 95).

The JNK orthologue Basket

JNK homolog, Basket in *Drosophila*

Similar to mammals, the JNK pathway is highly conserved in *Drosophila*, though only one JNK isoform exists in *Drosophila*, Basket, whereas ten JNK isoforms are found in mammals (96).

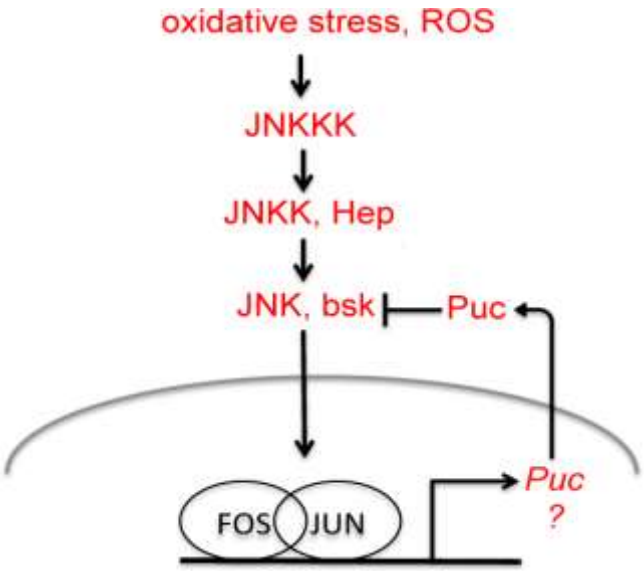

Figure 2.6 The JNK signaling pathway (modified from Valenza et al, BMRI 2018).

Thanks to low gene redundancy, *Drosophila* becomes an amazing model system to study JNK regulation in normal and stress conditions. ROS-induced signaling pathways include the activation of JNK, this event consists of a cascade of phosphorylation events starting with the activation of the JNKKK kinases, consisting of the Ask1 and Tak1, that activate MKK7, the ortholog of Hemipterous (Hep), and terminates with the activatioPn of the JNK kinase, encoded by the *basket (bsk)* gene in *Drosophila*, that is negatively regulated by Puckered (uc), a phosphatase, a target itself of the JNK kinase **(Fig. 2.6)** (97, 98).
In *Drosophila*, JNK protects the cells from oxidative stress and is able to extend lifespan in adult flies through the activation of autophagy, in particular JNK regulates the expression of Atg9, a component of the autophagy complex (99).

GST D1

As already mentioned above, GSTs existing in mammals, are generally divided into three categories: cytosolic, microsomal, and mitochondrial. Up to now, seven classes of mammalian cytosolic GSTs have been identified: Alpha, Mu, Pi, Theta, Omega, Sigma and Zeta (34, 35).
In *D. melanogaster*, the last four classes are present plus other two members, Delta and Epsilon, considered the most common classes of GST. In particular, in the last years, the Delta class is the best studied (100, 101).

Recently, it was reported that, four spliceforms of the Delta class of GST, like GST D1, D2, D3, D4, regulate in different manners JNK activity. GST D1 seems to inhibit JNK activity, whereas the other three isoforms activate it (102).

CncC orthologue of NRF2

The *Drosophila* CncC, ortholog to the vertebrate Nrf2, is a transcriptional factor, it is considered an important regulator of the cellular redox state both in vertebrate and *Drosophila*, whereas Keap1 is its negative regulator (103).

In normal conditions, CncC is normally present at the cytoplasmatic level, sequestered by its inhibitor Keap1, that induces CncC degradation throught ubiquitylation, but in conditions of elevated ROS, CncC is free to translocate into the nucleus, bind the Antioxidant Response Element (ARE) and induce the expression of key factors in the antioxidant processes like GST D1 (37, 104-106).

Previous studies have identified a number of reactive Cys residues in Keap1 which correspond to the sites of attack by ROS, in this way CncC is disassociated by Keap1 and can regulate the expression of target genes (106).

Although *Drosophila* is a well-known model for aging, the knowledge on NRF2 signaling is still not clear. Bohmann and Sykiotis have demonstrated that *Drosophila* CncC/Keap1 show function similar to human NRF2/Keap1; furthermore, they have shown that NRF2 pathway is involved in several cellular responses, it induces the expression of antioxidant enzymes like GSTD1, it is able to balance the intracellular redox, finally it regulates intestinal stem cell proliferation by reducing the age-related degeneration at the intestinal level.

2.2.4 Obesity and cancer in *Drosophila*

In the last years, the common fruit fly *Drosophila melanogaster* has become a predominal model system to study molecular mechanisms of human disease including cancer (46). Furthermore, up to 75% of human disease-causing genes have functional homolog in *Drosophila* (1) .

The molecular mechanisms that regulate growth, differentiation and metabolism are highly conserved in mammals and *Drosophila*, but the fruit flies have a genome less redundant than human genome, this allows simplifying genetic analyses for studying different human diseases. In particular, *Drosophila* is considered a cancer research

model with a focus on the roles of hemocytes to explain the interplay between inflammation and cancer (107).
Rudolf Virchow observed for the first time that leukocytes were associated with the neoplastic tissue by providing an evident link between cancer and inflammation. Subsequently, numerous experiments have been performed to convalided this discovery. Trincheri et al. proposed a possible connection of the inflammatory lesion that triggers cancer(108). Furthermore, it has been well-reported that the inflammatory process promotes tumor progression; in particular, it has been revealed that macrophages may be involved in cancer initiation or prevention (109, 110).
To better understand the real role of hemocytes in tumor progression or regression, several studies have been performed by using *Drosophila* as a tumor model: for example, Pastor-Pareja and colleagues have demonstrated through an innovative cancer model that a relationship exists between tumor and hemocytes (111). Research in *Drosophila* has documented the possible protagonists involved in the interactions between cancer and inflammation by including the JAK/STAT, JNK, TNF, Toll/Imd/TLR signaling pathways (112).
Thanks to these tools, *Drosophila* can be useful as a tumor model system to discover many signaling pathways conserved both in *Drosophila* and humans that regulates the immune system and its interactions with tumor cells.

2.2.5 *Drosophila* models to study obesity

Recently, *Drosophila melanogaster* has emerged as a powerful model organism to investigate the genetic mechanisms linked to obesity and other human metabolic disorders, because most of the human metabolic processes are conserved in flies. Furthermore, mammalian liver and adipose tissue show functionally analogies with the fly's fat body, considered a dynamic tissue involved in multiple metabolic functions like to store and release energy in the form of lipids and glycogen (113).
Here we report, two *Drosophila* established models used to study the metabolic changes and to chararcterize obesity-associated disorders.
1- HFD (High-Fat Diet) model consists of flies grown on specific diet enriched with 30% coconut oil as a source of saturated fatty acid that has been shown to cause a phenotype similar to obesity in humans, indeed HFD-induced obesity in flies show an increased triglyceride and glucose levels, resistance to starvation but at the same time this diet decreased lifespan, altering the positive intake of energy balance.

Recent publications report that HFD model in *Drosophila* causes heart dysfunction, typically found also in obese people (114, 115).

2- Genetic models, by genetic reduction of a key enzyme necessary for of energy storage, *Brummer lipase*[1] (*Bmm*[1]). The *Bmm* gene encodes for a lipase, homologue of the human adipocyte triglyceride lipase (ATGL), that determinates the lipolysis of TAG stored in lipid droplets in adipose tissue. The lack or malfunction of Bmm activity induces a deregulation of energy stored causing the obesity in flies. In *Bmm* mutants the animal continues to store energy but is not able to mobilize TAGs (116).

2.3 Anthocyanins

During my PhD project, I investigated the function of bioactive food like Flavonoids polyphenolic compounds, found in fruits and vegetables, components regularly present in human dietary, that have been recognized to possess a protective action against obesity and ATM phenotype, due to their anti-inflammatory and antioxidant activity. Over to these proprierties, they also show anti-diabetic, anti-cancer, anti-aging effects (117-121).

Until now, more than 9000 flavonoids have been identified in plants (122).

Flavonoids biosynthesis genes are conserved among various species and they are synthesized through the combination of aromatic amino acids like phenylalanine and tyrosin that linked with acetate units (123).

The basic flavonoids structure is characterized by two benzene rings linked throught an oxygen-containing pyrene ring, with several costituents that allow subdividing them into different classes among which Flavonols, Flavonones, Flavanols, Flavones, Isoflavones and Anthocyanins **(Fig. 2.7)** (124).

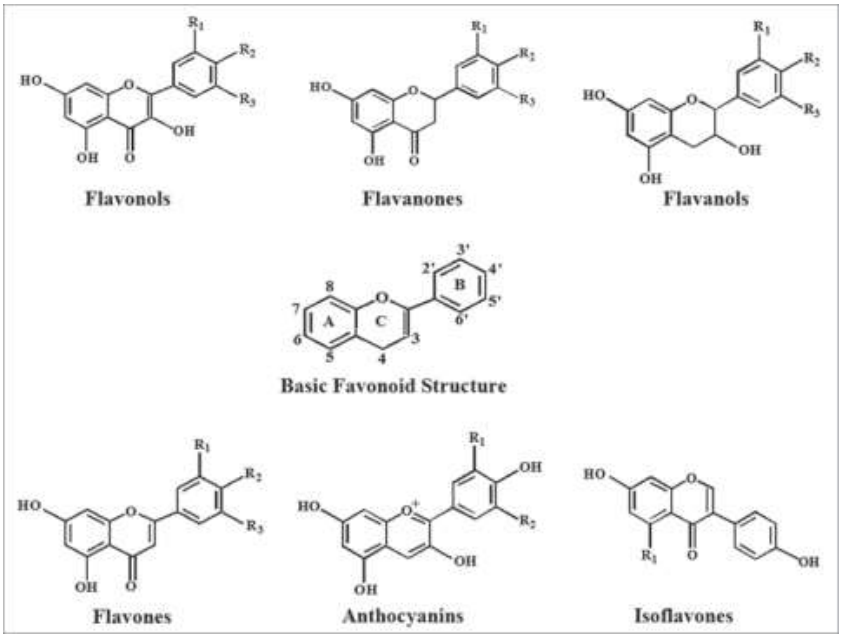

Fig. 2.7 Chemical structures of Flavonoids. The basic structure is constituited by three different heterocycle rings, thus that Flavonoids can be divided into six major subclasses: flavonols, flavanones, flavanols, flavones, anthocyanins and isoflavones (*Pandey KB et al., 2009*).

Anthocyanins's activity

In particular, the study has been focused on one class of flavonoids, Anthocyanins.
Anthocyanins are the red, purple and blue pigments found in flowers and the fruits of many plants. Recent studies have demonstrated that Anthocyanins-rich dietary reduces the risk to develop chronic diseases such as diabetes, obesity, cancer and cardiovascular diseases (125-127).
For what concerns the antidiabetic effect of Anthocyanins, it has been widely demonstrated that these bioactive molecules are able to increase insulin secretion in humans (128).
Furthermore recent studies, performed on model of diabetic mice, have reported that Anthocyanins ameliorate hyperglycemia and insulin sensitivity via adenosine monophosphate-activated protein kinase (AMPK) activation in adispose tissue and liver (129).
Anthocyanins also show anti-obesity proprierties. Clinical studies in humans have demonstrated that an higher consumption of anthocyanins, is associated with weight loss in both men and women (130, 131).
In addition they prevent weight gain and reduce the fat accumulation in obese mice

under High Fat Diet condition and in C57BL/6 obese mice (132-135).
Interestlingly, in our *Drosophila* model of obesity (described below), we find that anthocyanins ameliorate the obese phenotype by reducing the hemocytes migration and accumulation and phagocytic activity in larval fat body (136).
How Anthocyanins rescue the obese phenotype is still not clear, of course they act on inflammatory and oxidant processes.
The possible anti-inflammatory mechanism of anthocyanins, may include the inhibition of a several pro-inflammatory mediators like the nuclear factor-κB (NF-κB), Relish in *Drosophila* that, in response to external stimuli like oxidative stress and inflammation, triggers a large signaling cascade that culminates with the upregulation of proinflammatory cytokines/chemokines including TNF-α, IL-6, IL-1, IFN-γ, IL-8, the inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2). At the same time, the anthocyanins are able to act concomitantly with detoxification enzymes such as superoxide dismutase, catalase, glutathione peroxidase, GST, and glutathione reductase to reduce oxidation and activate antioxidant detoxifycation factors such as NRF2 called CncC in *Drosophila* (121, 137).
These finding suggest that anthocyanins can regulate the inflammatory responses, therefore they can be considered of the anti-inflammatory agents.

Many studies performed in animal models have demonstrated that Anthocyanins are also considered potential anticancer agents.
Anthocyanins have been isolated for investigating their anticancer proprierties on several tissues and organs like esophagus, colon, breast, liver and prostate (138).
Using mouse models, it has been discovered that Anthocyanins are able to inhibit angiogenesis and induce apoptosis and they possess anti-invasive proprierties by inhibiting the JNK pathway and Akt/mTOR signaling (139, 140).

Epidemiological studies show the link between Anthocyanin-rich diet and cardiovascular diseases. In vitro studies have demonstrated that Anthocyanins show anti-thrombotic effects, also observed in rats, in which Anthocyanins intake reduces the infarct size and modulates the antioxidant response by increasing the myocardial glutathione levels (141, 142).
Altogether, these studies confirm the beneficial effects on health of Anthocyanins.

2.4 *Drosophila P0206-Gal; UAS-Ni* obesity model

In this thesis, i focused on anti-inflammatory and antioxidant activity of Anthocyanins using a new *Drosophila* model of obesity.
In order to obtain this new model of obesity, we genetically reduced the size of the prothoracic gland, the endocrine organ, that produces the ecdysone hormone, that plays essential roles in coordinating developmental transitions such as larval molting and metamorphosis (143).
The genetic manipulation of the prothoracic gland causes the reduction of ecdysone levels (*E74b* target gene), resulting in block of the development, indeed the animal does not complete the cycle, it remains at third larva instar and continues to feed for 3 weeks with an increased in body weight (**Fig. 2.8 A-B).**
Furthermore, *Drosophila* fat body functions as storage for nutrients, which regulates the storage and release of energy in response to demand, whereas in an obesity condition the fat body continues to accumulate fat and sugars, indeed in our obese animals we observed that the size of the fat body cells from *P0206-Gal4; UAS-Ni* larvae increased **(Fig. 2.8 C)** also due to the accumulation of fats in lipid droplets visible by Nile Red staining **(Fig. 2.8 D)** (136).
In this way, *P0206-Gal4; UAS-Ni* animals acquired a phenotype that resembles to that seen in obese people, among which increased TAGs and glucose levels circulating in the hemolymph.

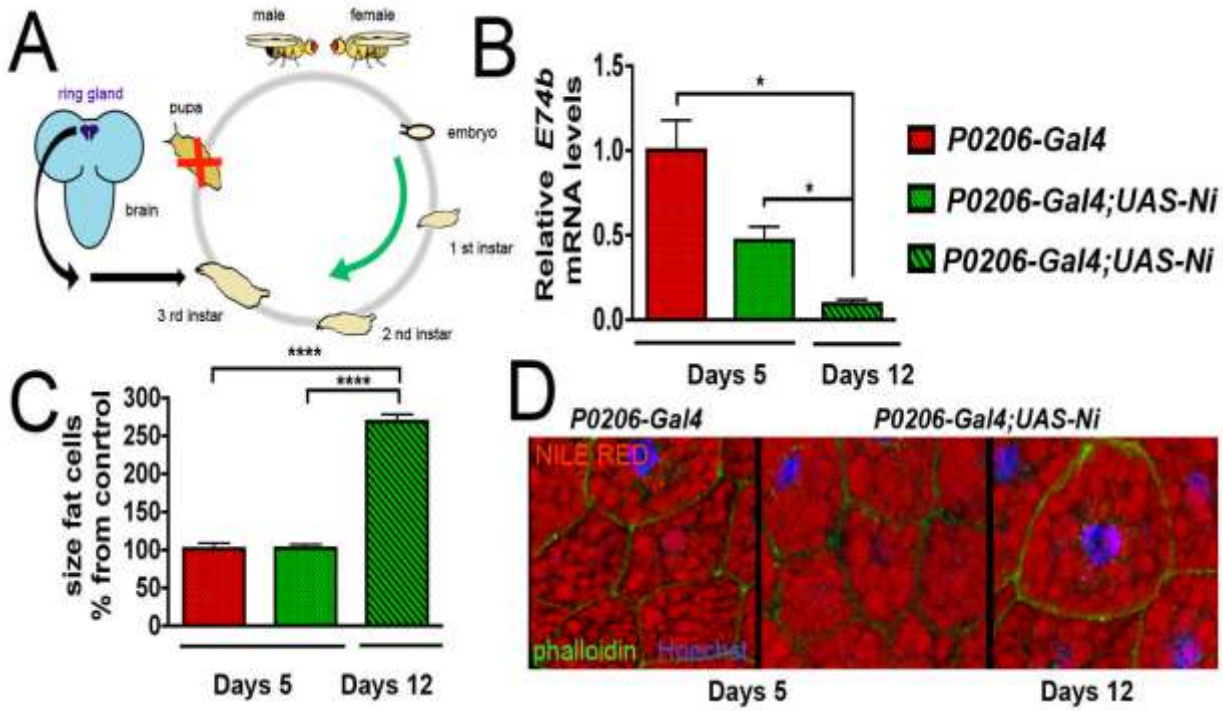

Figure 2.8 New Drosophila obese model. (A) the reduction of the prothoracic gland's causes the block of the puparation, (B)this event determinates the reduction of ecdysone levels in *P0206-Gal4; UAS-Ni* animals, (C) the size of fat body cells in *P0206-Gal4; UAS-Ni* animals increased, indeed through (D) Nile Red staining is possible to observe the accumulation of lipids in larval fat body.

Throught the binary system *UAS-Gal4*, we used *P0206-Gal4* driver for prothoracic gland-targeted RNA interference (RNAi) silencing the expression of CG7839 gene, ortholog of yeast Nok1, a ribosomial component, *UAS-Ni* (144).
In these conditions we blocked pupariation creating larvae *P0206-Gal4; UAS-Ni* with obese phenotype in the F1 generation (**Fig. 2.9)**.

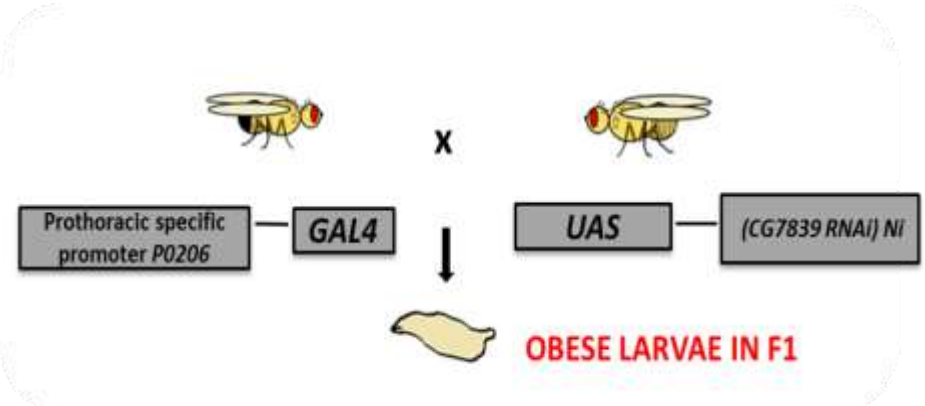

Figure 2.9 ***P0206-Gal; UAS-Ni offspring*****.** In our model, we obtained the reduction of ecdysone production through the binary system *UAS-Gal4* by regulating the expression of *UAS-Ni* (ortholog of yeast Noc1) in the prothoracic gland (using *P0206-Gal4*) obtaining larvae with similar features observed in obese people.

Moreover, in our *Drosophila* model hemocyte infiltration increases in larval fat recalled by pro-inflammatory cytochines. During this inflammatory state, hemocytes surround the fat cells, mimicking a process described in the fat of obese individuals suffering from chronic inflammation. Furthermore, these animals exhibit significant increase in ROS production over time, indicating the presence of an oxidative stress that may be responsible for the augmented phosphorylation of the JNK kinase (136). All these aspects demonstrate that our model can be used to investigate the mechanisms involved in obesity and analyze the anti-inflammatory and anti-oxidant effects of bioactive food like Anthocyanins.

Chapter 3

Manuscript published in BioMed Research International 2018 Volume 2018, Article ID 6413172

Anthocyanins function as anti-inflammatory agents in a *Drosophila* model for Adipose Tissue Macrophage infiltration

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Abstract

Epidemiological and preclinical studies have demonstrated that bioactive foods like flavonoids, polyphenolic compounds derived from fruits and vegetables, exert a protective action against obesity, cardiovascular disorders and Adipocyte Tissue Macrophage infiltration (ATM). All these pathologies are characterized by increase in reactive oxygen species (ROS) and in pro-inflammatory cytokines that have been shown to favor the migration of immune cells, particularly of macrophages, in metabolically active organs like the liver and adipose tissue, that in *Drosophila* are constituted by a unique organ: the fat body. This study, using a unique *Drosophila* model that mimics human ATM, reveals the beneficial effects of flavonoids to reduce tissue-inflammation. Our data show that anthocyanin-rich food reduces the number of hemocytes, *Drosophila* macrophages, infiltrating the fat cells, a process that is associated with reduced production of ROS and reduced activation of the JNK/SAPK p46 stress kinase, suggesting a fundamental function for anthocyanins as antioxidants in chronic-inflammation and in metabolic diseases.

Introduction

Obesity is a metabolic syndrome occurring worldwide, and often associated with other chronic diseases such as cardiovascular disorders, type II diabetes and cancer [1].
The onset of obesity is the result of multifactorial elements, including a sedentary lifestyle, genetic predisposition, ethnicity and environmental factors (such as organic pollutants) [2], these factors with a diet rich in fats and sugars and poor in phytonutrients may result in weight gain and subsequently lead to metabolic disorders [3,4]. Obesity is known to trigger a low-grade of inflammation in metabolically active tissues and in organs such as the liver and adipose tissue [5-8]. Inflammation is the result of cellular and humoral responses with the scope to protect the organism from various insults, including infection and tissue damage, in attempt to rescue tissue homeostasis [9,10].

In humans, the adipose tissue regulates lipid homeostasis, and in normal conditions controls the storage of energy reserves in the form of triglycerides as well as functioning as an endocrine organ, producing a variety of pro-inflammatory cytokines such as IL-1, 6, and 8, IFNγ, TNFα [11,12]. In pathological conditions, such obesity or metabolic syndrome, the adipocytes start to alter the production of these pro-inflammatory cytokines, which results in the activation of the innate immune system with recruitment of immune cells including macrophages leading to a state of chronic inflammation or ATM [7]. In addition, lipid accumulation and chronic inflammation in obese people are associated with a permanent increase of oxidative stress and with the production of high levels of reactive oxygen species (ROS) [13,14], which is often associated with the activation of the c-Jun-NH_2-terminal kinase (JNK/SAPK) p46, member of a mitogen-activated protein kinases (MAPKs) downstream of JNK signaling [15]. This pathway is highly conserved in *Drosophila*, and consists a cascade of phosphorylation events starting with the activation of the JNKKK kinases, consisting of the Ask1 and Tak1, that activate MKK7, the orthologue of Hemipterous (Hep), and terminates with the activation of JNK/SAPK p46 kinase, called *basket* (*bsk*) in Drosophila, that is a negatively regulated by *Puckered (puc),* a phosphatase, which itself is a target of JNK/SAPK p46 kinase (see Figure 3E) [16].
This pathological situation influences other organs by altering their functions. Furthermore the adipose tissue from obese individuals exhibits a reduced capacity

to store fat leading to an increase of circulating free fatty acids (FFAs) that promotes insulin resistance and damages the mitochondrial membrane thereby enhancing the production of ROS causing oxidative stress [17-19].
Epidemiological evidence suggests that a high intake of bioactive food is associated with a lower risk of developing chronic diseases like obesity [20]. Bioactive foods may influence the physiological and cellular activities of oxidative pathways and in recent years the attention has been focused on a class of secondary metabolites present in plant foods called flavonoids that seem to possess beneficial properties in preventing chronic diseases [21,22]. The possible health benefits of flavonoids are linked to their potent antioxidant and free radical scavenging activities demonstrated *in vitro* and *in vivo* using different animal models [23]. Among the different classes of flavonoids, anthocyanins represent the major red, purple and violet pigment in many plants and fruits. *In vivo* studies showed that anthocyanins added to the diet stimulate the secretion of insulin and decrease the generation of ROS [21,24]. Preclinical studies performed on human demonstrate that dietary-anthocyanins have a positive biological effect against obesity-induced inflammation and oxidative stress [24,25], which is associated with a lower risk of type 2 diabetes. This potentially important application creates a high interest in understanding the action of these natural bio-products in preventing metabolic diseases.

In order to study the mechanisms that control the anti-inflammatory response to flavonoids, we took advantage of a previously unrecognized conserved functional relationship between the immune cells, called hemocytes (macrophage like cells) and adipocytes (larval fat body, FB) [26]. *Drosophila* FB- a metabolic tissue with similar physiological functions to the mammalian adipose tissue and liver- acts as a functional unit to control key metabolic processes and the native immune response, in addition to storing fats and sugars [27]. In *Drosophila* the immune response is orchestrated by the hemocytes, that are circulating cells in the hemolymph, present at all stages of the life cycle and compose the fly's innate immune system [9,28-33]. Hemocytes are essential mediators in the cell-cell communication process: they have been shown to mediate a response between the fat body and tumor cells to control their growth [34] and to promote proliferation of epithelial cells in response to the release of ROS following cell death in cells of the imaginal discs [35].

Using our model of obesity, we observed that hemocytes infiltrate the FB of obese larvae mimicking the chronic inflammation present in human obesity (manuscript in submission). In this study we report that treatment with anthocyanin-enriched food results in a significant decrease in the number of hemocytes infiltrating the FB concomitantly to a reduction in ROS and of the phosphorylation of JNK/SAPK stress kinase.

Our data demonstrate that the mechanisms driving the protective role of bio-products like anthocyanins *in vivo* as anti-inflammatory and anti-oxidants are conserved in *Drosophila*. In addition, they highlight the potential use of our model to study the complex relationship between inflammation and obesity and corroborate the positive action of anthocyanins to combat chronic inflammation in humans.

Materials and methods

Fly stocks and husbandry. The Hml-RFP/CyO is a gift from Katja Brückner at UCSF.The *P0206-Gal4* from [36], *UAS-CG7839^{RNAi}* (BL 25992) is an RNA interference lines to reduce the expression of the *CG7839 gene,* encoding for the orthologue of a ribosomal protein Noc1, herein the *CG7839^{RNAi}* will be called Ni. Fly cultures and crosses were grown on standard fly food composed of yellow corn, sugar, yeast molasses-base at 25°C.

Feeding experiment and chemical compounds. Crosses were kept in culture bottles perforated to provide adequate air circulation and eggs were collected on a grape agar plate (5%) supplemented with dry yeast every 3 hours. First instar larvae were collected after 24 hours AEL (after Egg Laying) and shifted into vials containing different food. First instar larvae were reared with 2 g of standard food, hereafter Normal Food (NF) and 5 ml of each flavonoid (FL) extract, one containing only flavonoids (NF + FL) and another extract containing flavonoids and 0.24 mg/ml anthocyanins (NF + FL + ACN). All these phenolic compounds were extracted from the cobs of yellow and purple corn (gift from Katia Petroni and Chiara Tonelli, University of Milan), only the purple extract is rich in anthocyanins, while the content of other flavonoids is the same in both extracts (the content of flavonoids present in the extracts are reported in [37]).

Hemocytes quantification and size analysis in larval fat bodies. To label *in vivo* plasmatocytes, which comprise more than 95% of the hemocytes population in the *Drosophila* larva, we used the transgene *HmlΔ-DsRed (Hml-RFP)* that contains the

promoter for hemolectin, expressed in the hemocytes, fused with the Red Fluorescence Protein (RFP) [38]. Fat bodies from 20 larvae at 5 and 12 days AEL were dissected in phosphate-buffered saline (PBS) pH 7.4 and fixed in 4% paraformaldehyde (PFA) for 30 minutes. Hoechst 33258 (Sigma Aldrich) was added to stain DNA in a final concentration of 1 μg/ml. After washing with PBS, fat bodies were mounted onto slides with DABCO-Mowiol and images were acquired using an SP2-LEICA Lasertechnik GmbH confocal microscope. Images were analyzed with the ImageJ software. In the order to analyze the cell size, the larval fat bodies were fixed in 4% PFA, permeabilized with 0.2% Triton X-100 in PBS, rinsed in PBS 1X and membranes were stained with 1:100 Alexa Fluor 488 Phalloidin to visualize the cytoskeleton through the binding between Phalloidin and F-actin, and Hoechst 33258 for nuclei, then mounted onto slides with DABCO-Mowiol. Photographs were taken using confocal microscopy and the area of adipose cells for each fat body was calculated with ImageJ software. In order to visualize lipids, fat bodies were stained with Nile-Red (Sigma Aldrich) and with Alexa Fluor 488 Phalloidin following the protocol in [39].

In vivo detection of ROS using Dihydroethidium (DHE). DHE is used to detect cytosolic superoxides and radical oxygen species (ROS). The reaction between DHE and superoxide anions generates a highly specific red fluorescent product (ethidium), which intercalates with DNA. ROS levels were detected in live tissue as described in [40]. Briefly, larval fat bodies at 5 and 12 days AEL were dissected in Schneider's insect medium (GIBCO). After incubation in 30 μM DHE (Invitrogen) for 5-7 minutes in the dark at room temperature, fat bodies were washed three times with Schneider's medium and immediately mounted with VECTASHIELD Antifade Mounting Medium.

RNA extraction and quantitative RT-PCR. Total RNA was extracted from 8 whole larvae using QIAGEN RNeasy Mini Kit. 1 μg total RNA from each genotype was reverse-transcribed into cDNA using SuperScript IV MILO Master Mix (Invitrogen). The obtained CDNA was used as the template for quantitative real-time PCR (qRT-PCR) using SYBR Premix Ex Taq-Tli RnaseH Plus II (TaKara), analyzed on a RT-PCR BIORAD thermocycler machine. In these experiments, gene expression levels were normalized to *actin mRNA*, used as the internal control. The following primers for qRT-PCR were used: *actin5c* 5'-

CAGATCATGTTCGAGACCTTCAAC-3’ (R) and 5’-ACGACCGGAGGCGTACAG-3’ (F) and E74B 5’-GAATCCGTAGCCTCCGACTGT (R) and 5’-AGGAGGGAGAGTGGTGGTGTT (F) [39].

Protein extractions, and immunoblotting. The larval fat bodies (10 for each genotype) were dissected in Schneider’s medium serum-free and lysed in 80 µl of lysis buffer 1X (50 mM Hepes pH 7.4, 250 mM NaCl, 1 mM EDTA, 1.5% Triton X-100). Protease inhibitor cocktail (Sigma-Aldrich) was added to inhibit protease and phosphatase activities. Samples were sonicated two times for 10 seconds, and then centrifuged. Protein concentration was determined by Bradford protein assay (Bio-Rad). The samples were boiled in 1X SDS, then separated on 10% SDS-polyacrylamide gels and blotted. Membrane was incubated with primary antibody anti-phospho-p46 SAPK/JNK (Cell Signaling #9521) or anti actin (Hybridoma Bank) overnight at 4°C in blocking buffer then washed in 0.1% Tween 20 with TRIS-buffered saline (TBST). Appropriate secondary antibody, was incubated for 2 hours, followed by washing. The signal was revealed with ChemiDoc Touch Imaging System (Bio-Rad Lab).

Immunostaining. Dissected fat bodies from 20 larvae were fixed in a solution of 4% PFA/PBS for 40 minutes. After permeabilization with 0.3% Triton/PBS, tissues were washed in a solution of Tween 0.04%/PBS, saturated with 1% BSA/PBS and incubated over-night with anti-SPARC antibodies (1:400), a generous gift from M. Ringuette [41] and visualized using anti-Rabbit Alexa555 (Invitrogen).

Statistical analysis. The experiments were repeated at least three times and the statistical analysis among the various genotypes was examined by Student's t-test. Differences were considered significant if *p value* were less than 0.05 (*), 0.01 (**), 0.001 (***) and 0.0001 (****).

Results

Obese larvae have increased size of fat cells and increased hemocytes in the fat body.

In order to study the ability of hemocytes to infiltrate the fat cells, we blocked pupariation (Figure 1A) creating larvae *P0206-Gal4;UAS-Ni* where the reduction of the size of the prothoracic gland, the endocrine organ that produces ecdysone, resulted in reduced levels of ecdysone (Figure 1B), leading to animals that develop at almost normal rate and continue to feed until 3 weeks with an increased body weight (see method). *Drosophila* FB-cells function as storage for nutrients, which synthesize and release energy, and accumulates fat and sugars; in our obese animals we observed that the at 12 days AEL the size of the cells from the FBs from *P0206-Gal4; UAS-Ni* larvae increased (Figure 1C) due also to the accumulation of fats in lipid droplets visible by Nile Red staining (Figure 1D). Those *P0206-Gal4;UAS-Ni* animals acquired phenotypic characteristics of obese individuals, including increased triglycerides (TAGs), glucose circulating in the hemolymph, resistance of fat cells to stimulation with insulin and increased hemocytes in the FB (manuscript in submission).

Chronic inflammation in the adipose tissue is characterized by the infiltration of macrophages in the fat cells, we therefore analyzed if a similar event was present in the FB of our obese animals. We labeled the hemocytes *in vivo* using the *Hml-RFP* reporter line that specifically expresses Red-Fluorescence protein in hemocytes and introduced this transgene to our genetic background. *Hml-RFP* positive cells were monitored over time to visualize and quantify the number of hemocytes infiltrating the FB, from control and obese animals at 5 days AEL and at 12 days AEL in the obese larvae. These results showed that FBs from *P0206-Gal4/Hml-RFP;UAS-Ni* animals contain at 5 days AEL a small but significant higher number of hemocytes in their FBs (5.2%, $p<0.05$) as compared to control *P0206-Gal4/Hml-RFP* (Figure 1E), furthermore at 12 days the percentage of hemocytes in *P0206-Gal4/Hml-RFP;UAS-Ni* animals was drastically increased to (17%, $p<0.00001$). Hemocytes are characterized by the expression of high levels of the cell adhesion protein SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin or BM 40) [41], morphological analysis of FBs from 12 days *P0206-Gal4;UAS-Ni* animals, showed the presence of crown-like structures of hemocytes, positive with anti-SPARC antibodies, that surrounded the fat cells, mimicking similar structures described in the fat of obese individuals suffering from chronic inflammation (Fig. 1F).

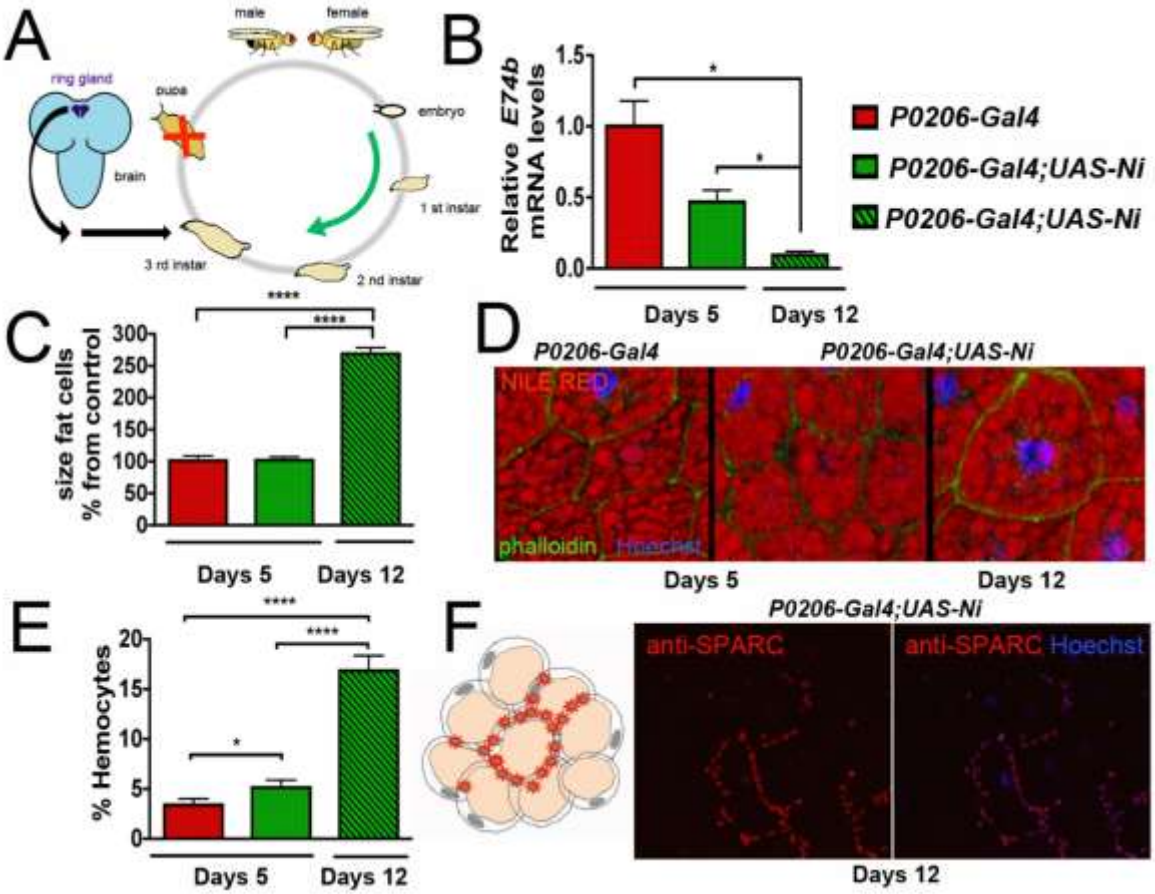

Figure 1: Obese larvae have increased hemocytes infiltrating the fat cells

(A) Ecdysone regulation of larval molting and metamorphosis. Reducing the size of the ring gland reduces ecdysone level in *P0206-Gal4; UAS-Ni* animals. (B) Quantitative RT-PCR in whole larvae of the indicated genotype showing the relative expression of *E74b mRNA. Actin5c* was used as control. (C) Relative size of cells from the FBs from animals of the indicated genotypes, at 5 and 12 days AEL. (D) Nile Red staining for lipids, Phalloidin for membranes, and Hoechst for nuclei, of FBs. (E) % of hemocytes infiltrating the FBs of animals at 5 or 12 days AEL, of the indicated genotype. Data are expressed as percentage of hemocytes in the cells of FBs. (F) Draw and confocal photographs of cell from the FB, showing hemocytes stained with anti-SPARC antibodies (RED), while nuclei are visualized using Hoechst (BLUE). Error bars represent SEM (standard error of the mean) of three independent experiments. * $P < 0,05$, *** $P < 0,001$, **** $P < 0,0001$.

Obese larvae have increased phosphorylation of JNK/SAPK and of ROS production in the FB.

Chronic inflammation in obese people is often associated with high levels of reactive oxygen species (ROS) and with the activation of the c-Jun-NH_2-terminal kinase (JNK/SAPK) p46 [15].

We therefore analyzed if in our *Drosophila* model of chronic inflammation, if there was an activation of JNK signaling by looking at the levels of phosphorylation of JNK/SAPK p46. Western blot analysis using extracts from FBs of larvae from *P0206-Gal4* at 5 days AEL or *P0206-Gal4;UAS-Ni* at 5 and 12 days AEL shows an increase in the phosphorylation of JNK/SAPK p46 kinase (Figure 2A).

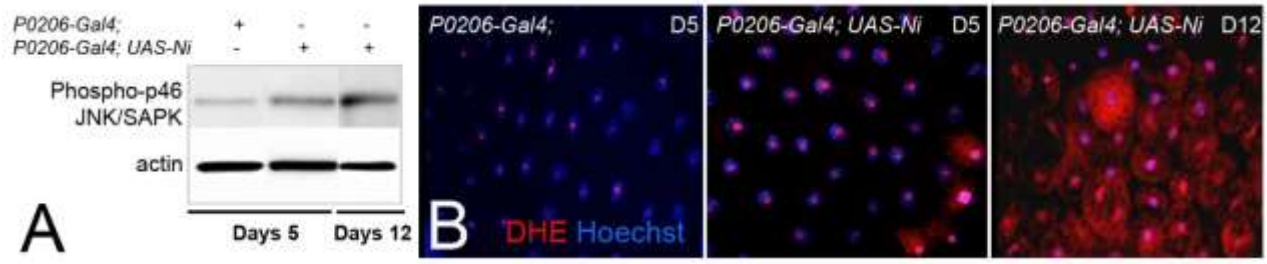

Figure 2: Obese larvae show activation of JNK/SAPK signaling and increased ROS production. (A) Western blot from lysates of FBs showing the level of phosphorylation of JNK/SAPN p46 kinase, in *P0206-Gal4* (control) and *P0206-Gal4;UAS-Ni* animals. Actin was used as control loading. (B) Confocal photographs (20x) of cells from FBs stained with DHE (red) for ROS, and Hoechst (BLUE) for nuclei.

Since ROS are known to induce the activation of the JNK pathway we then analyzed if in the FBs from the obese larvae there was an increase in ROS signaling, using DHE as a marker. These experiments show that at 5 days AEL, DHE staining increased in FBs from *P0206-Gal4;UAS-Ni* (Figure 2B, middle panel) animals as compared to control (Figure 2B, left panel), moreover DHE staining further increased at 12 days AEL (Figure 2B, right panel) suggesting that FBs form these animals exhibit significant increase in ROS production over time.

Dietary anthocyanins reduce hemocytes infiltration in FBs and phosphorylation of JNKSAPK p46.

Flavonoids (FL) and anthocyanins (ACN) are known to have antioxidant effects against inflammation-induced oxidative stress. Therefore, we analyzed if the presence of FL or ACN in the diet of the obese animals, had an effect on the chronic inflammation and stress phenotypes.
Staged first instar larvae were transferred to normal standard food (NF) or to food enriched with FL only or enriched with FL + ACN, herein called ACN (Figure 3A

and material and methods) and their effect on the migration of hemocytes in the FBs was quantified by visualizing the number of HML-RFP positive cells on dissected FBs using a fluorescent microscope. These experiments show that after 5 days of feeding with the different diets, only food containing ACN significantly reduces from 5.7% to 3.2% the presence of hemocytes in the FBs of *P0206-Gal4;UAS-Ni* animals, while treatment with FL did not have any effect (Figure 3B). At 12 days instead both, FL or ACN diets were able to significantly decrease the number of hemocytes (Figure 3B). In addition macroscopic analysis of the shape and number of hemocytes showed that at 12 days AEL both FL and ACN treatments were able to reduce the formation of crown-like structures of hemocytes surrounding the fat cells in *P0206-Gal4; UAS-Ni* animals (middle and left panel) Figure 3C. We then analyzed the effect of FL and ACN diets on the phosphorylation of the stress-response JNK/SAPK p46. FBs from animals growing in the different diets were dissected at 5 and 12 days AEL and phosphorylation of JNK/SAPK was analyzed by western blot. These experiments showed that at 5 days AEL, feeding with ACN significantly reduced the phosphorylation of JNK/SAPK p46 in FBs from *P0206-Gal4;UAS-Ni* (Figure 3D), while after 12 days AEL both diets with FL and ACN were able to significantly reduce JNK/SAPK p46 phosphorylation, suggesting a potential role at later points for FL in reducing oxidative stress.

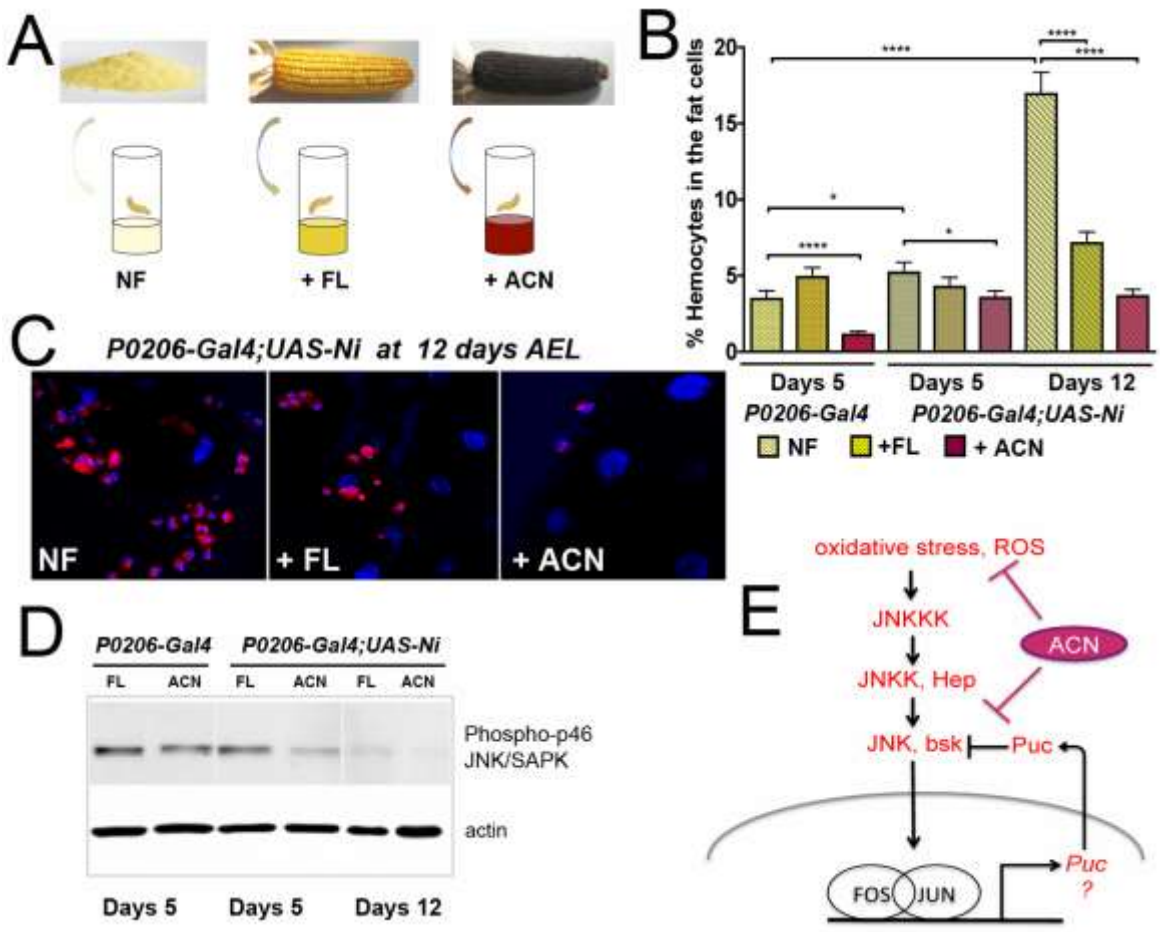

Figure 3: Anthocyanins-rich diet reduces the infiltration of hemocytes in the FBs and the phosphorylation of JNK/SAPK. (A) Scheme of the different diets NF: Normal Food, or enriched in FL: flavonoids, or ACN: anthocyanins. (B) % of hemocytes in the cells of the FBs from animals at of the indicated genotypes and fed with the indicated diets at 5 or 12 days AEL. (C) Confocal images showing hemocytes expressing Hml-RFP (RED) and nuclei stained with Hoechst (BLUE) from animals at 12 days AEL, upon feeding with NF, FL or ACN enriched diets. (D) Western blot from lysates of FBs showing the level of phosphorylation of JNK/SAPN p46 kinase, in *P0206-Gal4* (control) and *P0206-Gal4; UAS-Ni* animals fed in FL or ACN enriched diets. FBs were taken at 5 or 12 days AEL. Actin was used as control loading. (E) Model of JNK signaling and potential action of anthocianins. Error bars represent SEM (standard error of the mean) of three independent experiments. * $P < 0,05$, ** $P < 0,01$, *** $P < 0,001$, **** $P < 0,0001$

4. Discussion

Obesity and metabolic disorders are pathological conditions associated to our diet enriched of fats and sugars or to a sedentary life, but also to environmental factors that may pollute our food with chemicals that affect lipid metabolism. As

consequences we are seen an increase in cardiovascular diseases, type 2 diabetes and a chronic inflammation of the adipose tissue (ATM) induced by the persistent infiltration of macrophages into the fat cells, for which the mechanisms are not totally understood but have been associated with an oxidative stress condition present between the immune cells in the metabolic tissues. In order to study *in vivo* these relationship, we have taken advantage of the conserved relationship in *Drosophila* between the immune cells (hemocytes) and the fat body (adipose tissue) to study how bio-products like flavonoids in particular anthocyanins, that are known to act as antioxidants and that are naturally present in our food, may ameliorate or counteract the migration of the hemocytes into the FB using our animal model that mimics chronic inflammation in vertebrate (ATM).

Anthocyanins are a class of flavonoids classified as bioactive food have been shown to ameliorate hyperglycemia, insulin sensitivity and fat accumulation in obese mice fed to an high fat diet, while in vertebrates studies identify a beneficial effect by anthocyanins in combating inflammation-related diseases such as diabetes, cardiovascular diseases and obesity [25,42,43]. Moreover, clinical studies in humans demonstrate that higher consumption of anthocyanins is associated with weight loss in both men and women, and reduces the risk of developing chronic diseases with a mechanism poorly understood [24,44].

In this study, we are using our innovative model that mimic obesity in flies, where upon blocking growth by reducing ecdysone, the animals develop at almost normal rate but continue to feed with an increase in body weight and in the fat cell-size, these animals acquire the characteristics of obese people, with an accumulation of TAGs and insulin resistance (manuscript in preparation). Moreover, these animals present an infiltration of hemocytes (macrophage-like cells) within the cells of the FB that progressively increases until the formation of the typical “crown-like structures” described in obese patients suffering from ATM [5]. We demonstrate that in FBs from these obese animals there is increased production of ROS, indicating the presence of an oxidative stress that may be responsible to the augmented phosphorylation of the JNK/SAPK p46 stress kinase. Because the molecular mechanisms that regulate lipid metabolism are highly conserved between humans and flies [45,46] and hemocytes have been shown to be functionally equivalent to macrophages we can speculate that the mechanisms underline these humoral

responses are conserved also in flies. Therefore, we use our obesity model to investigate the antioxidant effect of flavonoids and anthocyanins to chronic inflammation. In our study, we find that a diet rich in anthocyanins reduces hemocytes migration in the larval FB and decreases the accumulation of TAGs in the fat cells (not shown) ameliorating several characteristics of the obese phenotypes. Moreover, we showed that anthocyanins reduce the production of ROS in cells of the FB, and significantly attenuate the phosphorylation of JNK/SAPK p46 kinase providing evidence that may play a key role in regulating the JNK-mediated cellular stress responses and to control ROS signaling.

The interplay of signals that regulate the non-autonomous responses between hemocytes and the cells of the FB is coming up as a new field for important studies, indeed recently hemocytes have been shown to be responsible of mediating an humoral immune response in a model for tumor growth, were they were shown to trigger signals responsible of killing the tumor cells through a non autonomous mechanism mediated by the activation of cytokines of the Toll and Eiger/TNFα by the fat body [34]. More recently, hemocytes were shown that upon stress conditions they are able to migrate near epithelial cells and to produce ROS to induced the release of Eiger/TNFα by the epithelial cells through the activation of the JNK signaling pathway, suggesting also in this case the presence of non-autonomous signals between the hemocytes and the cell of the epithelium necessary for tissue homeostasis [47] [35,48]. In a similar way, we can speculate that the hemocytes in the FB from obese animals maybe be activated by the oxidative stress signals (ROS), present in the FB, that trigger signals to induce the production of cytokines of the Toll and Eiger/TNFα that further aggravate the oxidative stress condition that attract the hemocytes that constitutively migrate into the fat cells causing a status of chronic inflammation.

In our experiments, we show that anthocyanins are able to reduce the activation of JNK/SAPK p46 stress kinase. As mention before, JNK pathway is activated upstream by ROS and by cytokines including Eiger/TNFα, this pathway is inhibited by a negative regulatory feed beck that induces the transcription of the phosphatase *puckered* (Figure 3E). In our model, we can speculate that anthocyanins may either directly block cytokines upstream of JNK signal, for example by controlling Eiger/TNFα signaling or they may contribute to the activation of the negative feedback that involved the activity of puckered.

Interestingly, anthocyanins were shown to act concomitantly with detoxification enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase (GST) and glutathione reductase to reduce oxidation. In *Drosophila Gst-D1* [49] and *jafrac*, an inhibitor of cell death, together with *puckered*, were shown to be transcriptional targets of *jun-fos* activity in response to the activation of JNK pathway, and these genes were shown to negatively counteract the oxidative stress response [50]. Our preliminary data however did not find any regulation in the expression of *GstD1* in the fat cells from the obese animals upon feeding with FL or ACN anthocyanins (data not shown) suggesting that probably this enzyme is not involved in the regulation of JNK signaling by anthocyanins in these cells.

In conclusion, with the present study we provide for the first time a strong evidence of the potential use of anthocyanins in the diet to control chronic- inflammation and provide a link to the oxidative stress that characterize the adipose tissue in obese animals. We were able to evidence the ability of anthocyanins to decrease *in vivo* the phosphorylation of JNK/SAPK p46 stress kinase, thus providing a new insight into the mechanism of phenolic compounds in the treatment of inflammation in adipose tissues, a field of currently study since the lack of a better knowledge of the mechanisms that regulate or control ATM in pathologies such as obesity and metabolic disorders.

Acknowledgements

We thank Sheri Zola and Maria Teresa Allocca for helpful discussion and critical reading of the manuscript. Miriam Ascagni for confocal facility. This work was supported by a Public Health Service grant from the NIH-DK085047 to PB and SZ. “Fondazione Cariplo 2014-0703” and “EHDN-689” to PB and AV.

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Chapter 4

Front. Genet., 01 March 2019
| https://doi.org/10.3389/fgene.2019.00051

Drosophila melanogaster: a model organism to study cancer

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Abstract

Cancer is a multistep disease driven by the activation of specific oncogenic pathways concomitantly with the loss of function of tumor suppressor genes that act as sentinels to control physiological growth. The conservation of most of these signaling pathways in *Drosophila*, and the ability to easily manipulate them genetically, has made the fruit fly a useful model organism to study cancer biology.

In this review we outline the basic mechanisms and signaling pathways conserved between humans and flies responsible of inducing uncontrolled growth and cancer development. Second, we describe classic and novel *Drosophila* models used to study different cancers, with the objective to discuss their strengths and limitations on their use to identify signals driving growth cell autonomously and within organs, drug discovery and for therapeutic approaches.

Keywords: *Drosophila cancer modeling*, cancer biology, oncogene, tumor suppressor, tissue growth, signaling, metabolism, therapeutic approaches

Introduction

The fruit fly, *Drosophila melanogaster*, is used as a model organism to study disciplines ranging from fundamental genetics to the development of tissues and organs. *Drosophila* genome is 60% homologous to that of humans, less redundant, and about 75% of the genes responsible for human diseases have homologs in flies (Ugur et al., 2016). These features, together with a brief generation time, low maintenance costs, and the availability of powerful genetic tools, allow the fruit fly to be eligible to study complex pathways relevant in biomedical research, including cancer. Indeed, publications that use flies to model cancer have exponentially increased in the last 10 years, as shown in the graph of Figure 1, suggesting the relevance of this model to cancer research.

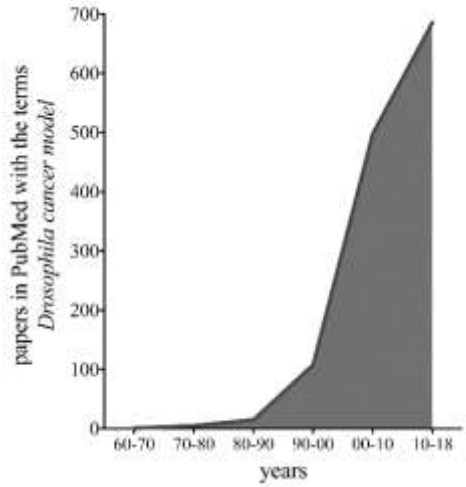

Figure 1: Graph representing the number of publications in PubMed found with the terms "*Drosophila* cancer model", in the last 48 years.

In this review we first describe the basic biological mechanisms responsible for uncontrolled growth conserved between humans and flies. We placed a particular emphasis on the characterization of epithelial tumors from most studied models (gut and brain), to novel approaches for studying tumor-induced angiogenesis, prostate, thyroid and lung cancers, with the goal to discuss their strengths and limitations. In the second part, we analyze few physiological mechanisms that uncover potential

non-autonomous mechanisms controlling growth, including the relation between the immune cells (macrophages) and the growth of epithelial cells, or the function of lipid metabolism in cancer growth. Finally, we discuss how *Drosophila* models are used to find novel interesting therapeutic approaches.

1.0 Properties of epithelial cancer cells

Cancer cells are characterized by unrestrained proliferation that results from defects in signaling driving cellular growth, apoptosis and changes in metabolic pathways. At cellular level, the hyperproliferative status of cancer cells is mainly due to the activation of growth signals induced by proto-oncogenes (e.g. the RAS/RAF/MAPK axis), which function downstream of receptor signaling cascades, and are deregulated in 25% of human tumors (Samatar and Poulikakos, 2014). Tumor cells escape the anti-proliferative effect of tumor suppressor genes, such as *RB* (retinoblastoma-associated) and *TP53* genes (Duronio and Xiong, 2013), through mutations in these genes, which result in uncontrolled growth (Hanahan and Weinberg, 2000, 2011; Hariharan and Bilder, 2006). Apoptotic cell death represents another physiological mechanism to maintain cellular homeostasis, and cancer cells have developed strategies to evade apoptosis, i.e. by increasing the activity of anti-apoptotic genes (*Bcl-2, Bcl-xL, Bcl-w*) and of pro-survival factors (*Igf-1, Igf-2*) or by downregulating the action of pro-apoptotic genes (*Bax, PUMA, Bin*) (Hanahan and Weinberg, 2011). Another characteristic of cancer cells is the reactivation of telomerase, present in 90% of human cancers, that allows them to replicate unlimitedly (Kumar et al., 2016).

Cancer cells also exhibit alterations in metabolic pathways that contribute to their survival. Rapidly proliferating cells have a high metabolic rate and suffer from low oxygen conditions (hypoxia). In epithelial tumors, this condition triggers the so-called angiogenic “switch” where the quiescent vascular network is induced to proliferate by the secretion of pro-angiogenic factors, such as VEGF (Vascular Endothelial Growth Factor) and FGF (Fibroblast Growth Factor) (Hida et al., 2018), allowing for the formation of new vessels that penetrate into the tumor mass to supply oxygen and nutrients (Carmeliet and Jain, 2011). Cancers cells also exhibit a metabolic switch where they reprogram their metabolism to use an alternative and less abundant anabolic pathway to sustain their growth. In particular they switch from oxidative phosphorylation to anaerobic glycolysis, where glucose is used to

produce lactate, through a process called the "Warburg effect" (Pavlova and Thompson, 2016; Vander Heiden and DeBerardinis, 2017). This metabolic switch is not yet completely characterized but is supported by the activation of oncogenes, including Myc that also activates glutaminolysis to fuel the TCA cycle with anaplerotic reactions to produce the intermediates necessary for cellular biosynthesis (Hsieh and Dang, 2016).

The last stage of tumorigenesis is represented by the invasive and metastatic capabilities of tumor cells to disrupt the apical-basal cell polarity, a process that is associated with the downregulation of cell-cell contact molecules and the release of metalloproteases (MMP1), lytic enzymes that degrade the extracellular matrix (ECM) allowing tumor cells to escape and colonize an environment that suites them and to acquire new oncogenic properties (Lambert et al., 2017; Massague and Obenauf, 2016). A variety of studies are now focused on how the tumor micro environment (TME), a specific niche composed of fibroblasts, lymphocytes and immune cells, that may shape pre-cancer cells for their progression into cancer cells and it may select the development of metastasis (Massague and Obenauf, 2016). Emergent evidence suggests also a key role for non-autonomous signals released by the cells composing the niche, particularly from cancer-associated fibroblasts (CAFs), that are essential to support the growth of cancer cells in this "new" metabolic environment (Lambert et al., 2017).

2.0 Cancer modeling in *Drosophila*

Most of the signaling pathways controlling cell growth and invasion in mammals have a conserved function in flies; allowing their modulation into several models that mimic a tumor's biology in a simple model organism like *Drosophila* (Millburn et al., 2016). The combination of genetic screens with the availability of powerful recombination techniques enabled also a rapid characterization of the primary function of conserved oncogenes and of tumor suppressor genes in a whole animal (Sonoshita and Cagan, 2017). In addition, recent studies using *Drosophila* imaginal discs explored the mechanisms that govern growth in epithelial tumors and their interaction with the local TME and stromal cells, including some steps in the recruitment of the immune cells (macrophages) to the tumor mass (Herranz et al., 2016).

3.0 Epithelial tumors in *Drosophila*

About 90% of human cancers are of epithelial origin (Hanahan and Weinberg, 2000). Epithelial tissues are characterized by a specific cell architecture composed of junctions and apical and baso-lateral membrane domains that are crucial for the maintenance of cell-physiological functions. Loss of cell adhesion and cell polarity, with an increase of cell motility, are indeed characteristic early cancer traits. In this context, *Drosophila* larval imaginal discs, are a monolayer epithelium that is limited apically by a squamous epithelium (peripodial membrane) and, basally to the notum, by a layer of myoblasts embedded in Extracellular Matrix, and constitute a perfect system in which to model the onset of epithelial cancer progression. These larval organs are indeed morphologically and biochemically comparable to mammalian epithelia (Wodarz and Nathke, 2007). Moreover, the prominent signaling pathways that regulate growth in humans are conserved in the fruit fly (Figure 2), allowing the use of this animal model to examine the hallmarks of cancer (St Johnston, 2002). During the last few years, the imaginal wing and eye discs have been used successfully to study tumor growth and invasion, to investigate the function of cancer genes, and to perform chemical screenings (Tipping and Perrimon, 2014). The imaginal discs also represent an excellent model to analyze oncogenic cooperation: thanks to the use of the MARCM system (Lee and Luo, 1999), it is feasible to induce simultaneously in single cells mutations in tumor suppressor genes (e.g. mutations in cell polarity genes and Hippo pathway components and interactors) and oncogenic activating mutations, or to overexpress specific genes (e.g. EGFR, Ras, Myc, Yorki), resulting in tissue overgrowth, alteration of the normal tissue architecture, disruption of the basement membrane, and invasive/metastatic behavior (Brumby and Richardson, 2003; Pagliarini and Xu, 2003a; Wu et al., 2010).

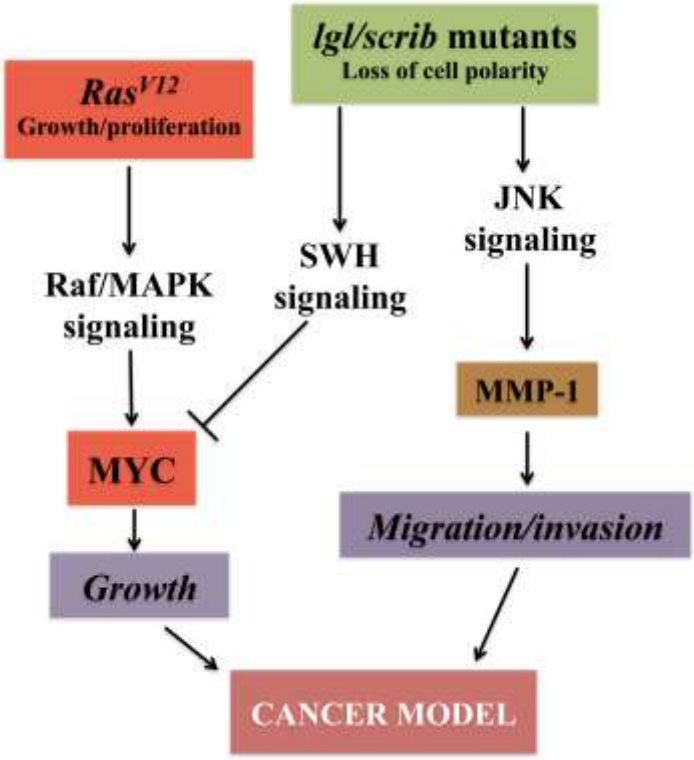

Figure 2: Major pathways converging on uncontrolled growth in *Drosophila* epithelial cells. The signaling pathways outlined confer growth, migration and invasive capabilities to epithelial cells both in vertebrates and flies. Models that mimic the growth of epithelial cancer cells and their ability to undergo metastasis in *Drosophila* have been established by inducing the cooperation between oncogenes (RED) like the active form of Ras (Ras^{V12}) together with the loss of function of cell polarity genes (GREEN) (Brumby and Richardson, 2003; Pagliarini and Xu, 2003b). Alteration of cell polarity with the downregulation of the SWH (Salvador-Hippo-Warts) pathway, together with Ras^{V12}, triggers downstream events, including activation of the MAPK signaling that stabilize Myc protein (Galletti et al., 2009) resulting in robust cellular growth. Activation of the JNK signaling, with the concomitant loss of cell polarity, induces metalloproteases (MMP-1) and confers to the epithelial cells the distinct characteristics of migration and invasion, hallmarks of tumor growth (Igaki et al., 2006; Ma et al., 2017; Uhlirova et al., 2005).

3.1 Marks of alteration in epithelial cells

3.1.1 Loss of cell polarity

Cellular junctions and a proper apical-basal cell polarity are fundamental for the maintenance of epithelial tissue architecture and function. During early cancer stages, tissues lose these properties and cells subvert their normal growth rate and

acquire invasive and migratory behaviors (Bryant and Mostov, 2008; Wodarz and Nathke, 2007). In *Drosophila*, three complexes establish and maintain epithelial polarity: the Crumbs/Stardust/PATJ/Bazooka, the Par6/aPKC (atypical protein kinase-C) and the Scrib/Dlg/Lgl (Scribble/Discs large/Lethal giant larvae) complexes, which are respectively placed at the apical, subapical and baso-lateral membrane domains. Alterations in these proteins provoke continued cell proliferation, loss of differentiation and complete loss of tissue architecture, resulting in neoplastic overgrowth (Bilder, 2004; Grzeschik et al., 2010; Johnson and Halder, 2014). *lgl* was the first neoplastic tumor suppressor gene discovered in *Drosophila* and its loss leads to an abnormal growth of the imaginal structures and the larval brain. In addition, *lgl* mutant tissues, and tissues bearing *dlg* or *scrib* mutation, have the ability to form secondary tumors in the thorax, brain, wings, muscles, intestine and ovaries (Woodhouse et al., 1998). The loss of cell polarity impacts cell proliferation through the deregulation of the Hippo (Hpo) pathway, a signaling cascade involved in organ size maintenance (Lu et al., 2010). It is not yet fully known how *lgl* activity interacts with the Hpo cascade, but it was observed that its downregulation up-regulates cell cycle genes (such as Cyclin E and E2F1) (Grzeschik et al., 2007) and permits the nuclear translocation of Yorkie (Yki), the downstream effector of the Hippo pathway, causing the activation of its target genes, including MYC, that was found to be important for the growth of *lgl* mutant clones in a competitive environment (Froldi et al., 2010). In humans, two *lgl* homologs have been discovered, *HUGL-1* and *HUGL-2*, with *HUGL-1* rescuing all the defects of the fly *lgl* mutant (Grifoni et al., 2004). *HUGL-1* loss of function has been associated with a series of human malignancies (Grifoni et al., 2007; Lu et al., 2009; Schimanski et al., 2005). Finally, while the human genome encodes for only one homolog of the tumor suppressor *scrib*, a number of homologs are known for *dlg* which have been implicated in different types of cancer (Halaoui and McCaffrey, 2015).

3.1.2 Growth Signaling

The **Salvador-Warts-Hippo (SWH)** tumor suppressor pathway was discovered first in *Drosophila* as a regulator of organ size (Pan, 2010; Yu et al., 2015) and later in humans, where it was found to be fundamental in the regulation of cancer growth (Harvey et al., 2013). The physiological activation of the Hippo (HPO) kinase,

(MST1/2 in human)(Harvey et al., 2003) consists in the phosphorylation of Warts (WTS), (LATS1/2 in human) (Genevet et al., 2010; Yu et al., 2010) and in the activation of the phosphorylated core complex, that includes Salvador (SAV in human)(Tapon et al., 2002) and Mob/MATS, that in turn, phosphorylate Yki (YAP/TAZ in humans)(Oh and Irvine, 2008). Phosphorylated Yki is sequestered and degraded in the cytoplasm, resulting in the inhibition of its nuclear transcriptional activity and oncogenic function (Harvey et al., 2013). Upstream, the Hippo cascade is regulated by components of cell junctions, including cell adhesion molecules such as Merlin, a homolog of the human Neurofibromatosis Type 2 (NF2) (Genevet et al., 2010; Yu et al., 2010), which acts as tumor suppressor; the cadherin Fat in complex with Dachsous; and by cell polarity regulators such as Crumbs (Harvey et al., 2013; Robinson et al., 2010). Alterations in the composition of the core proteins (HPO, WTS, SAV, MATS) of the pathway trigger Yki translocation into the nucleus that binds tissue-specific partners and induces the expression of its target genes, among them: CyclinE, dIAP1 and MYC (Harvey et al., 2003; Neto-Silva et al., 2010; Pantalacci et al., 2003; Ziosi et al., 2010). This articulated system is also tightly regulated by other signaling pathways: for example, in the *Drosophila* imaginal wing disc, Lgl or aPKC deregulation results in JNK activation to promote Yki nuclear translocation via phosphorylation of Ajuba (Jub), an upstream regulator of the cascade that binds to and inhibits Wts kinase activity (Sun and Irvine, 2013). In addition to the regulation of cell-cell interaction signals, components of the Hippo pathway have been found to be sensitive to mechanical stress (Panciera et al., 2017). This mechanotransduction function is critical in the control of physiological pathways, and its deregulation may contribute to the abnormal cell behavior in diseases such as cancer, where the cells in the tumor have to sustain physical forces generated by tissue overgrowth. Interestingly, this last function has shown differences in the behavior of Yki between human and flies: indeed, in *Drosophila* the Yki protein does not respond to integrin stimulation, while in mammalians integrin signaling promotes YAP/TAZ activity. One possible explanation for this different behavior may be that the N-terminus of Yki is missing a domain necessary to bind PDZ-containing proteins, which is found in its human counterpart YAP, and is necessary for the activation of the integrin-Src adhesion branch of the pathway (Elbediwy and Thompson, 2018). However, an interesting and potential explanation for this difference comes from a comparative analysis of the Yki protein and the evolution of the different epithelia: this analysis outlines how in *Drosophila* the

apical membrane of the columnar epithelium is well differentiated in its function to activate the Hippo pathway, whereas in mammals the multilayer of cells lacks a functional apical domain, and the activation of YAP/TAZ relies on the activation/signal from the integrin adhesion pathways of the stem cells on the basal layer of the epithelium (Elbediwy and Thompson, 2018).

The **RAS/RAF/ERK** signaling cascade is one of the most conserved pathways in all organisms, including *Drosophila*. This pathway is part of the MAP kinase signaling that, in addition to ERK1/2, also includes JNK1/2/3, p38/MAPK and ERK5, which mainly respond to stress activators (Morrison, 2012). Highly conserved in flies, ERK1/2 are activated by growth factors such as EGF or FGFs. These ligands bind to receptor tyrosine kinases (RTKs) to activate downstream signaling, in particular its core complex, which is represented by the guanidine exchange factor Son of Sevenless (SOS) that, in turn, activates the small G proteins RAS on the cell membrane. This leads to RAF activation and to the formation of the complex with the kinase D-Sor also called MAPKK or MEK that, upon phosphorylation of Rolled, the fly homolog of MAPK or ERK kinases, induces the activation of its final targets (Shilo, 2014). ERK in flies has much fewer targets than those described in vertebrates, the most common being the ETS-domain protein Pointed (Pnt). In particular PntP2, needs to be phosphorylated for its activation and is the principal activator of transcription downstream of many RTKs, and PntP1 is transcriptionally induced by MAPK (Shilo, 2014). A second transcriptional repressor is Capicua (Cic), an HMG box-containing protein highly conserved in vertebrates (Simon-Carrasco et al., 2018). Interestingly, in the last couple of years, this protein was found to possess oncogenic properties and be overexpressed in many tumors (Simon-Carrasco et al., 2018). In addition, Cic activity regulates co-target genes upon Yki activation, placing this protein at the crossroads of RTKs and SWH pathways (Simon-Carrasco et al., 2018).
Even though MAPK targets in *Drosophila* are less abundant than in mammals, its activation and translocation to the nucleus results in a growth phenotype mimicking a few characteristic steps of growth in tumor cells (Brumby et al., 2011). Activation of Ras is considered a cancer distinctive trait both in *Drosophila* and humans, and it represents one of the strategies to model human cancer in flies. In *Drosophila* there are three *Ras* genes but only *Ras1* has functional homology with mammalian *RAS*. In the epithelial cells of the wing imaginal disc, Ras1 activation triggers

hyperproliferation but also determines cell fate (Prober and Edgar, 2000). Ras activation is at the crossroads of other growth factor signaling cascades: recently, a link to Hpo function was shown in *Drosophila* epithelial cells, where Ras activation was able to induce the tissues to switch from a pro-differentiative to a pro-growth program by modulating SWH's transcriptional output (Pascual et al., 2017). Ras increases cell proliferation also through the transcriptional regulation of growth factors and their receptors. For example, it helps promote angiogenesis-like mechanisms in tracheal development through secretion of the FGF/EGFR molecules (Grifoni et al., 2015; Petit et al., 2002); its activation stabilizes pro-growth signals including MYC (Prober and Edgar, 2000), and inhibits pro-apoptotic molecules like Hid (Bergmann et al., 1998). Because of all these functional homologies to human RAS, its activation in *Drosophila* is considered a good method to establish models that mimic tumor growth.

The **JNK Signaling Pathway** is activated mainly by oxidative stress, producing reactive oxygen species (ROS), and by Eiger, the *Drosophila* homolog of TNF-□. Its function is variable and depends also on the cellular environment: it can indeed induce cell proliferation and migration, but its major role is to induce apoptosis (Igaki, 2009). The signaling core is characterized by Hemipterus/Hep (JNKK) (Glise et al., 1995), Basket/Bsk (JNK) (Stronach, 2005) and the AP-1 complex, that functions as negative feedback by up-regulating the expression of the Puckered phosphatase (Martin-Blanco et al., 1998). The AP-1 complex is composed of Fra (Fos-Related Antigen) and dJun (*Drosophila* Jun) and is the final effector of the cascade (Kockel et al., 2001). Upstream Hep is phosphorylated by many JNKK kinases (Tak1-12, Mekk1, Ask1, Slpr) and can also be activated by different indirect stimuli (e.g. RAS, JNKKKK/Msn, and Eiger). Cell death is induced by the expression of the pro-apoptotic genes *hid*, *reaper* and *grim*, whose activity inhibits the pro-survival protein dIAP1 (Weston and Davis, 2007). In *Drosophila* cancer cells, the JNK pathway plays a dual role, by suppressing or promoting growth depending on the context (Brumby and Richardson, 2003; Cordero et al., 2010; Uhlirova et al., 2005). *lgl*, *scrib* and *dlg* mutant cells undergo JNK-mediated apoptosis resulting in a mechanism of tumor suppression (Brumby and Richardson, 2003; Igaki et al., 2006; Uhlirova et al., 2005I). On the contrary, in tumor cells with active RAS, apoptosis is blocked and JNK signaling acts as a tumor promoter transcribing genes involved in growth and invasion such as MMP1 (Igaki et al.,

2006; Uhlirova and Bohmann, 2006). The overexpression of activated RAS together with Hep (*ras*^{v12}*hep*^{wt}) gives cells invasive and metastatic abilities, highlighting how these pathways converge to induce transformation in epithelia.

The **PI3K/Target of rapamycin (TOR)** signaling pathway is a highly conserved key regulator of growth.. The binding of insulin-like peptides (ILPs) (fly's insulin) to the receptor (InR) results in the phosphorylation of *chico*/IRS1-4, and the production of phosphatidylinositol-3, 4,5-triphosphate (PIP3) by PI3K, a reaction that is counteracted by the lipid phosphatase PTEN (Grewal, 2009). PIP3 recruits several Ser/Thr kinases to the plasma membrane, including Akt/PKB and PDK1 (3'-phosphoinosite-dependent protein kinase-1), while its activation results in the inhibition of Glycogen Synthase Kinase-beta (GSK3-□), a conserved kinase that not only controls energy metabolism by inactivation of Glycogen Synthase, but also regulates Wnt signaling by controlling □-catenin/*armadillo* (Xu et al., 2009) and Myc stability (Bellosta and Gallant, 2010). Activation of Akt also inhibits Tuberous Sclerosis Complex 1 and 2 (TSC1/2), a tumor suppressor binary complex that negatively regulates Rheb, a GTPase upstream of TOR kinase responsible for the activation of TORC1. TOR is found in two complexes: TORC1, which includes Raptor and LST8 adaptor molecules, is sensitive to amino acids and is inhibited by rapamycin; and TORC2, that is composed of LST8 and Rictor adaptor molecules, and does not respond to amino acids or rapamycin (Saxton and Sabatini, 2017). Activation of TORC1 results in phosphorylation of ribosomal protein kinase p-70-S6 (S6K) and of eukaryotic translation initiation factor 4E-binding protein 1(4E-BP1), thereby triggering protein synthesis and initiation of translation. Insulin and TOR activities are also balanced by a negative feedback mechanism that is activated when S6K is hyper-activated to counteract insulin activity. Under this condition, S6K phosphorylates IRS1-4/*chico* triggering its internalization and subsequent proteasomal degradation. This feedback mechanism is reduced in pathological conditions, such as the Tuberous Sclerosis Complex syndrome (TSC), where cells carrying *tsc1* or *tsc2* mutations display an abnormal increase in size and exhibit constitutive phosphorylation of S6K (Saxton and Sabatini, 2017). As members of PI3Ks and TOR signaling are frequently activated in human tumors, they are attractive targets for cancer treatment.

3.1.3 Myc and Cell Competition.

MYC is one of the most studied oncogenes, and its misexpression is associated with various tumor types including meningioma, Burkitt's lymphoma, medulloblastoma and hepatocellular carcinoma (Hsieh and Dang, 2016). *Drosophila* Myc is the sole fly member of the family of transcription factors that in mammals is composed of three genes (N-, L-, and *c-MYC*) (Gallant et al., 1996; Schreiber-Agus et al., 1997). Hypomorphic alleles of *myc* in flies are developmentally delayed and show a reduction in cell size resulting in smaller flies (hence the name of the mutant as *diminutive*= small) (Johnston et al., 1999), while null mutants die during larval stage (Pierce et al., 2004). Notably, ubiquitous expression of *myc* increases cell mass resulting in enrichment of genes encoding components of the nucleolus and of the ribosome; this evidence, concomitantly with Myc's ability to indirectly stimulate RNA pol I and III (Grewal et al., 2005; Hulf et al., 2005; Orian et al., 2005), contribute to revealing its role in the control of ribosomal biogenesis, thus mass and size. Myc activity is finely regulated, and while its expression is required at physiological levels during development, an excess of its activity triggers autonomous cell death and unbalanced growth (Grifoni and Bellosta, 2015). Therefore, Myc is strictly controlled both transcriptionally and post-translationally, where its protein stability is controlled by phosphorylation events downstream of RAS/ERK and GSK3 kinases with a signaling conserved in flies and mammals (Galletti et al., 2009; Parisi et al., 2011). Myc regulation of the cellular metabolic milieu is highly similar in *Drosophila* to the regulation found in tumor cells (DeBerardinis et al., 2008), indeed it was shown that in cells undergoing to a metabolic stress (starvation or competitive environment), expression of Myc switched their metabolism to increase glycolysis, glutaminolysis (de la Cova et al., 2014; Hsieh et al., 2015; Parisi et al., 2013), or lipid metabolism to favor survival by inducing autophagy (Paiardi et al., 2017; Parisi et al., 2013). Fascinatingly, these evolutionary functions of Myc to control mass and metabolism, resulted in the selective advantage of growth of epithelial cells described as cell competition and characterized in the monolayer epithelia composing *Drosophila's* imaginal discs Johnston, 2014). Briefly, cells expressing Myc create a competitive environment and they grow at the expense of *wild-type* cells that are killed by non cell-autonomous apoptosis (de la Cova et al., 2004; Moreno and Basler, 2004). Myc cells thus behave as "winners" and they are able to repopulate the space of the dying "loser" cells that are killed by unidentified Myc-dependent mechanisms (Johnston, 2014). Myc-

induced cell competition was also shown to be necessary in vertebrates to eliminate unfit cells (losers) during early embryogenesis (Claveria and Torres, 2016). More recently, evidence that sustains a central role for Myc-induced cell competition in the early steps of tumor formation have shown Myc present at high levels in cells surrounding the tumor near dying cells, potentially allowing the winner cells to expand and to eliminate the surrounding *wild-type* cells, thus establishing the first evidence of Myc involved in a tumor growth competitive environment (Di Giacomo et al., 2017; Johnston, 2014). Another form of cell competition was shown to be regulated by cell polarity genes *(lgl, scrib, dlg)* and by endocytic genes (such as *Rab5*). Cells mutant for these genes behave as losers and were eliminated by *wild-type* cells (Brumby and Richardson, 2003; Menendez et al., 2010); notably the expression of oncogenes in those loser clones provided them with super-competitive characteristics, i.e. *lgl* mutant cells over-expressing MYC sent death signals to the adjacent *wild-type* proliferating cells (Froldi et al., 2010), suggesting the presence of another mechanism of cell competition driven by different growth forces working in combination with cell polarity genes and oncogenic signals.

4.0 Organotypic *Drosophila* cancer models

4.1 Gut Cancer

Similar to mammalian counterparts, the *Drosophila* adult gut is specialized in the digestion of food, the absorption of nutrients, and for controlling the defense response against infection (Tian et al., 2018). Based on these distinct functions, the *Drosophila* gut is composed of three parts: foregut, midgut, and hindgut. Among them, the midgut has a distinct architecture that resembles the digestive tract of vertebrates. The epithelium is a monolayer that is replenished by Intestinal Stem Cells (ISCs) that differentiate to either enteroblasts (EB) or pre-enteroendocrine cells (pre-EE), that then differentiate into absorptive enterocytes (EC) or secretory enteroendocrine cells (EE). Thanks to significant similarities in the physiology between the *Drosophila* gut and the intestine of vertebrates (Apidianakis and Rahme, 2011), *Drosophila* adult midgut epithelium has been used to study the contribution of signaling pathways (i.e. EGFR, Notch, Hedgehog, and Wg/Wnt) to Intestinal Stem Cells (ISCs) renewal (Biteau and Jasper, 2011; Jiang and Edgar, 2009; Jiang et al., 2011).

In vertebrates, the majority of sporadic cases of colorectal cancer and familial adenomatous polyposis (FAP) cancer syndrome are associated with activation of Wnt signaling (Bienz and Clevers, 2000). In humans, abnormal expression of Wnt in ISCs promotes adenoma formation, while deletions in mouse ISCs of the tumor suppressor *adenomatous polyposis coli* gene *APC* triggers the initial step of colon-adenoma formation (Barker et al., 2009), underlying the relevance of both mutations in this malignancy. In *Drosophila,* loss of the *Apc* gene, leads to the over proliferation of ISCs in the gut, resulting in loss of epithelial cell polarity, hyperplasia and epithelial overgrowth resembling that of intestinal adenomas induced by the loss of *APC* (Yu et al., 1999). Remarkably, the over-proliferation of the *Apc* $^{-/-}$ cells was rescued by *lof* mutation of *Ras* (Wang et al., 2013). On the contrary *Apc*$^{-/-}$ cells expressing an active form of *Ras*^{v12} showed a malignant transformation including loss of cell polarity and invasive phenotype, highlighting the conserved functional cooperation between RAS and APC in controlling proper growth in the gut. In *Drosophila*, intestinal progenitors mutant for the *Apc* gene expand at the expense of the surrounding *wild-type* cells that die by apoptosis; because of this behavior these cells have been defined as "super-competitors" (Suijkerbuijk et al., 2016). *Apc* mutant cells exhibit higher Yki/YAP activity and increased JNK signaling, that was also detected at the border between *Apc*$^{-/-}$ and *wild-type* cell;. moreover, inhibition of apoptosis prevented *Apc* mutant cells from further expansion, suggesting that a competitive behavior in these cells is controlling *Apc* dependent tumor growth (Suijkerbuijk et al., 2016).

The JNK-Wg signaling is important to control the physiology and regeneration of intestinal cells, as ISCs damage leads to an overactivation of the JNK pathway and an increase in Wg ligand (Biteau et al., 2008; Cordero et al., 2012b).Wg activity in the enterocytes (ECs) indirectly drives the expansion of the ISCs by upregulating the JAK-STAT ligands Upd2 and Upd3, acting non-autonomously on ISCs proliferation (Tian et al., 2018). Moreover, activation of Wnt drives Myc upregulation in ISCs leading to non-autonomous upregulation of Upd3 in the ECs (Cordero et al., 2012a). Similarly, loss of *Apc1* in the midgut (ISCs) also results in JAK-STAT and EGFR pathway hyper-activation, and their removal suppresses the intestinal hyperplasia resulting from *Apc1* loss, revealing an underlying conserved signaling between flies and mammals that controls ISCs proliferation and gut homeostasis (Cordero et al., 2012a).

Another aggressive oncogene that is hyper-activated upon *Apc* loss, in mouse and human intestinal adenomas is the non-receptor tyrosine kinase *c-Src* (Yeatman, 2004). This proto-oncogene is amplified or activated in more than 20% of human tumors, and its activity has been demonstrated to play a central role in the formation of colorectal cancer (CRC). In mice, expression of *c-Src* increases in the proliferative progenitor cells of the "cripta" favoring hyperplastic adenoma formation (Cordero et al., 2014). In *Drosophila* the expression of c-Src orthologs (*Src42A* and *Src62B*) induces proliferation of the ISCs cells in *wild-type* animals, and reduction of their expression is sufficient to inhibit ISCs' hyper-proliferation of *Apc* mutant cells (Cordero et al., 2014). Notably, these results recapitulate an important part of the function of mammalian c-Src in the progenitor cells of the intestine during homeostasis and adenoma formation, suggesting a conserved role of this gene in flies in controlling proper ISCs proliferation.

Recently, *Drosophila* was also used to generate multigenic models of colon cancer using data from patients from The Cancer Genome Atlas. Interestingly, the outcomes of these models mimicked important properties of human cancers, and can be explored and used in chemical screens to find new combinations of cancer-relevant drugs (Bangi et al., 2016). Studies, using *Drosophila* models, to characterize intestinal human pathophysiology, revealed the high conservation between these species of the mechanisms underlaying colorectal tumorigenesis (Christofi and Apidianakis, 2013), and further revealed also the mechanisms that control the processes leading to bacterial-mediated inflammation (Lemaitre and Hoffmann, 2007).

4.2 Brain cancer

Meningioma are the most common intracranial tumors (Claus et al., 2005; Rogers et al., 2015) and frequently linked with mutations in the PI3K catalytic subunit p110α isoform encoded by the gene (*PI3KCA*), or in the *v-akt murine thymoma viral oncogene homolog 1* (*AKT1*) gene. Complex interactions were found between members of the PI3K/AKT/mTOR pathway and MAPK-, JAK/STAT and Notch-1-mediated pathways that contribute to meningioma progression (El-Habr et al., 2014). Increased risk of meningiomas was associated also with neurofibromatosis type II syndrome, where mutations within the tumor suppressor gene *Suppressor of fused*

(*SUFU)* was associated with hereditary meningiomas (Aavikko et al., 2012) and with medulloblastomas (Taylor et al., 2002). In *Drosophila* SUFU regulates Hedgehog (Hh) signaling (Ohlmeyer and Kalderon, 1998), with a similar function in humans, where loss of *SUFU* results in the aberrant activation of the Hedgehog (Hh) pathway (Aavikko et al., 2012).

Of all glioblastomas, the glioblastoma multiforme (GBM) is the most aggressive form of gliomas, accounting for approximately 50% of all glial tumors (Phillips et al., 2006). In GBM, Notch activity is associated with the control of Glioma Stem Cell (GSC), since its activity regulates asymmetric cell division and Notch unbalanced expression leads to uncontrolled growth and high malignancy (Mukherjee et al., 2016), Notch is an important target for therapeutic intervention in brain cancer treatment (Yuan et al., 2015). Several studies in flies demonstrate also un important role for Notch signal in controlling growth and stem cell maintenance in the brain (Song and Lu, 2011).

Moreover, the current understanding of asymmetric cell division and its relation to tumorigenesis is largely derived from studies on *Drosophila* neuroblasts (NBs), where mutation of a single gene, *brain tumor (brat),* was shown to alter asymmetric stem cell division in larval development, and to generate massive neoplastic growth and enlarged adult brain formed entirely of neoplastic NBs (Betschinger et al., 2006; Caussinus and Gonzalez, 2005). Suppression of *brat* expression was used to establish a model of glioma stemness in *Drosophila*, where the upregulation of Notch, induced by reducing *brat*, was the critical node to maintain self-renewal and proper stemness (Mukherjee et al., 2016). This observation was also confirmed in glioblastomas where the human orthologue of *brat*, the tripartite motif-containing protein-3 (TRIM3), was shown to be necessary to suppress NOTCH1 signaling and to control stem cell activity during development to reduce tumor growth (Chen et al., 2014; Mukherjee et al., 2016). Glioma stem cells divide asymmetrically under the guidance of cell polarity complexes that control the proper apical and basolateral polarization and cell division, a process that was originally identified in *Drosophila* and later confirmed for the mechanism driving differentiation in human glia for members of the *Hugl-1/Llgl-1* complexes (Prehoda, 2009). We recently developed a neurogenic brain tumor model by impairing asymmetric cell division through the loss of function of *lethal giant larvae* (*lgl*) the *Drosophila* orthologue of *Hugl-1*, in the type II NBs of the central brain (Paglia et al., 2017). In our model, PI3K activation mimics PTEN loss of function and hampers Lgl localization at the apical

membrane by aPKC cortical recruitment (Paglia et al., 2017). These data connect the function of *HUGL-1* in the maintenance of glioma stem cells with the loss of function of the tumor suppressor *PTEN* (Gont et al., 2013) and together with those in glioma (Read et al., 2009) show a conserved function for PI3K and EGFR overexpression in these tumors recapitulating many features of the neurogenic subtype of human glioblastoma. Inhibition of PI3K/Akt activity is currently used as a therapy in GBM (Zhao et al., 2017).
Other brain tumors such as oligodendrogliomas, that account for 10% of all cancers of the central nervous system, are characterized by mutations in the *capicua* (*cic)* gene (Bettegowda et al., 2011), a conserved transcriptional repressor that regulates MAPK effector genes downstream of receptor tyrosine kinase (RTK) (Simon-Carrasco et al., 2018). The development of correct animal model also for these tumors will be essential to develop specific treatments that can tackle these different brain tumors *in vivo*.

4.3 The paradigm for angiogenesis

In the fruit fly, the circulatory system is open, the heart pumps the hemolymph into the body cavities and the exchange of gases takes place directly within the organs (Medioni et al., 2009). Moreover, *Drosophila* is equipped with a complex branched system of interconnected tubules that is responsible for the oxygen transport, the tracheal system, an organ that is comparable in structure and function to the circulatory system of mammals (Affolter et al., 2009). In *Drosophila*'s epithelia, the induction of clones bearing *lgl*, Ras^{V12} mutations identified how tumors are able to recruit vessels to oxygenate the growing mass (Calleja et al., 2016; Grifoni et al., 2015). These tumor cells showed ectopic expression of *Bnl* (*branchless*), the *Drosophila* homolog of Fibroblast Growth Factors (FGFs,), and suffered from oxygen shortage (hypoxia). In addition, it was observed a trans-differentiation of tumor cells into pseudo-tracheal cells with and the formation of new vessels, mimicking human FGF-mediated vascularization in cancer (Grifoni et al., 2015).
Cell under hypoxia condition changes their cellular metabolism to favor growth, particularly in solid tumors (Pavlova and Thompson, 2016; Vander Heiden and DeBerardinis, 2017). Interesting studies in flies showed how reduction of the SCF (Skp/Cullin/F-box)-type ubiquitin ligase *Ago*, homolog of human *Fbw7,* increased tracheogenesis through up-regulation of the hypoxia-inducible transcription factor

Sima/dHIF and of its target, the FGF ligand Bnl (Mortimer and Moberg, 2013). Fbw7 is known to inhibit tumor growth by targeting proteins to the proteasome pathways, and is mutated in a wide range of primary human cancers, thus data suggests that its role as a tumor suppressor may be conserved also in the modulation of HIF-regulated angiogenesis in the tracheal system of the fly (Mortimer and Moberg, 2013). This process of neo-tracheogenesis is now considered a novel cancer hallmark in fly, which may help to explore the relation between angiogenesis and tumor growth in humans (Herranz et al., 2016) (Figure 3).

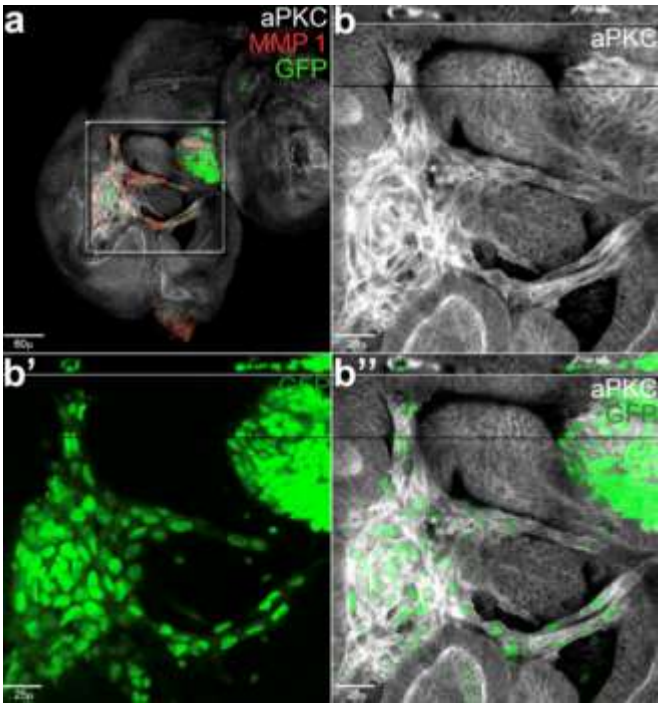

Figure 3: Cancer cells form branched and tubule-shaped structures (reproduced from (Grifoni et al., 2015) with permission). (a) An imaginal wing disc bearing *lgl*^{4}, Ras^{V12} clones induced in a wild-type background. (b-b") Magnifications of the central region squared in (a). Migrating tumor cells (GFP) are positive for the junctional marker aPKC (white) and secrete MMP1 (red). The reconstruction along the z-axis shown in the upper part of the magnified images reveals a tubule-shaped structure encircling a lumen, indicating these cells are forming tracheal-like structures.

4.4 Lung cancer

Lung cancer is a major cause of death in the world, and the standard therapeutic strategy used is chemotherapy because target therapies only decrease tumor growth and result in high toxicity. Recently, a new *Drosophila* lung cancer model was

developed exploiting the tubular structure of the tracheal network (Levine and Cagan, 2016), and considered functionally and anatomically comparable to the vertebrate airways (Andrew and Ewald, 2010). Both in *Drosophila* and mammals, airways is formed by interconnected branches that depends on the secretion of Bnl/FGFs by the neighboring cells (Ghabrial et al., 2003; Grifoni et al., 2015). Using a binary system, Ras^{V12} was ectopically expressed specifically in the tracheal cells while downregulating *PTEN*, a negative regulator of the PI3K/AKT signaling (Hafen, 2004; Ortega-Molina and Serrano, 2013). As a result, the cells of the tracheal branches over-proliferated to form tumors that ultimately killed the animals (Levine and Cagan, 2016). This model was successfully used in a screen for chemical compounds approved by the Food and Drug Administration (FDA), which resulted in the identification of several compounds able to reduce cell over-proliferation and to improve tracheal physiological functions (Levine and Cagan, 2016), further highlighting the strong potential of the use of fruit fly models for cancer-related chemical screens

4.5 Prostate and thyroid cancer

The prostate is an exocrine gland of the male reproductive system responsible for the maturation and production of the seminal fluid, with its activity depending on androgens mostly produced by the testis. During organogenesis, the differentiation of the prostate's epithelium occurs along with that of stroma and depends on the complex coordination of many transcription factors and hormones that control the maturation of the quiescent organ (Toivanen and Shen, 2017). The adult prostate epithelium has a low turnover rate and its hyperplasia characterizes the majority of benign prostatic tumors. On the contrary, adenocarcinoma of the prostate is an aggressive tumor that rapidly progresses to a metastatic stage that can be partially blocked by androgen therapy (Shiao et al., 2016). Studies on flies' male accessory gland revealed many parallels with the physiology of human prostate epithelium (Wilson et al., 2017), i.e. a genetic screen using the *Drosophila* accessory gland identified genes that promote growth and migration of the secondary cells as homologs of genes expressed in human prostate cancer (Ito et al., 2014).
Like in human prostate, *Drosophila's* accessory gland presents a secondary layer of epithelial cells that continue to proliferate; this homology allowed the development of models that mimic tumors of endocrine origin, including human prostate and

thyroid adenomas (Das and Cagan, 2013, 2018). For example, the multiple endocrine neoplasia type 2 (MEN2) syndrome, is characterized by different mutant-translocations involving the RET genes that result in multiple cancer phenotypes, including pheochromocytoma, parathyroid adenoma and the aggressive medullary thyroid carcinoma (MTC) (Das and Cagan, 2013). A recent study demonstrated that the papillary carcinoma of the thyroid (PTC), also caused by another genomic mutations of RET gene, can be profitably studied using the accessory gland of *Drosophila* to delineate and understand the mechanisms that characterize PTC in the context of the whole animal, including the relationship between tumor and normal cells in an environment that mimics tumor of endocrine origin in humans (Levinson and Cagan, 2016).

The prostate epithelium is characterized by the abundance of exosomes, microvesicles secreted from the endosomal multivesicular body (MVB) that fuse with sperm to modulate its activity and protect its homeostasis (Wilson et al., 2017). The exosomes are particularly relevant in cancer biology for their implication in tumor progression and survival, since they deliver survival factors, metabolites and miRNAs, that help creating a favorable microenvironment for cancer growth; in addition they also favor drug-resistance by activating mechanisms that favor the elimination of toxic chemicals such as chemotherapeutic products (Namee and O'Driscoll, 2018; Ruivo et al., 2017). Since the accessory gland has a similar structure as the prostate epithelium, characterized by the abundance of exosomes, it could be an optimal model to better study exosome biology in tumors of endocrine origins

5.0 Liquid tumors

The signaling pathways regulating blood cell differentiation are conserved from *Drosophila* to humans (Jung et al., 2005; Lebestky et al., 2003). In addition, fly macrophages originate via self-renewal from progenitor cells localized in the lymph gland, a specialized hematopoietic organ that can be compared to the hematopoietic stem cell niche of the mammalian bone marrow (Krzemien et al., 2007; Mandal et al., 2007). These similarities with vertebrate hematopoiesis outline the utility of using fly models to elucidate the basic mechanisms of hematopoietic differentiation and homeostasis responsible for severe diseases, including leukemia. *Drosophila* has already been used to study Acute Myeloid Leukemia (AML), a widespread form of

leukemia, and to identify the genes responsible for the disease. AML1 is a transcription factor, responsible for activating myeloid differentiation, which has a counterpart in the fly (Sinenko et al., 2010). In vertebrate tumors, the fusion of AML1 with the repressor ETO inhibits the differentiation of the multilineage progenitor cells, while their proliferation is activated, leading to AML. Interestingly, AML1 fused with ETO in *Drosophila* also causes the inhibition of hematopoietic cell differentiation, confirming that the fly is a good genetic model to study the mechanisms that drive leukemia in humans (Osman et al., 2009; Sinenko et al., 2010). Myeloproliferative neoplasms (MPNs) have also been reproduced in the fly through gain-of-function mutations in the JAK pathway, finding a role for the downstream effector of the SWH pathway Yki in priming the expansion of *Drosophila* blood cells, which undergo malignant behavior following JAK activation (Anderson et al., 2017).

6.0 Cancer and immune system

Inflammation in tumor development acts as "tug and war" since it may promote survival of tumor cells by favoring angiogenesis, by reducing the natural immune responses and by altering responses to chemotherapeutic agents (Mantovani et al., 2008; Wu and Zhou, 2009). The inflammatory response of cancer cells has been attributed to a response of the immune system to eradicate the tumor, but it can also be seen as a way to provide growth and survival, as inflammation contributes to genomic instability by releasing cytokines and through production of reactive oxygen species (ROS) that may induce genetic and genomic alterations (Negrini et al., 2010). Normal cells detect and repair DNA damage, ensuring the maintenance of the correct number of chromosomes and tissue homeostasis, instead often cancer cells have increased mutation-rates leading to high chromosomal instability (CIN) that triggers aneuploidy and advances tumorigenesis (Negrini et al., 2010). Chromosomal instability is a process conserved also in *Drosophila,* and it was shown to contribute to the invasive behavior of epithelial cells, with a mechanism called “compensatory proliferation” activated to counteract CIN-induced cell death (Benhra et al., 2018; Clemente-Ruiz et al., 2016).

The mechanisms controlling cancer immune response are somehow conserved also in flies as studies in *Drosophila* have shown that infiltration of macrophages (called hemocytes) in cancer cells requires the activation of the JAK-STAT, JNK, TNF-α, and Toll/Imd/TLR signaling pathways (Bangi, 2013). Of particular interest is TNF-

α that plays an important role in controlling apoptosis and the inflammation processes (Ham et al., 2016). TNF-α in tumors has distinct and overlapping functions to promote tumor growth and proliferation and to activate cell death, functions that are mainly mediated by the activation of TNFR1 that is ubiquitously expressed while TNFR2, mainly expressed on immune cells, is less well understood. Thus these opposite signaling pathways activated by TNF signals depend on the adaptor complexes recruited by the receptors and by the cellular context, and they may create a problem for the development of therapeutic strategies that target TNF signaling in tumors (Ham et al., 2016). In *Drosophila* the sole TNF- α , called Eiger (Egr), binds two receptors called Wengen (Kanda et al., 2002) and Grindelwald (Andersen et al., 2015), the latter shown necessary for the growth of $Ras^{V12}/scribble^{-/-}$ tumors (Andersen et al., 2015). An interesting mechanism links the possibility that ROS, induced by stress or local inflammation, triggers Egr expression in the hemocytes, to control JNK signaling, in a phenomenon called Apoptosis-Induced Proliferation (AIP), a sort of compensatory proliferative response of the epithelial cells that responds to cues from local "activated" hemocytes (Fogarty et al., 2016). Other studies highlighted the role of hemocytes in the interplay between inflammation and cancer, i.e. using a classic cancer model that recapitulates the hallmarks of epithelial cancer cells ($Ras^{v12}/scribble^{-/-}$), it was shown that cancer cells induce hemocyte's recruitment and proliferation *in vivo* by activating JNK signaling to cause the expression of JAK/STAT cytokines (Pastor-Pareja et al., 2008). Using a similar model it was shown that Egr expression was higher in the hemocytes derived from cancer animals, and that its activity was necessary to stimulate invasive migration of tumor cells (Cordero et al., 2010). On the contrary, Egr acts as a tumor suppressor to drive apoptosis in cancer cells upon activation of Toll/NF-κB signaling by the fat body (adipocytes) in response to the secretion of Egr by the circulating "activated" hemocytes (Parisi et al., 2014). Work using allograft transplantation experiments, identify also a function for the hemocytes in tumor initiation, that is independent on Eiger, but relays rather on the activation by external stimuli (i.e. CIN, abnormal growth) of JNK pathway and on the complex of non-autonomous and autonomous signals between tumor cells and those composing the tumor microenvironment; a similar mechanism has been proposed in vertebrates suggesting a conserved response for JNK signaling in fly to control initial tumor growth (Muzzopappa et al., 2017). .

In summary, all these data suggest the existence of conserved mechanisms between the immune and tumor cells in flies that may recapitulate some of the most evolutionary conserved aspects described in cancer cells.

7.0 Cancer and lipid metabolism, obesity

In tumor biology, evidences highlight the relevance of lipid metabolism in influencing tumor growth (Katheder and Rusten, 2017; Weber et al., 2017). In this context, a recent role was identified for adipose triglyceride lipase (ATGL) whereby it hydrolyzes triacylglycerols into fatty acids (FAs) that may act as signaling molecules to induce growth both cell autonomously and in neighboring cells (Walther and Farese, 2012). The contribution of ATGL to cancer growth is controversial, indeed several studies showed that its depletion reduced proliferation in colorectal cancer cells and in non-small-cell lung carcinoma (Ou et al., 2014; Zagani et al., 2015), and in breast and pancreatic carcinoma its upregulation contributed to tumorigenesis (Grace SA et al., 2017; Wang et al., 2017). On the contrary, lack of *ATGL* favored pulmonary neoplasia in mice and in few human tumors *ATGL* expression was found reduced highlighting the complex role of lipids in tumorigenesis (Al-Zoughbi Wael et al., 2016). Cancer cells activate *de novo* lipogenesis by upregulation of key enzymes in lipid metabolism, some of which, such as AcetylCo-A Lyase (ACLY), AcetylCo-A Carboxylase (ACC) and Stearoyl-CoA desaturase-1 (SCD), are targets of pharmacological inhibitors to decrease cancer proliferation (Peck and Schulze, 2016; Stoiber et al., 2018; Zaidi et al., 2012; Zu et al., 2013). Recent work associated the mechanism of lypolysis with the induction of autophagy, a mechanism used by the cells to re-cycle part of their cytoplasm or cellular content to survive when nutrients are reduced (Dall'Armi et al., 2013). The relevance in cancer of the link between lipids and autophagy was shown when ATGL-mediated lipolysis in a peritumoral area, increased autophagy and tumor survival using a non-autonomous mechanisms (Gnerlich et al., 2013; Martinez-Outschoorn et al., 2011). Interestingly, we observed that Myc in *Drosophila* induced autophagy in the fat body and this was enough to enhance survival of the whole animals upon starvation (Parisi et al., 2013). We linked this effect with the ability of Myc to increase desat1, a Stearoyl-CoA desaturase-1

(SCD1) key enzyme in the synthesis of lipids, that we found co-expressed with Myc in human prostatic tumors (Paiardi et al., 2017).

Metabolic disorders and obesity are associated with cardiovascular disease and type II diabetes (T2D), however numerous cohort studies reported that overweight people are more likely to develop certain types of cancer including endometrial, breast, liver, and ovarian cancer (Cancer, 2012; Chen et al., 2012; Dougan et al., 2015; Hirabayashi, 2016; Riboli, 2014; Wang and Xu, 2014). Obese people have often increased levels of circulating hormones like insulin that has been associated to higher levels of IGF-1 in colon, kidney, prostate and endometrial cancer (Gallagher and LeRoith, 2015; Roberts et al., 2010). Another hormone, leptin, a cytokine produced by the adipocytes to control satiety in a signaling circuit of the brain, has also been found up-regulated in tissues from obese people, particularly in women post-menopause, and increased levels of leptin have been associated with higher incidence of breast and other tumors (Ray, 2018). The adipose tissue produces pro-inflammatory cytokines including IL-6, IL-8, IFNγ and TNF-α among others (Arango Duque and Descoteaux, 2014; Scheller et al., 2011), and their over-production in fats from obese, activates the infiltration of macrophages into the adipose tissue inducing a low level of chronic inflammation or adipocyte tissue macrophage infiltration called ATM (Kuroda and Sakaue, 2017; Lafontan, 2014). This low level of inflammation increases the levels of ROS and induces DNA and protein damage that may increase the risk of cancer (Lafontan, 2014; Mraz and Haluzik, 2014). The role of the inflammatory response to combact infection and tissue injury, through the activation of the immune cells, is conserved also in *Drosophila's* circulating hemocytes (Lemaitre and Hoffmann, 2007), where most of the signals activated in the fat body results also in ROS production (Dionne, 2014; Vlisidou and Wood, 2015). Indeed, we showed, using a genetic model that harbors an inflammation state in the fat body of larvae that mimic ATM, that reduction of ROS, using exogenous anti-oxidants components like flavonoids and anthiocianins, decreased hemocyte's migration and JNK activation in the cells of fat body (Valenza et al., 2018), suggesting that the converging signaling between the fat body and hemocytes on lipid metabolism and ROS/cytokines in response to stress is conserved also in *Drosophila*.

8.0 Cancer stem cells

Cancer stem cells (CSCs) have more features than tissue stem cells because they are able to initiate the tumor growth and fuel its maintenance and metastasis (Kreso and Dick, 2014; Malanchi et al., 2011). In addition, CSCs are highly resistant to conventional therapy, both radiation and chemotherapy, and they are responsible for the recurrence of disease (Mueller et al., 2009). Since the mechanisms underlying the ability of stem cells to support cancer progression are still unclear, *Drosophila* is convenient to use as it provides many tools for genetic and molecular investigations. Adult stem cells are required for tissue homeostasis and repair after injury and in adult flies, populations of stem cells are present in the posterior midgut, testis, and ovarian follicle rendering it again a good system to dissect these stem cell programs (Hou and Singh, 2017). *Drosophila* was used to better understand the functions of the centrosome and microtubule-organizing center (MTOC) in the division of stem cells (Tillery et al., 2018). *Drosophila* and mammalian stem cells are similar and they are regulated by homologous signals corroborating the use of the fly in this field of tumor biology. CSCs can arise from normal stem cells whose long lifespan favors the accumulation of genetic mutations responsible for the malignant phenotype. The progression from normal progenitors to stem-like cancer cells was first explored in leukemia, although nowadays we know that several solid tumors such as brain, breast, lung and colon cancer originate from cells with stem features (Krivtsov et al., 2006). Several *Drosophila* models of stem cell tumors are now available, and a drug screening was successfully carried out highlighting several compounds active on the signaling promoting cancer growth (Markstein et al., 2014).

9.0 *Drosophila* cancer models for the identification of therapeutic drugs

Therapeutic drug discovery requires chemical screening, a procedure allowing for the identification of potential new drugs. The spread of sequencing, automation, and miniaturization has made High Throughput Screening (HTS) the leading contributor to early-stage drug discovery. HTS consists of random screening of chemicals to find an affinity for a specific protein or biological activity characteristic of a disorder. Once identified *in vitro*, the compounds need to be validated *in vivo* to assess efficacy and toxicity during a long and expensive period of drug development. The high throughput assays depend on the existence of a specific target, assuming an in depth understanding of a disease that is not always available. Phenotype

screening is an eligible option when the knowledge about the mechanisms underlying a disease process is not well defined. It is a process by which small molecules are screened for their effect on the phenotype in cells, tissue or whole animals, where a more physiological environment better describes the pharmacokinetics and toxicological effects of a drug. The great availability of genetic tools and the low cost of maintenance makes the fruit fly an ideal to model to study human diseases including cancer, in fact the fly has considerably contributed to understand tumor biology.

Chemical screens have been successfully performed in *Drosophila* for several disorders affecting the central nervous system, kidney and metabolism (Gasque et al., 2013; Hofherr et al., 2016; Whitworth et al., 2006), as well as for a type of thyroid cancer, the multiple endocrine neoplasia type 2A and 2B (MEN2) (Vidal et al., 2005). Regarding cancer, JAK- STAT, APC, Wnt, Notch and other signaling molecules, deeply characterized in *Drosophila* and shared with humans, are precious for cancer drug development. The availability of *Drosophila* models for multiple cancer types makes pharmacological screens possible against several drugs that aim to restrict proliferation and metastasis. The identification of anticancer compounds is possible using the adult fly, but also larvae, embryos and cells. The combined effect of anti-cancer drugs with radiation has been investigated in *Drosophila* larvae, producing similar findings to those observed in human cancer cells (Edwards et al., 2011). Moreover, *Drosophila* avatars, consisting of patient-specific tumors modeled in transgenic flies, are very promising for personalized medicine. *Drosophila* and other small model organisms are helpful to quickly analyze the mode of action of several active compounds *in vivo,* nevertheless mammalian models are indispensable in the successive phase of drug development to define important pharmacokinetic parameters such as absorption, distribution and metabolism.

10.0 Discussion

The communication between tumor cells and their microenvironment is largely implicated in neoplastic growth, hence the substantial difficulty to recapitulate the features of malignant transformation in cellular systems. Cancer research needs *in vivo* investigations, and the use of model organisms contributes to answer this

request. In this review we described most relevant approaches in *Drosophila,* used to explore cancer mechanisms and therapeutics that contribute to our understanding on tumor initiation and progression. In spite of some limitations, because of the anatomical differences between flies and humans, the use of *Drosophila*'s cancer models has been fundamental to understand some basic processes that regulate human cancers, such as the competitiveness of cancer stem cells (CSCs), the importance of tumor microenvironment, cancer cachexia, drug resistance and tumor-associated vasculogenesis, which was recently found to be functionally conserved in fly's cancer. Additional cancer hallmarks such as genomic instability, resistance to cell death, cell metabolism reprogramming, tumor-promoting inflammation and evasion from the immune system, have been studied and extensively characterized in *Drosophila*. Finally, although the evolutionary difference between *Drosophila* and humans certainly represents a restriction to the use of the fruit fly in drug discovery and development, phenotypic screenings have proven relevant to identify potential drugs that would elude the classic screens in the absence of targets. *Drosophila* is also offering a significant contribution to the investigation of organotypic cancers, since despite the evident differences at the macroscopic level, organ cells and functional units are usually well conserved at the biochemical and structural levels respectively. This conservation allowed to develop thyroid, lung, prostate, gut, brain and blood cancer models starting from the most characteristic genetic lesions found in the same human cancers. These models, as described in the review, are greatly helping in dissecting the contribution of specific molecular pathways to the final cancer phenotype. Given the heterogeneous nature of mammalian solid cancers, new strategies are being developed to decipher cancers at single-cell resolution. The international *Drosophila* community has always been engaged in the development of novel, sophisticated genetic tools, which allowed in the last 30 years to revolutionate functional gene analysis. For this reason, we anticipate that the use of the fruit fly will move fast into the field of precision medicine, contributing to seminal findings in this new era of cancer research.

11.0 Acknowledgments

We apologize to colleagues whose work was not cited in this manuscript due to space limitations. We thank all members of Bellosta's and Grifoni's laboratories and Sheri Zola for their comments on the review. Funding from Cariplo Foundation 2014703

and EHDN 689 for PB and AMV, AIRC-IG17252 for DG and MS.

12.0 Authors Contribution Statement

All the authors contributed to write the review manuscript.

13.0 Conflict of Interest

The authors declare no conflict of Interest.

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Chapter 5

Conclusions and future perspectives

During my PhD, I worked on an innovative project using *Drosophila* models to study human diseases.

The aim of this project was to characterize a new genetic model to study obesity with particular emphasis of the role of the hemocytes, the Drosophila's macrophages, and their migration into the fat body, Drosophila's adipose tissue, as a model mimicking chronic inflammation in obese people, and to analyze the effect of important compounds such Anthocyanins in the hemocytes infiltration in the obese Drosophila's larvae fat body.

The incidence of obesit drammatically grew in worldwide and it has become a global problem. Data reported by The World Health Organization (WHO) are alarming, in which the prevalence of childhood obesity increases with the risk to develop other pathologies (53, 145). The fruit fly, *Drosophila melanogaster*, is an excellent model for studying nutrient-sensor pathways and metabolic dysfuctions.
Recent studies performed on *Drosophila* obese models show that the molecular mechanisms that regulates the metabolic functions and processes are conserved between humans and flies (78).
Drosophila fat body is functionally analogous to the mammalian adipose tissue and liver, indeed the fly fat body contributes to the maintenance of energy storage in the form of lipids and glycogen, and it is involved in obesity-related disorders (146, 147). Furthermore, the *Drosophila* fat body is an organ that serves the roles of the immune system with recruitment of the hemocytes, equivalent to mammalian macrophages (107).

In this work, I demonstrated, for the first time, a strong evidence of the potential use of plant's derived Anthocyanins in the diet to control chronic inflammation and provide a link to the oxidative stress that characterizes the adipose tissue in obese animals. I was able to evidence the ability of Anthocyanins to decrease *in vivo* the phosphorylation of JNK/SAPK p46 stress kinase, thus providing a new insight into the mechanism of phenolic compounds in the treatment of inflammation in adipose tissues,

a field of current study since the lack of a better knowledge of the mechanisms that regulate or control ATM in pathologies such as obesity and metabolic disorders.
At the same time, Anthocyanins are able to activate antioxidant detoxifycation factors such as Nrf2. These finding suggest that Anthocyanins can regulate the inflammaroty responses, therefore can be considered as anti-inflammatory agents.
Furthermore, I evaluated the antioxidant power of Anthocyanins, in particular, I examined how the Anthocyanins extract modulates GST D1 mRNA expression level in our model.
Therefore, the mainly action mechanism of Anthocyanins in the cellular defence against inflammation-induced oxidative stress could be the regulation of the CncC/Keap-1 pathway, one possible way to increase the activity of GST D1, decrease JNK pathway activation and to fight the excess of ROS.
The present study confirms the great importance of Anthocyanins consumption and provides new insights into the mechanism through which these phenolic compounds regulate the specific signaling pathway involved in the different cellular processes.

In the future**,** we need to continue these experiments in order to better understand the mechanism that links Anthocyanins to antioxidant detoxifycation factors such as Nrf2 to discover new possible signals involved in inflammatory and oxidative process.

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Ph.D. Course in Environmental Sciences

PhD Student Final Report

PhD Student:	**Valenza Alice Maria**
PhD Course Cycle:	XXXI
Scientific Tutor:	Prof. Paola Bellosta, Dott. Maria Pasini
Thesis Project Title:	Anthocyanins rescue Macrophage infiltration in a *Drosophila* model of obesity
Project performed at:	University of Milan; Department of Bioscience.

List of Scientific Publications

Valenza A, Bonfanti C, Pasini ME, Bellosta P.
Anthocyanins Function as Anti-Inflammatory Agents in a Drosophila Model for Adipose Tissue Macrophage Infiltration.
Biomed Res Int. 2018 Mar 12;2018

Zhasmin Mirzoyan, Manuela Sollazzo, Mariateresa Allocca, **Alice Maria Valenza**, Daniela Grifoni and Paola Bellosta
***Drosophila melanogaster*: a model organism to study cancer**
Front. Genet.. 01 March 2019

List of attended Meetings and Congresses

AICH(Associazione Italiana Corea di Huntington) Hungtinton's day June, 1-10, 2016 Milan, Italy

Studi sulla disfunzionalità cardiaca e l'infiammazione indotta da obesità, usando *Drosophila* come modello sperimentale.
Mirzoyan Z[1], **Valenza A**[1], Pollard JB[2], Cassinelli M[2], Lupi V[2], Frattaroli M[2], Zola S[3], Kuo V[3], Pasini M[1], Arnaboldi L[1], Bellosta S[1], Bellosta P [1,3]
SISA (Societa Italiana per lo Studio dell'Arterioschlerosi) **POSTER Milano 16-17 Ottobre 2015**

***In vivo* effect of Flavonoids modulates ATM (adipocytes-macrophage infiltration) in *Drosophila*'s models of obesity.**
Valenza A., Mirzoyan Z., Bonfanti C., Pasini M.E., Bellosta P.
TALK IDRC ITALIAN DROSOPHILA RESEARCH CONFERENCE, BOLOGNA 14-16 Settebre 2016

Glutamine synthetase induces autophagy and neuronal survival in a Drosophila model of Huntington's Disease.
Luisa Vernizzi[1-2-3], Chiara Paiardi[1-2],Maria E. Pasini[1], **Alice Valenza[1],** Giusimaria Licata[1]Teresa Vitali[1-4], Maria A. Vanoni[1], Cinzia Gellera[5], Manuela Rizzetto[5], Franco Taroni[5] Caterina Mariotti[5], e Paola Bellosta[2]. [1]equally contributed; [2]Biosciences University of Milan; [3]UZH-Univesity of Zurich CH; [4]NIH, Bethesda [5]Neurological Institute C. Besta Milan. **POSTER European Huntington Disease Network (EHDN)-Plenary Meeting, September 16-18 The Hague, the Netherland**

Reducing ecdysone signaling affects fat metabolism an hemocytes mobility.
A. Valenza1, C. Bonfanti1, S. Zola2, Z. Mirzoyan1, M. PASINI1, P. Bellosta1-2
1Dept. of Biosciences, University of Milan, Italy 2Center for Integrated Biology-CiBio, University of Trento, Trento, Italy.
European Drosophila Research Conference (EDRC)-POSTER September 22-25 2017, London, United Kingdom

List of Seminars attended

"The p38/MAPK pathway: a key factor in cancer therapy and chemoresistance" Cristiano Simone, November 2015
"*V-ATPase and SNAREs: New functions for old lysosomal proteins****"*** *Thomas Vaccari, January 2016*
"Drosophila a model to study human diseases: obesity and related chronic inflammation" Paola Bellosta, Zhasmine Mirzoyan, January 2016
"Role Of Autophagy and mitochondrial shape in muscle physiology and disease" Marco Sandri, October 14 2016

"*Programming the celebral cortex: from development to brain organoids*" Paola Arlotta, November 2016

"Anthocyanins-rich corn in prevention of chronic diseases" Katia Petroni, May 2017

"*Approcci neuroanatomici per lo studio del sistema nervoso centrale in modelli sperimentali e nell'uomo****"***, June 2017

"Ethiovision XT" November 30 2017

"Meccanismi biologici del network neuro-endocrino alla base del controllo fame-sazietà" Carmela Asteria, Dicember 5,2017

List of Courses attended

«Comunicare la scienza» March 11-May 20 2016
«Analisi di immagini digitali di campioni biologici» April 20-May 25 2016
«Corso di matematica (ottimizzazione in più variabili)» May 10-June 21 2016
"Corso di statistica" September 5-28 2016
"Corso competenze trasversale"
"Environmental disasters and their ecological consequences" Dicember 13-15 2016
«Metodi molecolari applicati alla ricerca ambientale » May 30-June 8 2017
«Basi fisiche del sistema climatico e global warming» September 13-21 2017
" Stress ambientale e malattie legate allo stress" November 24 2017