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"Fatty Acid β-Oxidation Pathway in Canine Mammary Tumors: biochemical, immunohistochemical and pharmacological studies"

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This Ph.D. thesis is dedicated to my father, who has raised me to be the person I am today. You have been with me every step of the way, through good times and bad. Thank you for all the unconditional love, guidance, and support that you have given me, helping me to succeed and instilling in me the confidence that I am capable of doing anything I put my mind to. Thank you for everything.

Index

Acknowledgments

Acknowledgments

List of abbreviations

ACC	Acetyl-Coenzyme A Carboxylase
ACCS2	Acetyl Coenzyme Asynthase 2
Acetyl CoA	Acetyl Coenzyme A
ACLY	ATP Cytrat Lyase
Acyl CoA	Acyl Coenzyme A
AKT/PKB	Protein Kinase B
Annexin V/FITC	AnnexinV/Fluorescein IsoThioCyanate
ANOVA	Analysis of Variance
ATP	Adenosine Tryphosphate
CACT	Carnitine Acyl Carnitine Traslocase
cDNA:	complementary DNA
CMT	Canine Mammary Tumor
CPT1	Carnitine Palmytoil Transferase 1
CrAT	Carnitine Acetyl Transferase
CS	Carnitine System
DNLS	*De novo* lipid synthesis
ER	Oestrogen Receptor
ERK	Extracellular signal–Regulated Kinases
FADH2	Flavine Adenine Dinculeotide
FANS	Fatty Acid Synthases
FAO	Fatty Acid β Oxidation
FAS	Fatty Acid Synthesis
FBS	Foetal Bovine Serum
FCM	Flow CyotoMetry
FNAB	Fine Needle Aspiration Biopsy
G1 carcinomas	Well–differentiated Grade 1 carcinomas

List of abbreviations

G2 carcinomas	Moderately–differentiated Grade 2 carcinomas
G3 carcinomas	Poorly–Differentiated Grade 3 carcinomas
GH	Growth Hormone
GS	Grade System
HBC	Human Breast Cancer
HER2	Human Epidermal Growth Factor Receptor 2
HIF 1 α	Hypoxia Inducible Factor 1 alpha
IR score	ImmunoReactive score
LDs	Lipid Droplets
Malonyl Co A	Malonyl Coenzyme A
MECs	Myoepithelial cells
MG	Mammary Gland
NADH	Nicotinammide Adenine Dinucleotide
NADPH	Nicotinammide Adenine Dinucleotide Phosphate
NMG	Normal Mammary Gland
PBS	Phosphate Buffered Saline
PC	Prostate Cancer
PI	Propidium Iodide
PI3K/AKT	Phosphatidylinositol-3-Kinase/Protein Kinase B
PR	Progesteron Receptor
qRT–PCR	(quantitative) Real Time–Polymerase Chain Reaction
RT	Room Temperature
SCD	Steroyl-Coenzyme A Desaturase
SDS–PAGE	Sodium Dodecyl Sulfate–Polyacrillamide Gel Electrophoresis
SEM	Standard Error Mean

List of abbreviations

SREBPs	Sterol Regulatory Element–Binding Proteins
TBS–BSA	Tris Buffered Saline–Bovine Serum Albumin
TCA	Tricarboxylic acid
TNM	Tumor Node Metastasis
WB	Western Blot
WHO	World Health Organization

List of abbreviations

List of Figures

List of Figures

List of Tables

Abstract

Metabolic plasticity describes the ability of the cells to adapt their metabolic status in response to changes in the external microenvironment to support or allow rapid proliferation, continuous growth, and survival in adverse conditions. The metabolic activities in quiescent cells are totally different with respect to those of proliferating ones, in fact, under quiescent conditions, cells have a basal rate of glycolysis, converting glucose to pyruvate, which is then oxidized in the tricarboxylic acid cycle (TCA) into acetyl-CoA and carbon dioxide (CO_2) within the mitochondria. The acetyl-CoA then enters the TCA cycle (also known as the citric acid or Krebs cycle) where it is fully oxidized to CO_2 and H_2O, and produces huge amounts of energy during the process of oxidative phosphorylation (OXPHOS). Nevertheless, quiescent cells can also use fatty acids as building blocks to fulfill their physiological function and to maintain their structural integrity as well as redox and energy balance. On the contrary, during proliferation, metabolism turns towards an anabolic metabolism, with high glycolytic flux and lactate production. Tumor cells have a higher rate of glucose metabolism than their normal counterparts and preferentially use glycolysis instead of OXPHOS even under appropriate oxygen concentrations (Aerobic glycolysis or Warburg Effect). During aerobic glycolysis, glucose is converted to pyruvate, and the final product of this reaction is lactate, which is exported out of the cell, contributing to extracellular acidification. The resulting acidic environment promotes the degradation of the extracellular matrix by proteinases and increases angiogenesis through the release of vascular endothelial growth factor. Recently, it has been demonstrated that most cancer cells to fulfill their increased energy requests, can exploit other metabolic pathways, such as

Abstract

Fatty Acid β –oxidation which represents one of the most crucial mechanisms that can accompany cancer-associated metabolic reprogramming. The use of fatty acids as energy substrates requires about 25 different enzymes and transport proteins, which carry out fatty acids, import them into mitochondria, and facilitate the β-oxidation steps. In particular, the Carnitine System (CS) is considered as a gridlock to finely trigger the metabolic flexibility of cancer cells. The components of the CS play a crucial role in tuning the switch between glucose and fatty acid metabolism. Here, we report, in Canine Mammary Tumors (CMTs), the expression of the CS components in both canine tissue samples derived from bitches suffering from mammary tumors and CMT cell lines. In particular, the analysis of CMTs and mammary gland control specimens confirmed the aberrant expression of the four components involved in this transporting system (CPT1A, CACT, CRAT e CPT2) in primary tumors and especially in well-differentiated (grade 1) tumors in comparison to moderately- (grade 2) and poorly- (grade 3) differentiated tumors. The role of chemotherapy in bitches with malignant mammary tumors has not been clearly defined for all tumor types and, therefore, we have also tested the cytotoxic and proapoptotic effects of the CPT1A inhibitor ST1326 on CMT cell lines obtained from bitches with different mammary malignancies. Cell viability was evaluated in CMT cell lines (CMT-U309, P114, CMT-U27, CMT-U131 and CMT-U229) cells by using trypan blue staining. The exposure of the CMT cell lines to increasing concentrations of ST1326 [1-20 μM] for 48h has determined a significant reduced rate in cell viability. Furthermore, we have also evaluated whether the compound ST1326 is also able to induce cell death in CMT cell lines. CMT-U131 and

Abstract

CMT-U229 have been the cells with the greater sensitivity to ST1326 treatment in comparison to the other cell lines tested. The ability of ST1326 to induce apoptosis in CMT-U229 and CMT-U131 has been examined by employing Annexin V-FITC Assay using Flow Cytometry. The exposure of CMT-U229 cells to 10 μM ST1326 lead to a significant increase (8.40%) of cells in early apoptotic phase and also a significant increase (8.25%) at concentration of 20 μM in the late apoptotic phase, respectively. The treatment of CMT-U131 has also displayed a significant increase (13.8%) and (40.85%) in the late apoptotic phase at concentration of 10 or 20 μM ST1326, respectively. Finally, by western blot analysis we have investigated which molecular signaling pathways were involved in ST1326-mediated cell death. Our results have shown that ST1326 reduces, at least in part, the phosphorylated levels of serine/threonine-protein kinase Akt^{Ser473}, reduces the total levels of extracellular signal-related kinase (ERK) protein as well as increases the phosphorylated levels of ERK protein. Taken all together, our results identify CPT1A as a novel target for CMTs treatment and suggest new druggable pathways for prevention and treatment of these tumors, highlighting, for the first time, a new and emerging deregulated biochemical pathway in canine tumor metabolic reprogramming.

Abstract

Chapter1

Comparative oncology: integrating human and veterinary medicine

1.1 Comparative oncology: integrating human and veterinary medicine

Comparative oncology combines the study of naturally occurring cancers in animals with the study of human cancer biology and therapy [1]. Like humans, tumors in dogs are spontaneously occurring, rather than genetically induced as they are in mice, so canine tumors may more accurately mimic the situation in human cancer patients. The similarities between dogs and humans—with respect to anatomy, physiology, and tumor onset and progression—make canine tumor models a valuable tool for identifying new cancer-associated genes and for enhancing understanding of tumor molecular biology in humans [2]. With complete sequencing of dog genome an increased number of compelling evidences obtained from cross-species genomic studies confirm a high similarities rate between genomic profiles in canine and human cancers, supporting the idea that these illness share similarity also at genetic level [3]. Furthermore, alterations in different gene expression profiles as well as activation of different pathways associated with canine tumor progression have also been identified and, surprisingly, they are highly analogous to those identified in human counterparts [4,5]. Since dogs live in the same environment as humans, they may also act as epidemiologic or etiologic sentinels for the changing patterns of cancer development commonly seen in humans [6-10]. Importantly, in comparison to rodent models, spontaneous cancers in pet dogs recapitulate the biological complexity of human cancers in that they occur in the presence of an intact immune system and are characterized by tumor growth over long periods of time, inter-individual and intra-tumoral heterogeneity, development of recurrent or

resistant disease, and metastasis to relevant distant sites [1,11,12]. Most common tumor types include non-Hodgkin lymphoma, osteosarcoma, melanoma, prostate, lung, head and neck, and mammary carcinoma as well as soft-tissue sarcoma [13-19]. The majority of these tumors appear to be very similar to those of human counterparts, especially in relation to histological aspects, tumor genetics, molecular signaling patways alteration, biological behavior and response to conventional therapeutic schemes as well [20]. In comparative oncology, one of the best-studied examples of spontaneously occuring tumors is represented by tumors of the mammary gland [21], although in dogs it exists in two different forms, namely simple and complex [22]. During cancer progression, malignant carcinomas are characterized by a decreased oestrogen and progesterone recepor(s) expression [23,24]. This feature makes this tumor very similar to triple negative breast cancer Estrogen Receptor (ER) negative, progesterone receptor (PR) negative, HER2 (Human Epidermal Growth Factor 2) negative - normally found in human counterparts - known to have a poor prognosis and limited treatment options [25].

1.2 Anatomy of the canine mammary gland

The presence of mammary gland (MG), also known as (mamma, uber or mastos) is unique to mammals and forms the glandular tissue of a mamma [26,27]. In the bitches, the MG originates along the so-called milk line that extends parallel to the midline in the abdominal wall. Female dogs typically have five pairs of MGs which are composed of a corpus mammae (made up of 8–14 lobules, connective tissue, and skin) and a teat [26]. The cranial two pairs are named cranial and caudal thoracic MGs, the middle two pairs are termed cranial and caudal abdominal MGs, and the caudal pair is referred to as the inguinal MGs [28] (Figure 1).

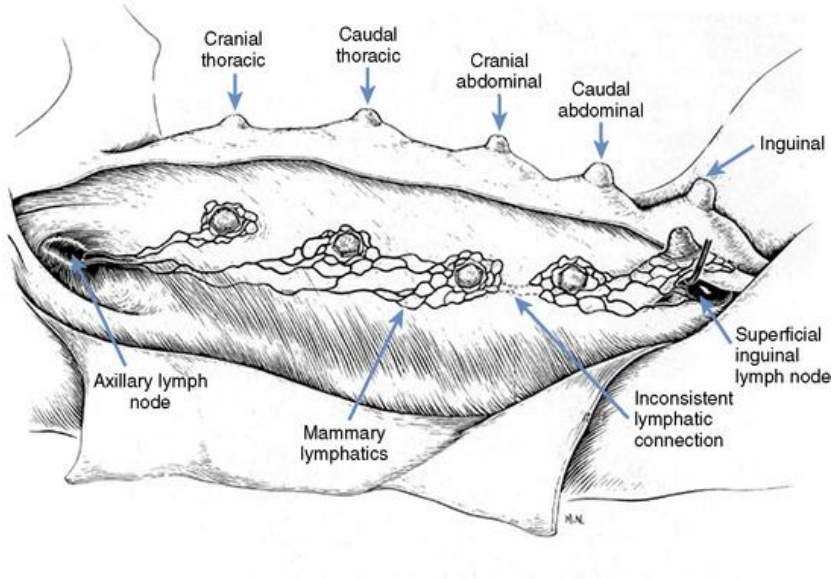


Fig. 1. Anatomical location of mammary gland in canine

Based on their anatomy, MGs are modified apocrine sweat gland, made up of a tubulo-alveolar structure [27,29], which is divided into lobules by

interlobular connective tissue. The MG is characterized by parenchyma (alveoli), stroma (connective tissue), ducts, vessels and nerves [28]. Canine MG is a dynamic organ susceptible of profound remodeling and differentiation throughout their entire reproductive life. Indeed, its full development is completed in the adult female, during pregnancy, where ductal epithelial cell proliferation and lobuloalveolar differentiation occur. At parturition, MG is characterized by a complex ductular-lobular-alveolar structure, associated with alveolar secretion [27].The epithelium of the ductal-lobular system consists of a dual-cell population of luminal epithelial and basal myoepithelial cells. Depending on the secretory activity, secretory alveoli are characterized by simple cuboidal to columnar secretory epithelium. Furthermore, the secretory epithelium is covered with myoepithelial cells (MECs), which in turn are lined by a basal lamina. Given their contractile force (due to the expression of the smooth muscle actin isoform), MECs are also responsible for milk ejection during lactation. MECs are also abundant in the ducts. Interalveolar tissue is relatively sparse, well vascularized and contains a considerable content of lymphocytes and plasma cells. Interlobular septa are characterized by dense irregular connective tissue with many elastic fibres [30]. Interlobular ducts have different linings: larger ducts are covered with a bilayered cuboidal to columnar epithelial lining, whereas the smaller ducts have a single layer of cuboidal epithelium, which may be secretory in lactating MGs [27]. As previously stated, the canine MG is a hormone-dependent organ whose cyclic activity is linked with consecutive development and regression phases that differ between single glands and also within each gland [31,32]. For example, during diestrus, it is possible to observe the

complete mammary lobuloalveolar and secretory differentiation [31,32], which is related to the long-lasting and functional corpus luteum that is able to produce high progesterone levels regardless to pregnancy [32]. As canine MG has homology with a single human breast [26] then similar developmental characteristics exist [33]. This feature, along with others, makes the dog a reliable model for the study of human breast cancer (HBC) [33].

1.3 Incidence of CMTs

Canine mammary tumours (CMTs) are the most common type of cancer in intact female dogs. They represent about 50% of all cancers and the second most important tumor in the canine species, preceded exclusively by skin tumors[34]. A study focusing on the incidence of CMTs found tumors in approximately 0.05% of females that were spayed before their first heat cycle. This figure increased to 8% or 26% when the animals were spayed after their first or second heat, respectively [35]. When the animals are spayed later, the risk of developing malignant tumors is the same as for an intact bitch [36]. The real incidence of mammary cancer and the relationship between benign and malignant lesions is difficult to determine because small benign tumors escape the attention of the veterinary surgeon or are not subjected to surgical removal. However, several lines of evidences have reported an incidence rate of about 200 ⁄ 100 000 dogs⁄ year [37-39]. High prevalence rates (more than 70%) have been reported in Colorado, United States of America (USA), in a life-span observation study including 672 female beagles that were experimentally exposed to radiation [40]. However, other results obtained from clinical cases in the USA also

referred an incidence of 51% *per* year for CMTs [6]. Although these evidences, it is to note that the incidence of CMTs is diminishing in the USA, as well as in other and some countries in Western Europe, due to the common practice of performing ovari(ohyster)ectomy at an early age, varying from 8 weeks to 7–12 months of age [41,42]. On the contrary, in regions such as Scandinavia and Spain, the incidence of CMTs is much more increased due to the fact the ovari(ohyster)ectomy practice is not frequently performed [41-43]. In fact, another study including more than 80,000 insured bitches has shown an incidence rate of approximately 111/10,000/dogs/year [41]. In Europe, results obtained from clinical reports about CMT, point out that the incidence rate ranging from 26.5% to 70% [38]. Dobson et al (2002) have also reported that, increased incidence rate (205 cases/100,000 dogs/year) has also been observed in the United Kingdom [37]. The increasing of malignant diagnoses can be linked to the environmental pollution and to chemical exposure or to the habits of pet owners [44]. Moreover, it is extremely probable that diet, body mass and estrogens may be the cause of mammary tumours in female dogs, as in humans [35].

1.4 Aetiopathogenesis and Risk Factors of CMTs

1.4.1 Hormonal Influence

The mammary tumours occurring both in dogs and humans are tightly controlled by hormones, and similarities in “hormones addiction” can be noted between HBC and CMTs [45]. Under physiological conditions, ovarian steroids (oestrogens and progesterone) may induce the growth of normal mammary tissue both in bitches and in womens. Thus, the potential

proliferative effect exerted by these hormones on the breast and mammary epithelium generates the optimal conditions for neoplastic tranformation [46-48]. It has been reported that such event occurs at every oestral cycle making the bitch strongly susceptible to tumorigenesis [49-51]. Oestrogens is involved in the prolifeartion of ducts, while progestins are involved in the lobuloalveolar development of the MG [50,52]. The overexpression of growth hormone (GH) production within the MG has been described as a possible mechanism involved in progesterone-induced CMTs [53,54]. It is also supposed that GH induces the proliferation of mammary stem cells during the process of mammary carcinogenesis [53,55]. The effect of ovarian steroids in bitches is controlled by receptors expressed within the mammary tissue. ER and PR are found in both normal and neoplastic tissues [49,56-62]. Compelling evidences have shown that ER is higher expressed in benign tumours with respect to malignant ones [49,56-58,61-64]. Similarly, PR expression is found to be progressively decreased from hyperplastic/dysplastic over benign to malignant canine mammary lesions [49,57,59]. The decreased expression of ER and PR has found to be also inversely correlated with a worse prognosis [49,56,60,65] but not all studies are in agreement with these results. For example in another study, any statistically significant correlation has been found between the loss of ER and PR expression and a worse prognosis [59]. Moreover, dogs that have been spayed more than 2 years before tumour surgery are more likely to have an ER-negative tumour and therfore to have a decreased benefit from ovari(ohyster)ectomy at the time of tumour surgery [47]. Bitches that are spayed before the first oestrus have a 0.5% risk of developing mammary tumours in comparison to sexually intact ones; those spayed before the

second oestrus have an 8% risk, and those spayed after the second oestrus and before two and a half years of age have a 26% risk [36].

1.4.2 Age

According to several studies, CMTs occur in middle-aged and old bitches [66-68]. The median age of development ranging from 8 to 10 years [66,68,69]. As a matter of fact, a research involving a great closed beagle colony have shown that mammary cancer develops mainly at 8 years of age [70]. It has been found also differences in the development of benign and malignant neoplasia. Indeed, dogs with benign tumours have a mean age of 8.5 years, while dogs with malignant neoplasia present a mean age of 9.5 [71]. The presence of malignant tumours before 5 years of age is scarce [72,73]

1.4.3 Breed Predisposition

As far as breed predisponition is concerned, purebred dogs have been reported to have a higher representation among cases of CMTs compared to mixed breed [69,73-75] Several breeds, e.g. Poodles, English Springer Spaniels, Brittany Spaniels, Cocker Spaniels, German Shepherds, Maltese, Yorkshire Terriers and Dachshunds, seem to have an increased risk of developing a mammary tumour [75,76]. The English springer spaniel has an incidence of 319 /10,000 dogs/ year, while the Rough Collie has an incidence of 5/ 10,000 dogs/year [41]. According to the studies, conflicting results have been reported. For example, MacEwen and Withrow (1996) report a decreased risk for Boxers, Chihuahuas and mixed breeds, instead

others report Boxers as a high-risk breed and Collies, Shetland Sheepdog and Bernese Mountain dogs as low-risk breeds [72]. In addition, Boxers and Bernese Mountain dogs are represented as high-risk breeds [77].

1.4.4 Obesity

In dogs, obesity – especially at young age – represents a major problem and a common risk factor for CMT development [78-80]. Dogs diagnosed as obese at one year of age or if they present a severe obesity at one year before the diagnosis of CMT, have a higher prevalence of MG tumour with respect to those that do not [73,75]. Dogs that are thin at 9–12 months of age have decreased risk of CMT development [80]. Obesity has been also identified as risk factor for HBC development due to increasing local and free circulating oestrogen [81-83]. Interestingly, obesity in dogs may influence the development of MG tumour *via* the same mechanisms [27]. Prolonged beef intake, pork and a diet poor in consumption of chicken has been linked to a high incidence of MG tumour in dogs [78].

1.5 Clinical Presentation

Typically, the owner of the dog present their pet dogs to the veterinarian after having observed the presence of lump(s) on the MG (Figure 2). Along with these, other clinical signs such as ulceration around the affected gland(s), anorexia, pyrexia, emaciation, change in gait, signs of pain and other signs of systemic illnesses can occur. The glands most frequently affected are represented by the caudal abdominal and inguinal

MGs compared to other glands [71]. Furthermore, low incidence of MG tumour in male dogs could be due to the absence of the influence of ovarian hormones (oestrogen and progesterone) on the mammary tissue, and a smaller amount of susceptible tissue, which are both characteristics in female dogs [70]. A dog could have more than one tumour in its MG, freely movable or fixed, presented with ulcerative surface, small or large [27].

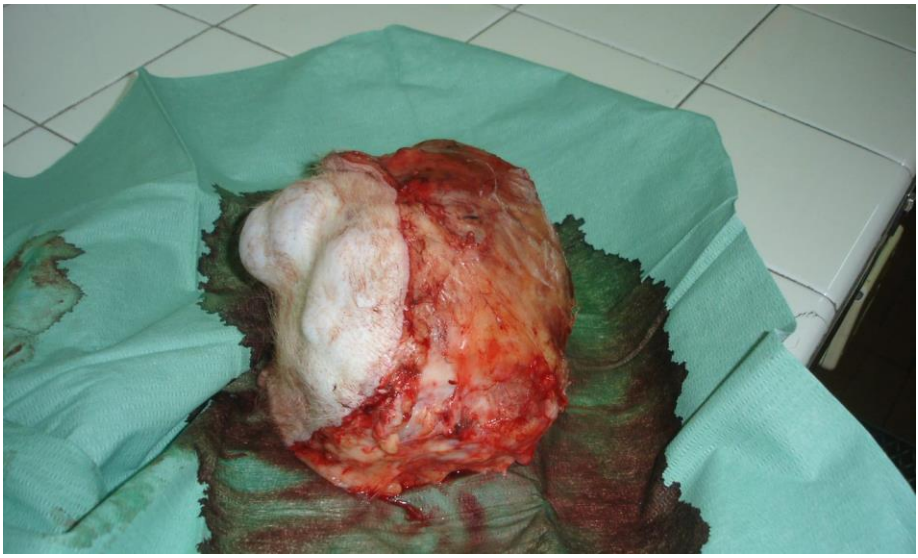


Fig. 2. Example of spontaneously developed mammary gland tumour in dog.

1.6 Histopathology

Histopathology represents the "gold standard" method for obtaining an accurate diagnosis for CMTs [84]. Histopathology is routinely performed using hematoxylin and eosin staining protocols, which - if adequately performed – are appropiate for CMT diagnosis. However, since selected

subtypes or histopathology variants of CMT exist, it is difficult to diagnose them using routine staining and therefore special staining or immunohistochemistry (IHC) techniques for an accurate diagnosis are demanded [85]. In 1974 and in 1976, the World Health Organization (WHO) presented the "International Histological Classification of Tumors of Domestic Animals that included canine MG tumours and dysplasia. In 1999, a revision (Table 1) of the classification system of MG tumours was published [86,87] because, over the time, the knowledge in the veterinary oncology field has been increased. These have been the basis for the classification of tumours in domestic animals including CMTs. Recently, several modifications to the classification systems for CMTs (Table 2) have been proposed due to the need to include newly described histologic subtypes of CMTs [22]. The different tumour types according to the WHO classification and a brief descrition are described below.

Table 1 The World Health Organization (WHO) classification (1999) of canine mammary gland tumours

Tumour type	*Tumour sub-type*
1. Malignant tumours	1.1 Non-infiltrating (in situ) carcinoma
	1.2 Complex carcinoma
	1.3 Simple carcinoma
	1.3.1 Tubulopapillary carcinoma
	1.3.2 Solid carcinoma
	1.3.3 Anaplastic carcinoma
	1.4 Special types of carcinomas
	1.4.1 Spindle cell carcinoma
	1.4.2 Squamous cell carcinoma
	1.4.3 Mucinous carcinoma
	1.4.4 Lipid rich carcinoma
	1.5 Sarcoma
	1.5.1 Fibrosarcoma
	1.5.2 Osteosarcoma
	1.5.3 Other sarcomas
	1.6 Carcinosarcoma
	1.7 Carcinoma or sarcoma in benign tumour

2. Benign tumours	2.1 Adenoma 2.1.1 Simple adenoma 2.1.2 Complex adenoma 2.1.3 Basaloid adenoma 2.2 Fibroadenoma 2.2.1 Low-cellularity fibroadenoma 2.2.2 High-cellularity fibroadenoma 2.3 Benign mixed tumour 2.4 Duct papilloma

3. Unclassified tumours	
4. Mammary hyperplasia and dysplasia	4.1 Ductal hyperplasia 4.2 Lobular hyperplasia 4.2.1 Epithelial hyperplasia 4.2.2 Adenosis 4.3 Cysts 4.4 Duct ectasia 4.5 Focal fibrosis (fibrosclerosis) 4.6 Gynecomastia

1.6.1 Benign Mammary Gland Tumours

Adenoma. This type of tumour is composed of well differentiated epithelial or MECs. It is classified as simple tubular type. The solid nodes are composed of fusocellular cells and are called myoepitheliomas. It is a rare tumour in dogs.

Complex Adenomas or Adenomyoepitheliomas. The origin of this tumour is from continuous proliferation of epithelial cells and MECs without forming myxoid matrix. It is characterized by having a capsule, no necrosis, atypia and low mitosis.

Basaloid Adenoma. This type of tumour consists of uniform cords on basaloid monomorphic epithelial cells nest. The cells of the periphery are arranged side-by-side manner and are oriented against a thin basal lamina. In most cases the tumours are small.

Fibroadenoma. This type of tumour originates from the proliferation of stromal and epithelial elements. Two sub types are described as: (1) pericanicular fibroadenoma (the stroma surrounds the epithelium) and (2) intracanicular fibroadenoma (the stroma compresses and deforms the epithelium).

Benign Mixed Tumour. This type of tumour consists of proliferated cells that appear either fusiform or stellate, morphologically resembling mesenchymal cells and epithelial components, producing adipose tissue and/or cartilage and/or bone, sometimes with fibrous tissue. The cells are sometimes embedded in abundant myxoid matrix. There is some level of pleomorphism and atypia. It is the most common benign tumour in dogs.

Ductal Papilloma. This type of tumour is lobed or ramified in a distended duct. There is proliferation of the epithelium of ducts on a fibro-vascular axis. Cellular atypia is evident on the epithelium, and nuclear hyperchromasia. There is minimal mitotic activity and the epithelium is distributed as a single layer on a layer of MECs.

1.6.2 Malignant Mammary Gland Tumours

Ductal Carcinoma *In Situ (DCIS).* This is the most common of the two types of carcinoma *in situ*, the other type is lobular ductal carcinoma *in situ*. It is often associated with invasive canine mammary carcinomas. The tumour develops in the extra- or intra-lobular ducts. DCIS is characterised by the proliferation of epithelial cells in more than two ductal units in one histological section. The cellular architecture of the cells is atypical, characterized by connecting bridges in the ductal lumen. There is also polarization of epithelial cells in a layer associated with another continuous layer comprising MECs. There are micro-calcifications seen in the ductal lumen.

Lobular Carcinoma In Situ. In this type of tumour, epithelial proliferation causes the filling and expansion of the terminal lobular units. About 50% of the lobe is affected and the lumen is completely lost, but the basement membrane is maintained. The cells have the same shape, with small and spherical nuclei. The nuclei are small with discrete and uniform nucleoli. There is noticeable invagination of the cytoplasmic membrane due to a single vacuole around the nucleus.

Carcinoma in a Mixed Tumour. Mixed tumours exhibit a complex histological pattern, having cellular components from epithelial and mesenchymal origin. Some of the cells can turn malignant, giving rise to carcinoma in mixed tumours. Carcinoma in mixed tumours composed of

nodules or foci of highly pleomorphic epithelial cells with atypical mitoses, arising in benign mixed tumours.

Complex Carcinoma or Malignant Adenomyoepitheliomas. This tumour is composed of epithelial and MECs proliferation. However, myxoid matrix is not evident. Other features include atypia, necrosis, and absence of a capsule and high mitotic activity.

Papillary Carcinoma. Papillary aborescent epithelial proliferation with a central fibrovascular stroma characterizes this tumour. These lesions are classified as papilloma, carcinoma *in situ* in papilloma, papillary carcinoma *in situ*, invasive and non-invasive papillary carcinomas. In benign tumours, neoplastic papillae contain MECs that could be observed between basement membrane and the epithelial cells. This characteristic is not seen in the malignant variant.

Tubular Carcinoma. Predominantly tubular arrangement of the proliferated epithelial cells qualifies this type of tumour. The amount of stroma is variable. With or without necrosis, peritumoural lymphocytes can be observed. The rate of tissue and vessel invasion is high in these tumours.

Solid Carcinoma. This is a common cancer of dogs that usually occurs when the tumour has stayed for a long time without surgical intervention. On histopathology, the epithelial cells are solidly arranged in chords, sheets or clusters. The cells are not differentiated, with small hyperchromatic nuclei with a high mitotic index. In some cases, the cells

will show vacuolated cytoplasm. There is variable amount (small to moderate) of stroma and areas of necrosis.

Micropapillary Carcinoma. Microscopically, in this tumour type it is possible to note the presence of cystic spaces that look like lymphatic vessels distributed within the MG tissue. Within the spaces, a micropapillary pattern which is morule like, is assumed by clusters of epithelial cells. The cytoplasm is eosinophilic and abundant. The nucleus is vesicular and pleomorphic with prominent nucleoli. Lymph node metastasis is common and the mitotic index results variable.

Invasive Lobular Carcinoma. This type of CMT shows small cells in a linear arrangement, which are non-polar and are uniform in size. The tumour is diffusely invasive with large amount of fibrous stroma. Solid foci may be formed by the tumour cells, containing mucin and having a signet appearance, or arranged around benign ducts in a parallel way.

Pleomorphic Lobular Carcinoma. This tumour type is a result of the dispersal of epithelial cells in the stroma, or an irregular outline of the cells in a linear pattern. The cytoplasm is abundant and eosinophilic with accentric and pleomorphic nuclei. Cytoplasmic vacuoles are sometimes seen.

Secretory Carcinoma. With fine needle aspiration biopsy (FNAB), the cells are round to oval and in the form of clusters. The nucleoli are fragmented and the chromatin is irregularly distributed. The cells have a

clear and abundant cytoplasm with the nucleus pushed to the periphery by secretory vacuoles. Histopathologically, this tumour appears as an infiltrative carcinoma, with the neoplastic cells showing peripherally disloged nucleus by large vacuoles and a clear cytoplasm. The proliferation pattern could be solid and/or tubular with eosinophilic spaces filled with secretion.

Mucinous Carcinoma. The presence of abundant extracellular mucinous material characterizes this tumour type. It is also known as gelatinous carcinoma. The proliferated cells may form solid, tubular or papillary structure. Large amount of mucinous eosinophilic secretions fill the spaces in these structures. The secretion is also positive in diastase and alcian blue. The accumulated mucin is mostly located in the intraductal structure. When the mucoid content leaks from the intraductal structure, it then becomes invasive mucinous carcinoma.

Lipid-rich Carcinoma. This tumour is uncommon in dogs and is composed of an expansive growth. The stroma separates the nests and cords of neoplastic cells. The cytoplasm of the cells is vacuolated and the nuclei are round to flat. There may be peripheral displacement of the nucleus by vacuoles. When 80% of the tumour cells are lipid producing, the diagnosis is confirmed.

Squamous Cell Carcinoma. This tumour is composed of areas of squamous differentiation in the solidly arranged sheets and cords of tumour cells. Keratin layers are found in the centre of the more

differentiated tumour. Invasion of the lymphatics in these tumours is not uncommon.

Spindle Cell Carcinoma. This tumour is not very common in dogs. Histopathologycally, there is presence of spindle cells in bundles or in a circular pattern. The cytoplasm of the cells appears eosinophilic and might be vacuolated. The nuclei could also be vacuolated with a fragmented chromatin. These features should be noted in at least 80% of the tumour section in order to confirm the diagnosis.

Anaplastic Carcinoma. This histologic subtype of CMT is highly aggressive with early metastasis and recurrence and is considered to have the worst prognosis. This tumour is diffusely infiltrative. The proliferating epithelial cells are large, atypical with linear outline. The stroma is loose, abundant and reactive, with individual cells invading it. The cells are also anaplastic, with one or two prominent nucleoli and chromatin fragmentation. The tumour has a high mitotic index with marked anisocytosis. Blood and lymphatic vascular structures invasion by neoplastic cells could be observed and one of the peculiar features of this tumour is inflammation.

Fibrosarcoma. These tumours are malignant and are characterized by fibroblasts with varying amounts of collagen. Collagen-producing spindle-shaped cells are arranged as reticular fibres form these tumours. Fibrosarcomas are among the most encountered mammary sarcomas in the dog.

Osteosarcoma. This tumor type is composed of the formation of bone and/or osteoid by the neoplastic cells. Osteosarcoma could occur as combined or non-combined (pure). The combined shows both osseous and cartilaginous malignant tissues. There is high mitoses and pleomorphism.

Carcinosarcoma. In the dog, the features of carcinosarcoma resemble those described in humans. The cut surfaces of these tumours are firm to bony with a clear delineation. The cells are epithelial-like and well delineated. The type of differentiation varies including solid, adeno, mucinous, anaplastic, squamous, and sarcomatous areas with fibroblastic, chondroblastic and osteomatous differentiation. When present, metastasis is of mixed type, sarcomatous or carcinomatous.

Other Sarcomas. Other sarcomas that could develop in the mammary gland comprise chondrosarcoma, haemangiosarcoma and liposarcoma. These are very uncommon and appear similar to those observed in other organs.

Table 2. Proposed histologic Classification 2010 from Golschmidt [22]

Malignant Epithelial Neoplasms
Carcinoma–in situ
Carcinoma–simple
a. Tubular
b. Tubulopapillary
c. Cystic-papillary
d. Cribriform
Carcinoma–micropapillary invasive
Carcinoma–solid
Comedocarcinoma
Carcinoma–anaplastic
Carcinoma arising in a complex adenoma/mixed tumor
—The benign counterpart is still detectable in the section.

Carcinoma–complex type
—The epithelial component is malignant, and the myoepithelium is benign.
Carcinoma and malignant myoepithelioma
—The epithelial and myoepithelial components are malignant.
Carcinoma–mixed type
—The epithelial component is malignant; the myoepithelial mesenchymal component is benign; and the mesenchymal component is cartilage or bone.
Ductal carcinoma–malignant counterpart of ductal adenoma
Intraductal papillary carcinoma–malignant counterpart of intraductal papillary adenoma

Malignant Epithelial Neoplasms—Special Types

Squamous cell carcinoma
Adenosquamous carcinoma
Mucinous carcinoma
Lipid-rich (secretory) carcinoma
Spindle cell carcinomas
Malignant myoepithelioma
Squamous cell carcinoma–spindle cell variant
Carcinoma–spindle cell variant
Inflammatory carcinoma

Malignant Mesenchymal Neoplasms—Sarcomas
Osteosarcoma
Chondrosarcoma
Fibrosarcoma
Hemangiosarcoma
Other sarcomas

Benign Neoplasms
Adenoma–simple
Intraductal papillary adenoma
Ductal adenoma
With squamous differentiation (keratohyaline granules)
Fibroadenoma
Myoepithelioma
Complex adenoma (adenomyoepithelioma)
Benign mixed tumor

Hyperplasia/Dysplasia
Duct ectasia
Lobular hyperplasia (adenosis)

Regular With secretory activity (lactational)
With fibrosis–interlobular fibrous connective tissue
With atypia
Epitheliosis
Papillomatosis
Fibroadenomatous change
Gynecomastia

Neoplasms of the Nipple
Adenoma
Carcinoma
Carcinoma with epidermal infiltration
Hyperplasia/Dysplasia of the Nipple
Melanosis of the skin of the nipple

Carcinoma–Cribriform

Cribriform carcinoma, which is uncommon, is characterized by the proliferation of a population of neoplastic epithelial cells forming a sievelike arrangement. The lumina formed are very small and often round, and they are surrounded by bridges of neoplastic cell. The surrounding interstitial connective tissue is sparse. Neoplastic cells vary from columnar to polygonal and often have scant homogeneous eosinophilic cytoplasm. Anisokaryosis and anisocytosis are moderate, and the number of mitoses is variable.

Carcinoma–Micropapillary Invasive

Micropapillary invasive carcinoma is a type of mammary neoplasm that has been described in dogs. Within the mammary tissue are present one or more nodules characterized by an intraductal neoplastic population forming small intraluminal irregular aggregates and small papillae that do not have a supporting fibrovascular stalk and are surrounded by empty

lacunar spaces. The neoplastic cells grow along ductal walls and infiltrate the periductal collagenous stroma. Cells are pleomorphic and cuboidal to polygonal and have scant eosinophilic cytoplasm. The nuclear cytoplasmic ratio is high, and nuclei are central and oval, with a large central nucleolus and hyperchromatic granular chromatin. Anisokaryosis and anisocytosis are moderate to severe, and the mitotic index is high. The infiltrating micropapillary areas may be seen in association with tubulopapillary or solid areas. This tumor frequently shows vascular invasion and possesses a high metastatic potential.

Inflammatory Carcinoma

The term inflammatory carcinoma denotes a clinical entity characterized by a very fast clinical course, edema, erythema, firmness, and warmth of the mammary glands, with or without mammary nodules. Histologically, the invasion of dermal lymphatic vessels by neoplastic emboli represents the major characteristic feature. These neoplastic emboli cause a blockage of the superficial lymphatics causing severe edema of the region.

Although many efforts have been made to find a correlation between histologic type and clnical prognosis, no reliable criteria have yet been established. The differences in the prognosis among histopathological subtypes of adenocarcinomas are not significant. However, animal with solid carcinomas, anaplastic carcinomas and squamous cell carcinomas have a survival rates of less than 2 years. On the contrary, bitches with tumors including a myoepithelial cell profliferation (complex type carcinomas) have a significative higher survival rate than those with

tumors consisting of secretory cells alone (simple carcinomas). Moreover simple type carcinomas often invade the lymph vesses and frequently metastatize to lymph nodes and distant organs, whereas complex carcinomas less frequently give metastases.

1.7 Staging of CMTs

The TNM staging system is the most common way that veterinary clinicians used in order to stage canine mammary gland tumours. TNM stands for Tumour (describes the size of the tumor), Node (describes spread of cancer to nearby lymph nodes), Metastasis (spread of cancer to other parts of the body) [72,88]. The original WHO staging system of Owen (1980) is more sophisticated because the T and M categories include an additional subsection for fixation. In 1996, MacEwen and Withrow have modified this original staging system that comprises five stages instead of four as reported in the original system. Taking into consideration the original staging system, dogs with a small tumour (T1) along with the involvement of lymph nodes are categorized as stage II, as well as dogs with a larger tumour (T2) regardless of the lymph node status. Moreover, dogs with a large tumour (T3) are considered as stage III, regardless of the lymph node status. Dogs with distant metastasis are classified as stage IV, independent of the tumour size or lymph node status. On the contrary, according to the modified system, dogs without regional lymph node involvement and without distant metastasis are categorized in stages I, II and III, depending on their tumour size, respectively. Each patient with regional lymph node involvement will be classified as stage IV and each

patient with distant metastasis as stage V, regardless the tumour size [72]. It has been reported that staging of CMT correlates with the prognosis of disease, namely an advanced stage confers a worse prognosis [89]. For CMTs classified as stage 1, namely small- non-invasive- or well-differentiated-tumours, surgery alone represents a valid therapeutic treatment choice and could be curative, whereas for larger tumours with advancing stage and ill-defined margins need other forms of therapies such as, chemotherapy, hormonal therapy and/or radiotherapy [27].

1.8 Histologic Grading of CMTs

Malignant CMTs are graded histopathologically to provide important prognostic information to the clinician. Tumours with a higher grade have a worse prognosis [22,90,91]. Even though any specific grading system (GS) exists for CMT, different GSs are used in veterinary medicine. Gilbertson et al. (1983) [92] adapted a human classification system [93]. Misdorp (2002) used a method similar to the Bloom and Richardson method, another human grading method [94]. Increasing growing body of works applies the Elston and Ellis method [90] (also called Nottingham method), a modification of the Bloom and Richardson method, which is frequently used in human medicine [65,90,95-100]. Gilbertson et al. (1983) based their classification method on numeric grading of three parameters: the degree of duct epithelial proliferation and of atypia in non-invasive neoplasms, the degree of nuclear differentiation in malignant proliferative lesions and the intensity of lymphoid cellular reactions [92]. Both the Bloom and Richardson and the Elston and Ellis methods apply similar parameters: tubule

formation, nuclear pleomorphism and mitotic counts. The Elston and Ellis method (1991)[90] represents the best method to choice because allow a more specific and objective assessment of the various paramenters. Each of the parameters is scored based on a scale from 1 to 3. The total score is then converted to the grade of malignancy: 3–5: grade I (well-differentiated carcinoma), 6–7: grade II (moderately differentiated carcinoma) and 8–9: grade III (poorly differentiated carcinoma) [90,94]. These parameters have been shown to be of prognostic significance in the evaluation of mammary carcinomas and their survival two years after surgery. Karayannopoulou *et al.* found significant differences in survival between cases with different tumor grades [91]. Survival is worse in dogs with grade III carcinomas than in those with grade I or grade II [91]. Bitches with simple carcinomas have a worse prognosis in comparison to other carcinomas; any significant difference in survival between grade II and grade III cases, since both have a poor prognosis [91]. Less differentiated carcinomas (grade III) present an increased risk of death when compared with more differentiated carcinomas (grade I and II) [91]. Lymphatic/vascular invasion and lymph node metastasis are obviously related to a poor prognosis [22] .

1.9 Therapeutic Modalities for CMTs

The surgical excision of CMTs represents the “gold standard” procedure and is regareded to be the single most effective method to achieve local tumor control except for the presence of distant metastases. Apart from improving survival time and quality of life in cancer patient, surgical

removal or resection of tumours enables an accurate and detailed histopathological observation [101]. The surgical procedures rely on the tumour size, location and patient status [102]. The different surgical techniques used include lumpectomy, simple mastectomy, regional mastectomy, unilateral mastectomy and bilateral mastectomy [102]. Although the surgical removal of the entire mammary chain (mastectomy) is recommended in order to prevent recurrence or even the development of a newly tumor mass formation, it has been reported that the adopted kind of surgery does not affect neither the recurrence rate nor survival time [103,104]. When subjected to mastectomy, as in women, several dogs with malignant CMTs present micrometastases, hence despite surgery, the disease move forward [92]. Although do not exist alternative established guidelines for treatment apart from surgery, dogs with advanced-, metastatic- or aggressive histological type of CMTs could take advantage from adjuvant therapy such as radiotherapy, anti-Cox-2 treatment, chemotherapy, desmopressin, hormonal therapy, chemotherapy or anti-angiogenic therapy [105-113]. Carboplatin is another cytotoxic drug that have been employed in the treatment of CMTs showing positive results especially for the treatment of CMT with advanced clinical staging [114]. Another study has reported the use of gemcitabine in the treatment of CMTs. In this study the authors have demonstrated that high-dose gemcitabine treatment is well tolerated with an unexpected low incidence of adverse effects and the quality of life is maintained, if not improved in all treated dogs [115].

Chapter 2

Metabolic alterations as hallmark of cancer cell

2.1 The Warburg Effect as a hallmark of cancer

Cancer is a complex disease consisting of distinct histological and genetic features [116]. Nevertheless, all cancers share common biological traits among which uncontrolled proliferation is the most important ones [117]. Cancer cells undergo an extensive metabolic MR to fulfill the increased demands of macromolecules and energy for proliferation [118]. Progressively, the concept of altered metabolism as a distinctive characteristic of cancer cells has become widely accepted and Hanahan and Weinberg edited the list of cancer hallmark to include the reprogramming of energy metabolism [119] (Fig. 3).

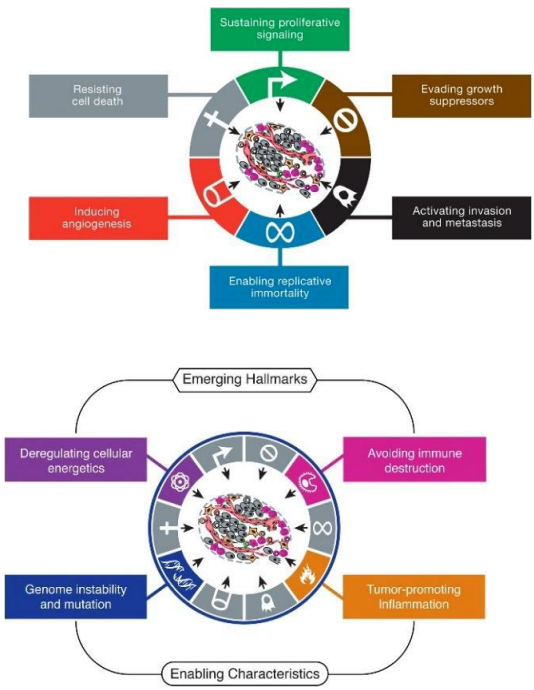


Fig.3. Cancer hallmark as described by Hanahan and Weinberg in the year 2000 and updated in 2011. From Hanahan and Weinberg [119].

Metabolism consists of two distinct biochemical reactions that occur within a cell or an entire organism, which are essential to sustain life [120]. Cell metabolism can be divided into two major categories: *catabolism*, which refers to the breakdown of molecules into smaller metabolites in order to release energy and *anabolism*, which describes the consumption of energy in order to build macromolecules needed for most cellular functions [120]. The most common feature shared by the vast majority of cancer cells is the propensity to show an altered metabolism [121,122]. In particular, one of the major hallmarks of cancer cells is the increased addiction on glucose that represents the main fuel for mammalian cells, providing not only energy but also metabolites for different anabolic processes [123]. Glucose is taken up into the cell by glucose transporters and subsequently converted to pyruvate in the cytoplasm *via* the process of glycolysis, which allows Adenosine Tryphosphate (ATP) production. In normal (quiescent) cells, the glycolysis-derived pyruvate is predominantly imported into the mitochondrial matrix where it is oxidized to acetyl coenzyme A (acetyl CoA) which is then fed into the tricarboxylic acid (TCA) cycle, followed by oxidative phosphorylation (OXPHOS) for high-efficiency ATP generation. The full oxidation of one molecule of glucose produces up to 38 ATP molecules (including 2 ATP generated by glycolysis). The hypothesis that cancer cell metabolism, in comparison to their normal (non-transformed) counterparts is enhanced do not represent a novelty. Indeed, it was only at the beginning of the 20th century, due to the tremendous advances carried-out in the field of biochemistry, that the energy demands of cell proliferation were initially taken into consideration. The first that hypothesize such link was the German bacteriologist von

Wassermann, who supposed that cancer cells are able to increase oxygen consumption due to their increased proliferation rate. As a result of this hypothesis, he sought to target cancer cells by using a derivative of selenium, a drug that disrupts cell respiration [124]. Despite very promising results in rodents, this drug showed toxicity in humans, and other studies also revealed that selenium was not as effective in killing cancer cells as initially thought, suggesting that Wassermann's observations were merely coincidental [125]. Detailed study of cancer cell metabolism was performed few years later through the meticolous work of the German physiologist Otto Warburg. In his forays into research, Warburg observed that cancer cells employ large amounts of glucose even in the presence of oxygen, unlike differentiated cells which predominantly metabolize glucose to carbon dioxide (CO_2) by oxidation of glycolytic pyruvate in the mitochondrial TCA cycle when oxygen is available. This reaction produces Nicotinamide adenine dinucleotide (NADH), which subsequently fuels OXPHOS to maximize ATP production, with minimal production of lactate (Fig. 4).

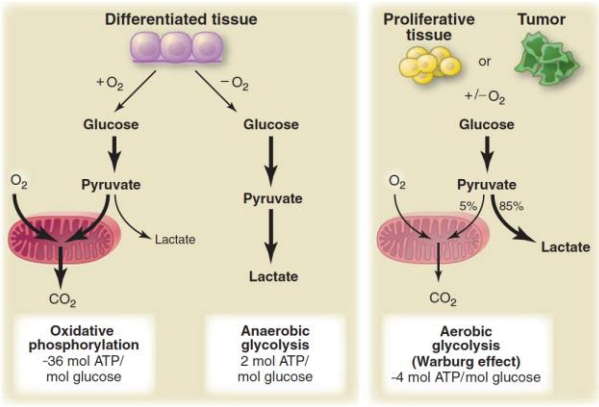


Fig. 4. Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect). From Vander Heiden et al. [123].

Under anaerobic conditions, most differentiated cells produce large amounts of lactate. Conversely, most cancer cells produce considerable amounts of lactate despite the presence of adequate oxygen availability and therefore their metabolism is often called “aerobic glycolysis” or Warburg effect [123]. However, for Warburg this phenomenon remained unanswered and unsolved. At the beginning, Warburg supposed that the lactate production in cancer cells was caused by an impairment of OXPHOS due to inherent defects in mitochondrial function [126]. However, other investigations revaled that mitochondrial function is not impaired in the majority of cancer cells, proposing other explanations for aerobic glycolysis in cancer cells [127-129]. As far as ATP production is concerned, it is to note that aerobic glycolysis represents a less efficient metabolism, generating only 4 mol of ATP/mol of glucose with respect to 36 mol/mol generated by OXPHOS. Why cancer cells prefer a less efficient metabolism in terms of ATP production? Different speculations have been proposed for the maintenance of this apparently wasteful catabolic state. One such speculation is that the mitochondrial respiration malfunction and enhancement of glycolysis are believed to be a metabolic advantage under the intermittent hypoxic conditions experienced by pre-malignant and malignant tumor cells [130,131]. However, aerobic glycolysis is not only encountred in cancer cells, but is also found in rapidly dividing normal cells under conditions of normoxia as well [132]. Furthermore, it has been also observed that the glycolysis rate enhances in order to satisfy the increased anabolic needs of the rapidly proliferating cells for precursor metabolites [123]. Nevertheless, a focus on the cell's anabolic needs alone overlooks two important points: first, in addition to precursor metabolites,

rapidily growing/proliferating cells need ATP to fulfill the energy requests of biosynthetic pathways. Second, by definition, the Warburg effect denotes the increase in the glycolysis rate resulting in the production of lactate as final product, which is not involved in the production of precursor metabolites. Actually the molecular mechanisms involved in the enhanced aerobic glycolysis are increasingly well recognized. For example, in physiological conditions, the phosphatidylinositol-3-kinase/Protein Kinase B (PI3K)/(AKT) o signaling pathways play a critical role in promoting aerobic glycolysis in activated T cells in response to growth factors or cytokines stimulation [133]. During tumorigenesis, defects of mitochondrial respiration due to mitochondrial DNA mutations/deletions represents a major important contributing factor [134-137]. Furthermore, the oncosuppressor p53, one of the most frequently cancer-related mutated genes, can act both as a positive regulator of mitochondrial respiration [138] as well as a negative regulator of glycolysis [139]. These events, along with the concomitant activation of hypoxia-inducible factor 1α (HIF 1α), a transcription factor activated by hypoxic-, oncogenic-, metabolic- and oxidative stresses, often lead to the upregulation of the glucose transporters to promote the retention of glucose inside cells in addition to upregulating hexokinase 2, the enzyme that catalyzes the first step of glycolysis and different glycolysis pathway enzymes or isozyme subtypes, thus explaining the increased glucose uptake and altered utilization [122,140]. The studies carried-out till date suggest that the presence of aeroblic glycolysis represents a peculiar feature of rapidly proliferating cells, and that also it may give a growth advantage to both rapidly proliferating normal cells,

e.g., during development and tissue regeneration, and to cancer cells during tumor development.

2.2 The role of lipid metabolism in cancer

The study of cancer cell metabolism has grown exponentially during the last decade and it is commonly accepted that cancer cells rely on increased glycolytic activity and and impaired OXPHOS (Warburg effect) [141]. However, intense researches - especially with the use of lipidomic-based technologies - have shed-light of how lipid metabolism plays an important role in cancer biology [142-144]. To date, compelling evidences suggest that the reprogramming of cellular lipid metabolism is directly involved in malignant transformation and cancer progression [145,146]. For example, rapidly proliferating tumors, through *de novo* lipid synthesis (DNL), can obtain a constant lipids supply for membrane biogenesis of new daughter cells [147,148]. Furthermore, the upregulation of mitochondrial fatty acid β-oxidation (FAO) can support tumor cell energetics and redox homeostasis [149], while lipid-derived messengers can regulate major signaling pathways or coordinate immunosuppressive mechanisms [150-152]. Therefore, lipid metabolism has been involved in a broad range of oncogenic processes, such as metastatic colonization, drug resistance, and cell differentiation [151,153-157]. Lipids are heterogeneous biological molecules which comprhend phospholipids, fatty acids, triglycerides, sphingolipids, cholesterol, and cholesteryl esters [158]. Lipids have a wide distribution in cellular organelles and represent crucial components of all membranes [159-164]. In addition to their role as structural cell and organelle membrane elements, lipids also

display many pivotal roles. For example, lipids can act as second messengers to transduce signals within the cells, and can also serve as energy sources when nutrients availability is limited [165-168]. Lipids are intermediates and even signaling molecules of metabolic pathways that can be broken down into bioactive lipid mediators in order to regulate a large number of neoplastic processes, such as cell growth, cell migration and metastasis formation [169,170]. Mammalian cells were found to readily obtain lipids by using two distinct mechanisms: i.e., DNL synthesis and uptake. While the DNL synthesis has found to be low in normal adult – except for lipogenic tissues such as liver, adipose tissue, or mammary epithelium during lactation) – a growing body of evidences have shown that lipid metabolism is considerably reprogrammed in cancers [171-176]. On one hand, the increased DNL synthesis not only facilitate the formation of membrane lipid bilayers but also allows the cells to alter their membrane composition in favor of oxidative damage-resistant saturated fatty acids as means of adapting to oxidative stress [147]. Indeed, it has been also shown that rapidily growing cancer cells show a strong lipid and cholesterol avidity, which they fulfill by either increasing the uptake of exogenous lipids and lipoproteins or overactivating their endogenous synthesis (lipogenesis and cholesterol synthesis). High content of lipids and cholesterol in cancer cells are stored in lipid droplets (LDs), and excessive amount of LDs and stored-cholesteryl ester content in tumors have been considered as hallmarks of cancer aggressiveness [177-183] (Fig. 5).

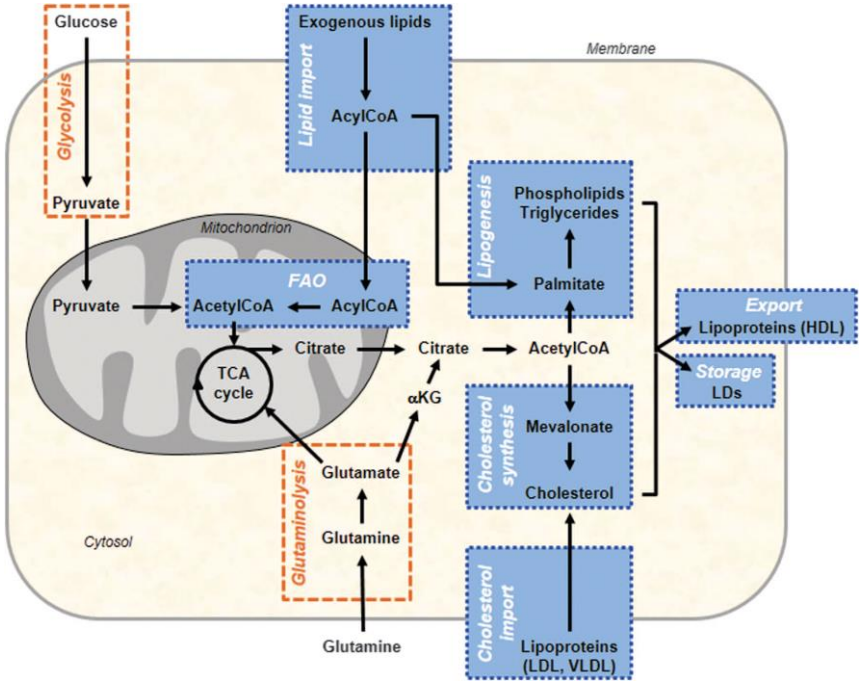


Fig. 5. A simplified map of the main altered lipid metabolic pathways in cancer cells. Lipid metabolic network (blue) includes import/export and catabolic pathways (FAO) as well as de novo synthesis pathways, such as lipogenesis (that is, synthesis of TGs and PLs) and cholesterol synthesis. Glucose- and/or glutamine-derived citrate, provided by the increased glycolysis and/or glutaminolysis, are common precursors of lipogenesis and cholesterol synthesis. Cancer cells can also take up exogenous cholesterol, transported by LDL and very-lowdensity lipoproteins (VLDL), to meet their cholesterol requirement. When cholesterol, PLs and TGs are in excess in tumors, they are exported into circulation as high-density lipoproteins (HDLs) or locally stored into LDs. Exogenous FAs taken up by cancer cells are broken down to produce energy through mitochondrial FAO process. TCA cycle, tricarboxylic acid cycle αKG, α-Ketoglutarate. From Beloribi-Djefaflia S et. al, 2015 [145].

Breast, prostate and colorectal cancer(s) are characterized by an aberrant lipid metabolism which involves fatty acid synthesis (FAS) and FAO,

upregulating fatty acid synthases (FANS), acetyl-CoA carboxylase (ACC) and ATP citrate lyase (ACLY), respectively [167,184,185] (Fig.6).

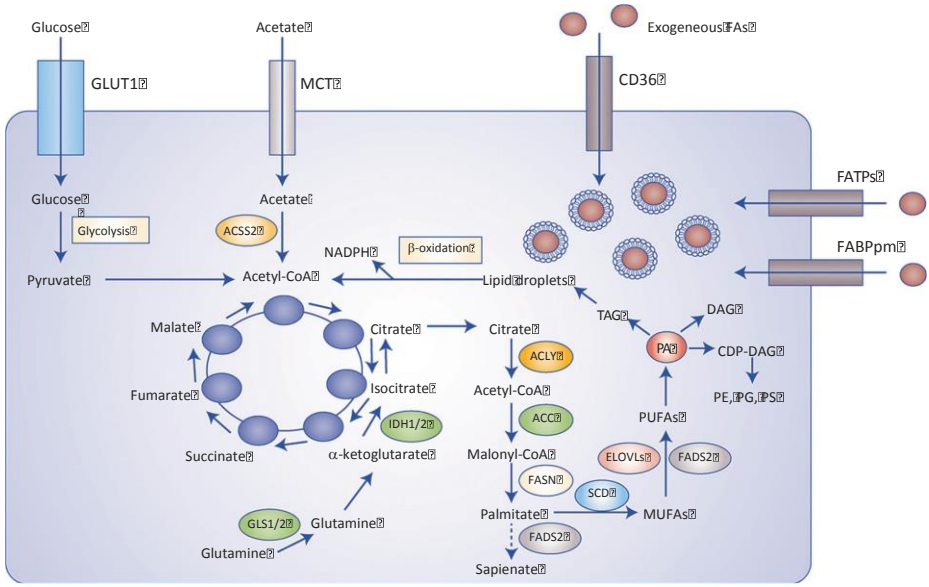


Fig. 6. Cancer cells obtain fatty acids (FAs) from de novo lipogenesis and exogenous uptake. The exogenous uptake of FAs from the surrounding microenvironment is facilitated by specialized transporters, including CD36, FATPs and FABPpm. FAs and their synthetic products can be subsequently stored as LDs, and used for NADPH and acetyl-CoA production through β-oxidation. In terms of carbon sources for de novo lipogenesis, cancer cells rely on glucose, glutamine and acetate to synthesize citrate. Palmitate is ultimately generated from citrate through the enzymatic activities of ACLY, ACC and FASN, and can subsequently be desaturated and elongated to form a diverse group of lipid species. An alternative pathway for palmitate desaturation exists, which generates sapienate through FADS2, instead of palmitoleate. Abbreviations: GLUT1, glucose transporter 1; MCT, monocarboxylate transporter; CD36, cluster of differentiation 36; FATPs, fatty acid transport proteins; FABPpm, fatty acid-binding protein; GLS, glutaminase; IDH1/2, isocitrate dehydrogenase; ACLY, ATP–citrate lyase; ACSS2, acyl-CoA synthetase short-chain family member 2; ACC, acetyl-CoA

carboxylase; FASN, fatty acid synthase; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SCD, stearoyl-CoA desaturase-1; FADS2, fatty acid desaturase 2; ELOVLs, elongation of very long-chain fatty acid protein; PA, phosphatidic acid; TAG, triacylglycerol; DAG, diacylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS; phosphatidylserine. From Nikos Koundouros and George Poulogiannis, 2020 [186].

The primary source for the initial step of *DNL* synthesis involves Citrate. Its synthesis occurs in the mitochondrial TCA cycle involving a two-step reaction: decarboxylation of pyruvate to form acetyl CoA with subsequent condensation of the acetyl-group with oxaloacetate to form citrate (Fig. 6). The synthesized citrate is excreted back into the cytosol where it reacts with acetyl CoA *via* ACLY for the growing lipid acyl chains. In addition, acetyl CoA can be synthesized from acetate via acetyl CoA synthetase 2 (ACCS2). The carboxylation of acetyl CoA to malonyl CoA (malonyl Coenzyme A) by ACC represents the first reaction of fatty acid synthesis. Subsequently, malonyl-CoA is converted by FASN through repeated condensation reactions of acylgroups to form a 16-carbon saturated fatty acid, namely palmitate. The latter can then be modified to give rise to other saturated and unsaturated fatty acids with more than 16-carbons. The desaturation process is catalyzed by stearoyl-CoA desaturase (SCD) [187]. Acetyl CoA can also be used to generate cholesterol, an important building block for steroid synthesis and membranes. Specific components of the lipogenic machinery have been shown to play a protumorigenic role in several cancers. For example, FASN results to be upregulated in several human malignancies [188-190], thus suggesting its involvement in cancer development and also a useful pharmacological target [167]. At the same time,

it has been shown that ACLY plays a pivotal role in human breast cancer since its involvement is required for transformation, migration and invasion of breast cancer cells *in vitro* along with tumor growth *in vivo* [191]. Lipids uptake and storage are also upregulated in several malignant tumors [155,178,192,193]. For example, sterol regulatory element-binding proteins (SREBPs) are key transcription factors that play a critical role in regulating the expression of genes involved in both lipid synthesis (e.g, cholesterol synthesis and uptake under physiological and pathological conditions[194-196]. Several lines of evidence suggest that SREBPs are involved in various metabolic and cancer disease(s) [197-200]. However, while fatty acid synthesis represents a well-investigated complex metabolic network, the specific role of FAO, namely as source of energy and elements for sustained proliferation, still remains a field to finely explore, especially in CMTs.

With respect to the lipogenic phenotype, the role of mitochondrial FAO in tumors has been also investigated [201,202]. Although previous studies of cancer bioenergetics have paid attention on the Warburg effect [123,126], FAO also represents a major energy source in terms of ATP production [203]. Apart from this, another main function of FAO is the production of cytosolic Nicotinammide Adenine Dinucleotide Phosphate (NADPH) [204], an essential source reducing power to support biosynthesis and for neutralizing oxidative stress [205]. FAO is a multi-step catabolic process occurring in the mitochondria characterized by repeated series of reactions to yield fatty acids shortened by two carbon atoms. The overall set of these reactions ends up with the production of NADH, FADH2 (Flavin Adenine Dinucleotide) and acetyl CoA in each round, until the last cycle - where the catabolism of a four-carbon fatty acid forms - two acetyl CoA

molecules [206]. NADH and FADH2 generated by FAO process enter the electron transport chain to produce ATP [207] (Fig. 7). Additionally, the FAO-derived NADPH represents an essential element for cancer cells to quench reactive oxidative stress [208].

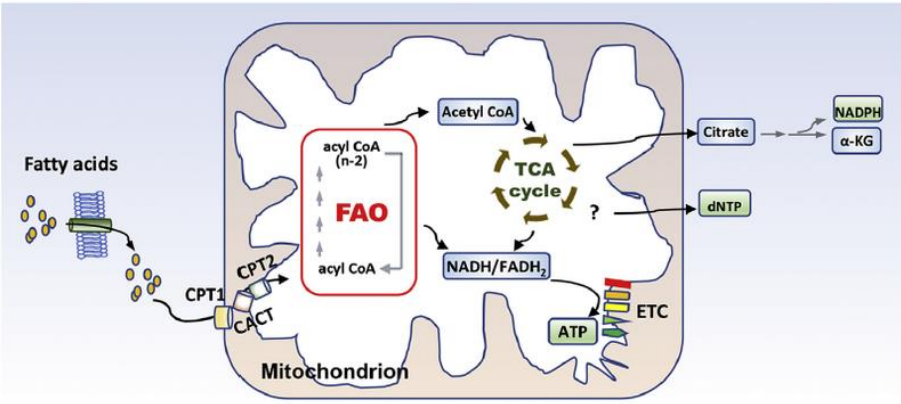


Fig.7. FAO basics

Long chain fatty acids enter cells via fatty acid transport proteins and then shuttled into the mitochondrion by the carnitine shuttle system. In the mitochondrion, fatty acids undergo oxidative removal of successive 2-carbon unit in the form of acetyl-CoA. Acetyl-CoA will be oxidized to CO2 through the TCA cycle. Both FAO and the TCA cycle produce reduced electron carriers (NADH/FADH2), which will pass electrons to ETC to yield ATP. Apart from bioenergetic production, the carbon and hydrogen sources of FAO-generated acetyl CoA can be exported out of the TCA cycle to the cytoplasm to engage in NADPH and dNTP production. From Ma Yibao et.al, 2015 [209].

To date, it is well known that the so-called oxidative tumors (non-glycolitic tumors), such as prostate and B-cell lymphoma need the FAO

pathway to fulfill their energetic requests [201,210]. Moreover it has also been reported that FAO can be considered as a druggable metabolic pathway for cancer treatment [209]. For example, it has been reported that the pharmacologic inhibition of FAO decreases energy metabolism in MYC-overexpressing in triple-negative breast cancer cells [211]. Again, the inhibition of FAO induces apoptosis in leukaemia and glioblastoma cells [204,212]. Finally, FAO has been also implicated in several aspects of metastaic process, due to its ability to promote invasion of tumor cells to neighboring tissues, intravasation of cancer cells into blood or lymphatic vessels, survival of cancer cells in the circulatory and lymphatic systems, extravasation, colonization of cancer cells in other organs and for its pivotal role in regulation of cancer stem cells [155].

2.3 The involvement of Carnitine System (CS) in Cancer

For being used as energy substrates, fatty acids require approximately 25 different enzymes and carrier proteins which are involved in different functions, such as the cellular uptake and activation of fatty acids, their import into the mitochondria and the FAO steps. Fatty acids must be activated to fatty acyl Coenzyme (acyl CoA) by fatty acyl CoA synthetase before they can be shuttled into the mitochondria. This is an important prerequisite, as the mitochondrial inner membrane is impermeable to fatty acyl CoA thioesters and, thus, the carnitine system (CS) is responible for fatty acids tranport across mitochondrial membranes [213]. CS is composed of both enzyme able to catalyze the acylCoA+carnitine↔CoA+acylcarnitines reaction and carrier(s) involved

in the bi-directional transport of acyl moieties from cytosol to mitochondria and *vice-versa* [203]. Three components take part to this system: the carnitine palmitoyltransferase 1 (CPT1), the carnitine acylcarnitine translocase (CACT) and carnitine palmitoyltransferase 2 (CPT2). Furthermore, carnitine acetyltransferase (CrAT) closes the carnitine cycle, allowing the acetyl CoA export produced during FAO as acetylcarnitine in the cytosol (Fig. 8).

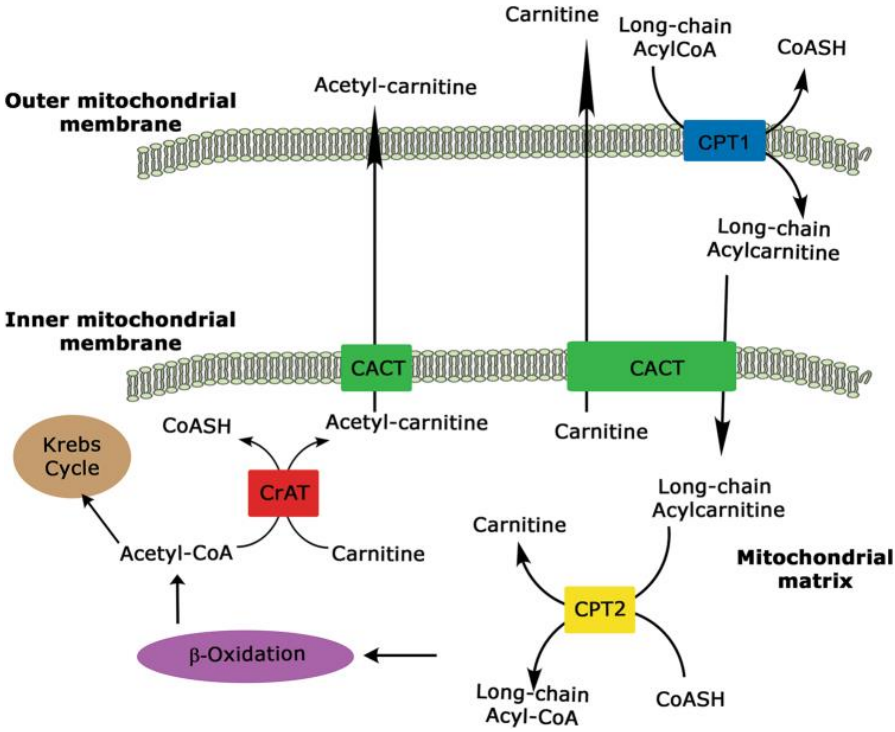


Fig. 8. Schematic view of carnitine system.

The long-chain fatty acid-CoAs are converted into the carnitine derivatives by CPT1, located on the outer mitochondrial membrane of the contact sites. A specific carnitine-acylcarnitine translocase (CACT) catalyzes the mole-to-mole exchange of

carnitine/acetylcarnitine and acylcarnitines promoting the import of the acylcarnitines through the mitochondrial membranes. In the mitochondrial matrix, long-chain acylcarnitines are reconverted to the respective long-chain acyl-CoAs by carnitine-palmitoyltransferase-2 (CPT2) and undergo β-oxidation to produce acetyl-CoAs. Finally, CrAT converts short-chain acetyl-CoAs to their membrane permeant acetylcarnitine counterparts, allowing CACT to export them from mitochondrion to cytoplasm. From Melone M.A.B et al., 2018 [203].

CPT1 localizes on the outer mitochondrial membrane and works by converting long chain-fatty acyl CoA to fatty acylcarnitine derivatives. Three subtypes of CPT1 exist: CPT1A is mainly expressed in liver [214], whereas CPT1B is present in tissue characterized by a high rate of FAO such as, muscle tissue and brown adipose tissue [215]. CPT1C represents the third and final isoform, which is normally encountred only in the brain [216]. In physiological settings, all these three isoforms are inhibited by malonyl-CoA, but due to the greater binding efficiency of CPT1A to malonyl CoA, it is found to be the isoform with the greatest capacity to perform the rate-limiting step of FAO [217,218]. CACT, grapples on the inner mitochondrial membrane and acts by shuttling acylcarnitine into the mitochondrial matrix. Mutations in CACT gene have been linked to a severe carnitine-acylcarnitine translocase deficiency, leading to pathological disorders including muscle weakness, cardiac arrest, hypoglycemia, hepatic dysfunction and hepatomegaly, which is even lethal in infants [219]. In the mitochondrial matrix, CPT2 acts reconverting long-chain acylcarnitines to the respective long-chain acylCoAs. Lastly, CrAT acts by catalyzing the reversible transfer of acyl groups from a short-chain acyl CoA thioester to carnitine and regulates the ratio of acylCoA/CoA in the subcellular

compartments [220]. Defects in CRAT gene result in muscle insulin resistance due to accumulation of long-chain acylcarnitines [221]. Growing body of evidences has linked CPT1 family members to tumorigenesis [222]. For example, the expression of CPT1A, CPT1B, and CPT2 has been found to increase 3- to 4-fold in chronic lymphocytic leukemia cells compared to normal stromal cells [222]. Interestingly, the increased CPT1A expression found in acute myeloid leukemia and ovarian cancer also correlates with poor patient outcomes [223,224]. Other studies have shown that inhibition/depletion of CPT1 leads to apoptosis and suppression of cancer cell proliferation and chemoresistance [225]. Furthermore, Ricciardi MR *et al.* (2015) in their elegant study have shown that the CPT1A inhibitor ST1326 possesses marked cytotoxic activity against different leukemia cell lines and primary cells obtained from patients with hematologic malignancies [226]. They have demonstrated that ST1326 inhibited FAO in leukemia cell lines by causing a concentration- and time-dependent cell growth arrest, mitochondrial damage, and apoptosis induction [226]. Moreover, in another study, Pacilli and *coll.* (2013) have shown that ST1326-mediated FAO inhibition is due to the concomitant blocking of CPT1A- and CACT-activity [227]. In closing, taken all toghether, these studies clearly point out that FAO pathway is an emerging metabolic feature of certain tumor type, especially for those with a "lipolytic phenotype" and a novel druggable therapeutic target.

Chapter 3

Aim of the study

3. Aim of the study

CMTs are the most common type of neoplasia in female dogs; 50% of which are malignant [35]. Tumors originating from the mammary gland in dogs and in humans display many clinical and molecular similarities [14]. Therefore, spontaneous canine mammary cancer could represent an excellent animal model for studying several complex molecular mechanisms that occur during malignant transformation and thus, may be helpful for improving prognosis and treatment of human breast cancer [14]. Malignant transformation produces metabolic alterations that can contribute to cancer development and progression. Metabolic reprogramming refers to the ability of cancer cells to alter their metabolism in order to support the increased energy requests due to continuous growth, rapid proliferation, and other characteristics typical of neoplastic cells [203]. To date, metabolic adaptations are considered a hallmark of cancer [119]. Despite it has been proposed that altered oxidative stress and carbohydrate metabolism represent a clear and defining metabolic network in CMTs [228], it still remains unknown how and if other metabolic changes occur and whether they confer the aggressive phenotype to tumor cells. In this study, we aim to verify if the components of the Carnitine System (CS), regulating fatty acid mitochondrial import/export, are expressed and therefore implicated in CMTs metabolism. For this reason, we evaluated CPT1A, CACT, CPT2, and CRAT expression in CMT cell lines and, additionally in canine mammary specimens derived from bitches suffering from CMTs. Furthermore, we have also tested the tumoricidal potential of the CPT1A inhibitor ST1326 in CMT cell lines and also investigated the

molecular signaling pathways involved in both cell apoptosis and survival pathways. In closing, this thesis work aims to demonstate, for the first time, the involvement of FAO pathway (*via* the CS components expression) in CMTs. The results of this research also point out new druggable pathway for the prevention and treatment of CMTs. In fact, the preclinical evidence for the anticancer potential of CPT1A inhibitors may give a great likelihood for those veterinary patients suffering from CMTs who do not benefinit with conventional chemotherapeutics.

Chapter 4

Materials and Methods

4.1 Cell cultures

Cell lines used in this study were: P114 (canine mammary anaplastic cells), CMT-U309 (spindle-cell mammary tumor cells), CMT-U27 (simple ductal mammary carcinoma cells), CMT-U229 (atypical benign mixed tumor), CMT-U131 (infiltrating ductal carcinoma of scirrhous type), CF33 (canine mammary carcinoma) and MDCK (normal canine epithelial cells). P114 cells were a kind gift of Dr. Gerard Rutteman (Department of Clinic Science and Companion Animals, University of Utrecht, The Netherlands) and cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) (Euroclone, Milan, Italy). CMT-U309, CMT-U27, CMT-U229 and CMT-U131 cells were a generous gift of Prof. Dr. Eva Hellmen (Department of Anatomy, Physiology and Biochemistry, Uppsala University, Sweden) and maintained in Roswell Park Memorial Institute (RPMI) medium (Euroclone). MDCK and CF33 cells were a gift of Dr. Serena Montagnaro (Department of Veterinary Medicine and Animal Productions, University of Naples) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Euroclone). All the cell lines were supplemented with 10% foetal bovine (FBS) serum (Euroclone), 1% antibiotics (penicillin and streptomycin) (Euroclone) and 1% glutamine (Euroclone). Cells were grown at 37 °C in a 95% air and 5% CO_2 atmosphere.

4.2 Mammary tissue samples

Detailed information is listed in Table 3. 47 CMT samples were used for immunohistochemical analysis of CPT1A protein, whereas 22, 29 and 25 CMT samples were used for immunitochemical analysis of CACT, CPT2 and CRAT proteins, respectively. The CMT samples were obtained by routine surgical excision, carried-out on 47, 22, 29 and 25 female dogs, for tumor treatment with owner's consent at the Veterinary Teaching Hospital of the Naples Federico II University. The research protocol was approved by the Ethical Animal Care and Use Committee of the University of Naples Federico II (Prot. N° PG/2019/0121361). Immediately after the surgical resection, the specimens were routinely divided into two aliquots and stored under appropriate conditions based on the diagnostic analyses to be performed. In addition, and as controls, six macroscopically and histologically normal mammary gland (NMG) tissue samples were used which were excised routinely during tumor surgery according to the standard surgical treatment protocol; these control tissues, were obtained from those cases where the entire chain of MGs was excised. For hematoxylin and eosin staining and immunohistochemical analysis, samples were fixed in 10% neutral buffered formalin and then paraffin-embedded, while for western blot (WB) analysis, samples were weighed, washed in Phosphate Buffered Saline (PBS) and stored at -80°C. Histological diagnosis and grading on mammary tissue samples were performed according to Goldschmidt's updated classification and criteria [22]. Tumors were divided in 3 malignancy grades: 14 well (G1), 14 moderately

(G2) and 14 poorly (G3) differentiated. For CPT1A protein expression five benign tumors were also included in this study.

Table 3: Main characteristics of normal and neoplastic canine mammary tissues and CPT1A immunoreactivity cells, staining intensity and immunoreactive (IR) score

N°	Breed	Age (years)	Histotype	Tumor grade	CPT1A immunoreactive cells	Staining intensity	IR score
1	MIXED BREED	9	NORMAL MAMMARY GLAND	Normal gland	51,2 %	2	6
2	GERMAN SHEPHERD	7	NORMAL MAMMARY GLAND	Normal gland	48,4 %	3	9
3	MIXED BREED	4	NORMAL MAMMARY GLAND	Normal gland	60.3%	2	8
4	POODLE	11	NORMAL MAMMARY GLAND	Normal gland	0%	0	0
5	JACK RUSSEL TERRIER	5	NORMAL MAMMARY GLAND	Normal gland	57,2%	3	9
6	MIXED BREED	9	NORMAL MAMMARY GLAND	Normal gland	0%	0	0
7	AMERICAN TERRIER	6	ADENOMA SIMPLE	Benign	75%	3	12
8	MIXED BREED	11	COMPLEX ADENOMA	Benign	56,4%	3	9
9	MIXED BREED	8	ADENOMA SIMPLE	Benign	70,8 %	2	8
10	MIXED BREED	9	BENIGN MIXED TUMOR	Benign	52,3 %	3	6
11	YORKSHIRE TERRIER	10	COMPLEX ADENOMA	Benign	52,6 %	3	9
12	MIXED BREED	9	TUBULAR CARCINOMA	1	27,5 %	3	6
13	BEAGLE	6	SIMPLE CARCINOMA	1	78,4 %	3	12
14	COCKER SPANIEL	9	TUBULOPAPILLARY CARCINOMA	1	75,4 %	3	12
15	MIXED BREED	14	TUBULAR CARCINOMA	1	70,4 %	3	12
16	MIXED BREED	8	TUBULOPAPILLARY CARCINOMA	1	28,0 %	2	4
17	MIXED BREED	11	SIMPLE CARCINOMA	1	51,9 %	2	6
18	SHIH TZU	9	TUBULOPAPILLARY CARCINOMA	1	87,5 %	3	12
19	IRISH SETTER	7	MIXED TYPE CARCINOMA	1	90,0 %	3	12
20	YORKSHIRE TERRIER	8	COMPLEX TYPE CARCINOMA	1	79,2 %	2	8
21	PINSCHER	9	COMPLEX TYPE CARCINOMA	1	69,5 %	3	12
22	YORKSHIRE TERRIER	13	COMPLEX TYPE CARCINOMA	1	47,9 %	3	9

23	JACK RUSSEL TERRIER	3	MIXED TYPE CARCINOMA	1	52,4 %	3	9
24	YORKSHIRE TERRIER	8	COMPLEX TYPE CARCINOMA	1	84,3 %	3	12
25	MIXED BREED	7	TUBULOPAPILLARY CARCINOMA	1	54,5 %	3	9
26	MIXED BREED	9	CYSTIC PAPILLARY CARCINOMA	2	39,2%	2	6
27	EPAGNEUL BRETON	10	TUBULOPAPILLARY CARCINOMA	2	12,3%	2	4
28	MIXED BREED	6	MIXED TYPE CARCINOMA	2	47,5	2	6
29	COCKER SPANIEL	10	TUBULAR CARCINOMA	2	59.8%	3	9
30	MIXED BREED	11	TUBULAR CARCINOMA	2	65,9%	2	8
31	MIXED BREED	9	SOLID CARCINOMA	2	32,1%	2	6
32	MIXED BREED	9	TUBULAR CARCINOMA	2	25,1%	2	6
33	MIXED BREED	13	TUBULAR CARCINOMA	2	25,1%	2	4
34	SHIH TZU	9	TUBULAR CARCINOMA	2	21,2%	3	6
35	MIXED BREED	9	TUBULOPAPILLARY CARCINOMA	2	22,4%	3	6
36	COCKER SPANIEL	6	COMPLEX TYPE CARCINOMA	2	21,7%	1	2
37	MIXED BREED	10	TUBULOPAPILLARY CARCINOMA	2	65,7%	3	12
38	MIXED BREED	13	TUBULAR CARCINOMA	2	72,0%	3	12
39	POODLE	15	COMPLEX TYPE CARCINOMA	2	90,0%	2	8
40	MIXED BREED	13	TUBULAR CARCINOMA	3	51,9%	2	6
41	MIXED BREED	12	TUBULAR CARCINOMA	3	12,0%	3	6
42	MIXED BREED	11	COMPLEX TYPE CARCINOMA	3	6,4%	2	2
43	SHIH TZU	9	COMPLEX TYPE CARCINOMA	3	7,4%	1	1
44	MIXED BREED	12	MIXED TYPE CARCINOMA	3	10,2%	1	2
45	GREYHOUND	9	SPINDLE CELL CARCINOMA	3	5,7%	3	3
46	MIXED BREED	13	ADENOSQUAMOUS CARCINOMA	3	4,7%	1	1
47	MIXED BREED	9	CARCINOMA AND MALIGNANT MYOPITHELIOMA	3	28,4%	2	4
48	PINSCHER	7	COMPLEX TYPE CARCINOMA	3	8,2%	1	1
49	MIXED BREED	13	MIXED TYPE CARCINOMA	3	27,7%	3	6
50	SIBERIAN HUSKY	11	COMEDOCARCINOMA	3	5,6%	1	1

51	GERMAN SHEPHERD	4	TUBULAR CARCINOMA	3	32,6%	1	3
52	BEAGLE	6	TUBULAR CARCINOMA	3	31,0%	2	6

4.3 Immunohistochemistry (IHC)

Mammary tissue sections were dewaxed in xylene, dehydrated in graded alcohols and washed in 0.01 M PBS pH 7.2–7.4. Endogenous peroxidase was blocked with hydrogen peroxide 0.3% in absolute methanol for 30 min. The immunohistochemical procedure (streptavidin biotin-peroxidase method LSAB Kit; Dako, Glostrup, Denmark) was described elsewhere [229]. Primary mouse anti-human CPT1A or primary rabbit anti-human CACT, CPT2, or CRAT antibodies were diluted in an antibody diluent (Dako) and applied for two hours at RT. The immunolabelling procedure included negative control sections incubated with normal serum IgG (Dako) instead of the primary antibody. A mixture of biotinylated anti-mouse and anti-rabbit immunoglobulins (LSAB Kit; Dako), in PBS, were used as secondary antibody and applied for 30 min. After washing in PBS, sections were incubated in streptavidin conjugated to horseradish peroxidase in Tris–Cl buffer containing 0.015% sodium azide (LSAB Kit; Dako) for 30 min. To reveal immunolabelling, diaminobenzidine tetrahydrochloride was used as a chromogen and hematoxylin and used as counterstain.

4.3.1 Scoring of immunoreactivity

Immunohistochemical analysis was carried-out by semiquantitative method in which the intensity and the percentage of cells showing positive

staining were taken into consideration. The scoring was performed blinded by two pathologists (BR and MS). The percentage of immuno-positive cells weas obtained from 10 random fields *per* case and *per* section, using a 40 × objective lens and quantified by counting the number of CPT1A, CACT, CPT2 or CRAT positive cells and by arbitrarily assigning scores as follows: score 0: no positive cells, score 1: < 10% positive cells, score 2: 10–30% positive cells, score 3: 31–60% positive cells, score 4: > 60% positive cells, as previously reported [230,231]. Staining intensity was scored: 1 for weak, 2 for moderate and 3 for strong staining. Then, a combined immunoreactivity (IR) score, ranging from 0 to 12, was calculated for each specimen by multiplying the values of these two categories (Table 2).

4.4 Samples preparation, protein extraction and western blot (WB)

WB analysis, carried out in order to validate antibody cross-reactivity, employed complementary tissues from three canine NMGs and 11 CMTs (4 G1, 4 G2 and 3 G3) that were snap frozen upon collection and stored at −80°C. Cells were resuspended in lysis buffer containing: 25mM Tris-Cl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1× protease/phosphatase inhibitors (Roche, Basel, Switzerland). Mammary tissues were homogenized, using an ultra Turrax ™ (Ika-Werke GmbH, Staufen, Germany), in the same lysis buffer and sonified 3 × 15 s and kept on ice for 30 min. Then, the tissue or cell lysates were spun at 16,000 × g for 20 min and protein content was determined using the BCA assay kit (Thermo Fisher Scientific, Waltman, USA). Equal amounts of protein lysates were separated by 8-

15% Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane (Perkin Elmer, Boston, USA). After blotting, membranes were blocked for 60 min with Tris Buffered Saline-Bovine Serum Albumin (TBS-BSA) solution containing 25 mM Tris pH 7.4, 150 mM NaCl and 5% BSA. Thereafter, membranes were incubated with the primary antibodies against CPT1A (8F6AE9 Abcam, Massachusetts, USA), SLC25A20 (CACT) (NBP1-86690, Novus Biological, Colorado, USA), CPT2 (NBP1-85471, Novus Biologicals), CRAT (ab153699, Abcam), p-ERK Thr202/Tyr204 (#9101, Cell Signaling Technology Inc., Danvers, MA), total ERK (#9102, Cell Signaling Technology), p-Akt Ser 473 (#9271, Cell Signaling Technology Inc.), total Akt (#9272, Cell Signaling Technology Inc.), Vinculin (sc-7649, Santa Cruz Biotechnology Inc.), β-actin or GAPDH (Santa Cruz Biotechnology, California, USA) at room temperature (RT) for 60 min or overnight at 4°C on a rocker. Subsequently, membranes were washed three times with TBS buffer containing 0.05% Tween® 20 and incubated with the horseradish peroxidase-conjugated secondary antibody (1: 10000) at RT for 60 min. Immunoreactive bands were visualized by using an enhanced chemiluminescent kit (Thermo Scientific, Rockville, USA) and quantified by using Image J software (National Institutes of Health, Bethesda, USA).

4.5 Isolation of RNA and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from CMT cell lines and normal epithelial cell lines and extracted according to Trizol procedure. The quality and the quantity of total RNA were assessed by using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltman, USA). Briefly, 1 µg total RNA was reverse-transcribed into complementary DNA (cDNA) by using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) following the manufacturer recommendations. The resulted cDNA was amplified in 96-well plates on LightCycler 480 RT-PCR System with SensiFAST SYBR® Hi-ROX Kit (Bio-Line, London, UK). Primers used were: CPT1A-Fw-5'-TTCCATTCTTTCCCTTTCCAC-3', CPT1A-Rv-5'-GTCTCCGTCCTCCCTTCG-3'; HPRT-Fw-5'-TGCTCGAGATGTGATGAAGG-3', HPRT-Rv-5'-TCCCCTGTTGACTGGTCATT-3'. Both genes were amplified by using the following thermal cycling conditions: 2 minutes of incubation at 95°C, followed by 40 cycles of 5 sec. denaturation at 95°C and 15 sec. annealing and elongation at 60°C. Data were collected at the end of elongation step. The relative changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method [232]. Primers for qRT-PCR analysis were designed using Primer3 software (http://bioinfo.ut.ee/primer3/).

4.6 Assessment of Cell Viability by Trypan blue assay

For the determination of cell viability, CMT-U309, P114, CMT-U27, CMT-U229 and CMT-U131 CMT cells were seeded at appropriate density

in 24 well tissue culture plate and cultured for 24h hours at 37°C. Thereafter, the cells were treated with increasing amounts of ST1326 (1-20 μM) in a medium containing 1% FBS for further 48 hours. The cultures were harvested and washed twice with PBS; the cell pellet was then suspended with 0.5 ml PBS. Then 20 μl of cells was mixed with equal volume of 0.4 % trypan blue (Sigma-Aldrich, USA). Cell counts were performed in triplicates using a digital cell counter (Bio Rad TC20, Hercules, CA, USA) with trypan blue 0.4% v/v exclusion dye to identify viable cells. The total numbers of viable cells in each experiment were compared with those of the parallel-untreated control cell counts performed simultaneously in three independent experiments.

4.7 Evaluation of Apoptosis by Flow Cytometry (FCM)

The apoptosis rate of CMT-U229 and CMT-U131 cells induced by ST1326 was measured by using FCM. Cells were double stained with Kit Annexin V-Fluorescein Isothiocyanate (Annexin V-FITC) and Propidium Iodide (PI) apoptosis assay (Dojindo Molecular Technologies Inc., Munich, Germany). Cells were seeded into 100 mm dishes at density of 6×10^5 and 4×10^5 in RPMI medium containing 10% FBS, respectively. After 24h, the cells were treated with increasing amount of ST1326 (1-5-10-20 μM) in a medium supplemented with 1% FBS for 48 h. After that, the cells were detached with trypsin and centrifuged at 400 × *g* for 5 min, washed twice with 1 mL PBS, and the supernatant was discarded. Subsequently, the cells were resuspended with 400 μL of binding buffer. Then, 5 μL of Annexin V-FITC and 5 μL PI staining solution were added into the suspension and

incubated at RT for 15 min. The apoptosis rate of the cells was analyzed within 30 min with BD FACSCanto II system and elaborated using the DIVA software (BD Biosciences, Milan, Italy). For each condition, at least 10,000 events were recorded.

4.8 Statistical analysis

Statistical analysis was carried-out by using GraphPad Prism v5.0 Software (La Jolla, California, USA). Data are presented as mean ± standard error mean (SEM) of indicated replicate experiments. Data analysis was performed using Student's *t* test for the comparison between two groups, whereas one-way Analysis of Variance (ANOVA) with Tukey's *post* test was used for the comparison of multiple groups. Results with $p < 0.05$ were considered statistically significant.

Chapter 5

Results

5.1 Carnitine System in Canine Mammary Tumors (CMTs)

5.1.1 CPT1A is overexpressed in CMT cell lines

We have examined CPT1A expression by WB analysis in CMT cell lines and we have found higher levels of CPT1A expression in all three CMT cell lines analyzed (CMT-U27, P114 and CMT-U309) compared to the immortalized normal epithelial cell line MDCK (Fig. 9a). MDCK cells have beel used as comparator according to a previous paper which used this cell line as control [233]. Densitometric analysis of WB has revealed a significant increase in CPT1A by 7.31-, 5.26- and 7.53-fold in CMT-U309, P114 and CMT-U27 cells, respectively (Fig. 9b). In addition, we have also verified whether the changes observed in protein expression are also detected at mRNA levels. Specifically, as shown in Fig. 9c, we have found that, in comparison to MDCK cells, the CPT1A mRNA expression increases of 15.1-, 8.8- and 12.3-fold in CMT-U309, P114 and CMT-U27 cells, respectively.

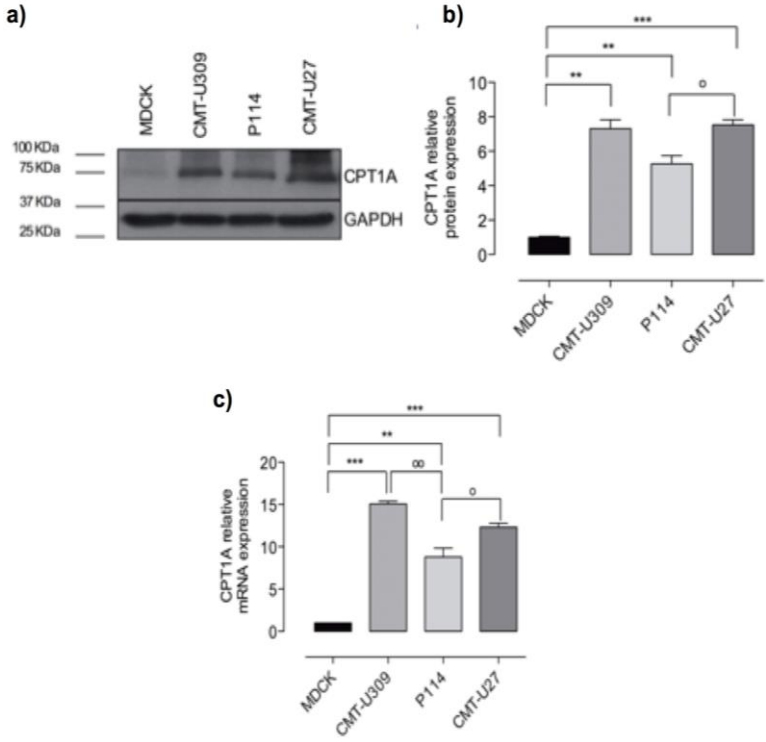


Fig.9. Expression of CPT1A protein and mRNA in canine mammary cell lines

*a) Representative western blot analysis and b) densitometric values of CPT1A protein expression in normal epithelial cells (MDCK) and three canine mammary tumor cells (CMT-U309, P114 and CMT-U27). GAPDH was used as an internal control to verify equal protein loading. Each bar represents the mean ± SEM of repeated independent experiments (ANOVA) *p<0.05 **p<0.01 and ***p<0.001 vs control (MDCK cells); o p<0.05 vs P114 cells b) Representative qRT-PCR analysis of CPT1A mRNA expression in normal epithelial cells (MDCK) and three canine mammary tumor cells (CMT-U309, P114 and CMT-U27). Each bar represents the mean ± SEM of repeated independent experiments (ANOVA); **p<0.01 ***p<0.001 vs control (MDCK cells); oo p<0.01, o p<0.05 vs P114 cells.*

5.1.2 CPT1A protein expression correlates with malignancy grades in CMT specimens

Considering our results on CMT cancer cells, we have then assessed the expression of CPT1A in CMT and in NMG specimens. WB analysis has revealed higher CPT1A protein expression levels in CMT samples compared to those of canine NMG (Fig. 10a) being the CPT1A protein expression 55-fold higher in cancerous tissues compared to healthy ones (Fig.10b). IHC analysis has revealed that only ductal and lobular epithelial cells showed positive CPT1A immunostaining. Moreover, MECs cells are negative whereas inflammatory cells have showed a weak CPT1A immunoreactivity. Specifically, four out of six NMGs (67%) showed moderate (mean immunoreactive (IR) score = 4.83±1.60, range 0-9) CPT1A immunostaining characterized by few and small cytoplasmic granules (Fig. 10c). All benign tumors (5/5; 100%) showed strong (mean IR score 8.80±0.970) CPT1A immunostaining with few and small cytoplasmic granules (Fig.10d). All G1 carcinomas (14/14; 100%) displayed strong (mean IR score 9.43±0.724, range 4-12) CPT1A immunoreactivity characterized by large granules throughout the cytoplasm and mostly present in several intra-tumoral areas (Fig. 10e). All G2 carcinomas (14/14; 100%) showed strong (mean IR score = 6.79±0.757, range 2-12) immunoreactivity for CPT1A but restricted to regions where mammary glandular morphology was still preserved (Fig. 10f). Thirteen out of 14 poorly G3 differentiated tumors (92%) (mean IR score = 3.00±0.593, range 0-6) were weakly stained for CPT1A even if a moderate immunostaining was present in less phenotypically aggressive

counterparts (Fig. 10g). Interestingly, in four out of 14 G3 carcinomas (29%), strong CPT1A immunoreactivity was observed only in neoplastic cells infiltrating the surrounding tissue (Fig. 10h). Based on a semi-quantitative assessment of CPT1A immunoreactive cells, the CPT1A protein expression levels correlates inversely with the degree of tumour differentiation (Fig. 10i).

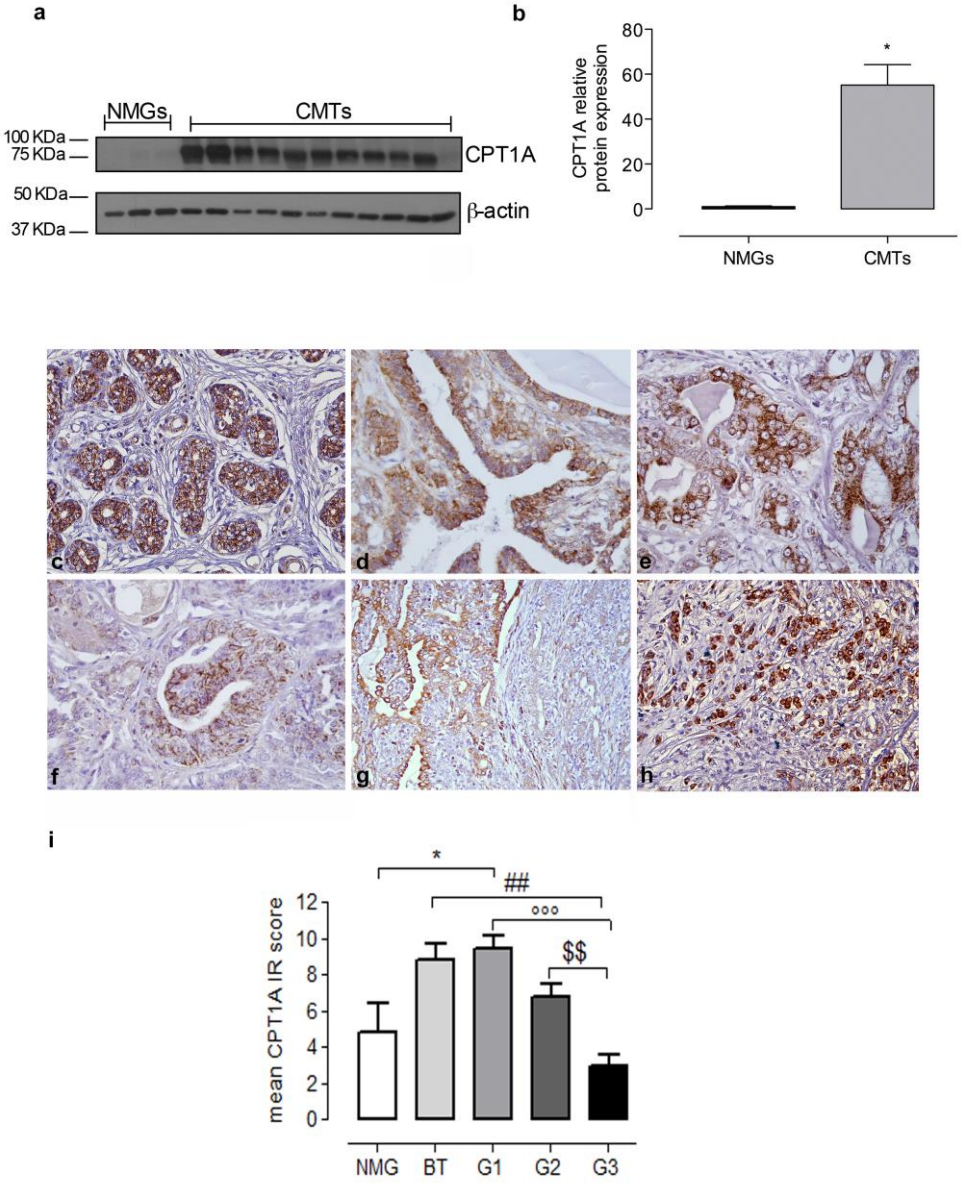


Fig.10. Expression of CPT1A in canine mammary samples. a) Western blot analysis of CPT1A protein expression in three normal mammary glands (NMGs) versus 11 canine mammary tumor tissues (CMTs). β-actin was used as control to verify equal protein

*loading. b) Graph shows the Mean ± SEM of relative CPT1A protein expression in CMTs vs NMGs (Student's-t-test). c-h) Immunohistochemical labelling of CPT1A in canine normal and neoplastic mammary glands: c) normal mammary gland tissue. IR (immunoreactive) score=8: CPT1A moderate immunostaining characterized by few and small cytoplasmic granules are evident in epithelial cells. Stromal and myoepithelial cells are negative. d) Simple adenoma case number 9, IR score=8: strong CPT1A immunostaining characterized by few and small cytoplasmic granules is evident in 70.8% of neoplastic cells. e) G1 Mixed Type Carcinoma, case number 12, IR score=9: CPT1A diffuse cytoplasmic strong immunostaining is observed in 52.4% of neoplastic epithelial cells and mainly localized in some neoplastic areas. f) G2 Cystic-Papillary Carcinoma, case number 15, IR score=6: 39.2% neoplastic cells with moderate CPT1A immunostaining surround a central tubule-papillar area with strong CPT1A immunostaining. g) G3 Tubular, case number 41, IR score=6: more differentiated neoplastic area with CPT1A strong immunostaining (left part) and less differentiated neoplastic area (right part) with CPT1A weak immunostaining are shown. h) G3 Mixed Type Carcinoma, case number 38, IR score=6: strong CPT1A immunostaining in peripheric infiltrating neoplastic cells is shown. i) Graph shows the Mean ± SEM of Immunoreactive (IR) score for the expression of CPT1A in normal and tumoral samples with different malignancy grade (ANOVA) * $p<0.05$ NMGs vs G1 carcinomas; ## $p<0.001$ between benign tumors vs G3 carcinomas; °°° $p<0.0001$ between G1 vs G3 carcinomas; $$ $p<0.001$ between G2 vs G3 carcinomas.*
NMG: normal mammary gland; BT: benign tumor; G1: grade 1 carcinomas; G2: grade 2 carcinomas; G3: grade 3 carcinomas.

5.1.3 Expression of CACT in CMTs

Antibody cross-reactivity was assessed on three NMGs and seven CMTs with different histological grades by WB. Immunoreactive bands were observed at the expected molecular weight range (around 33 kDa), with different signal intensities, both in normal and in mammary tumor tissues (Fig. 11a). Furthermore, WB analysis has also revealed that the expression

of CACT protein was higher in CMT samples compared to NMG ones (Fig. 11b). Different signal intensities have been also observed in CMT cell lines (CF33, CMT-U27, CMT-U309 and P114) used as *in vitro* model (Fig. 11c). Upon immunohistochemical analysis, all G1 carcinomas (15/15, 100%; mean IR score=6.67±1.01 range 1-12) showed strong CACT immunostaining diffused throught the cell cytoplasm (Fig. 11d) whereas all G2 (8/8 100%; mean IR score=4.63±1.24 range 1-12) (Fig. 11e) as well as three out of six (50%) (mean IRS=1.83±1.45 range 0-9) were weakly immunostained for CACT protein showed lower IR score values compared to G1 carcinomas (Fig. 11f). Based on a semi-quantitative assessment of CACT immunoreactive cells, the CACT protein expression levels correlates inversely with the degree of tumour differentiation (Fig. 11g).

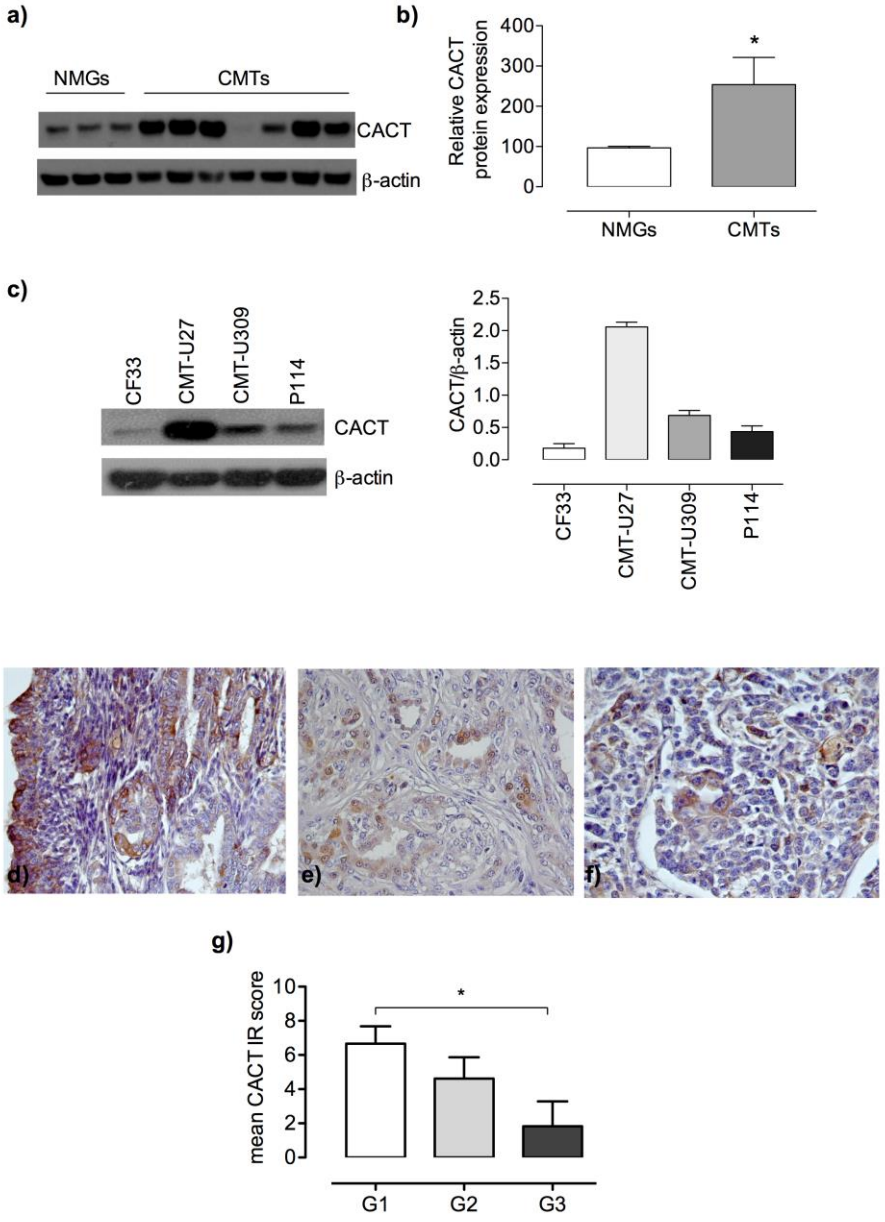


Fig. 11. Expression of CACT in CMT samples and CMT cell lines

a) Western blot analysis of CACT protein expression in three normal mammary glands (NMGs) versus seven canine mammary tumor tissues (CMTs). β-actin has been used as

*control to verify equal protein loading. b) Graph shows the Mean ± SEM of relative CACT protein expression in CMTs vs NMGs (Student's-t-test). (b, left panel) Representative western blotting showing expression of CACT protein in four CMT cell lines of different tumor origin (CF33: mammary adenocarcinoma; CMT-U27: simple ductal carcinoma; CMT-U309: spindle-cell carcinoma and P114: anaplastic carcinoma). (b, right panel) Densitometric analysis of CACT protein expression levels expressed as CACT/β-actin densitometric ratios for each cell line. β-actin has been used as loading control. c) Representative CACT immunohistochemical expression in CMT glands. Cytoplasmic expression of CACT in ductal epithelial cells of G1 carcinomas (**d**), G2 carcinomas (**e**), G3 carcinomas (**f**). (**g**) Graph shows the Mean ± SEM of Immunoreactive (IR) score for the expression of CACT in tumoral samples with different malignancy grade (ANOVA) * $p<0.05$ G3 vs G1 carcinomas; G1: grade 1 carcinomas; G2: grade 2 carcinomas; G3: grade 3 carcinomas.*

5.1.4 Expression of CPT2 in CMTs

Antibody cross-reactivity was assessed on three NMGs and seven CMTs with different histological grades by WB. Immunoreactive bands have been observed at the expected molecular weight range (around 71 kDa), with different signal intensities, both in normal and in mammary tumor tissues. (Fig. 12a). Furthermore, WB analysis has also revealed a slightly increase of CPT2 protein in CMT samples compared to NMG ones (Fig. 12b). Different signal intensities were also observed in CMT cell lines (CF33, CMT-U27, CMT-U309 and P114) used as *in vitro* model (Fig. 12c). Upon immunohistochemical analysis, all G1 carcinomas (9/9, 100%; mean IR score=7.33±1.14 range 2-12) showed strong CPT2 immunostaining diffused throught the cell cytoplasm (Fig. 12d) whereas all G2 (5/6 83%; mean IR score=2.83±0.87 range 1-6) (Fig. 12e) as well as one out of seven

(14%) (mean IR score=1.29±1.29 range 0-9) are weakly immunostained for CPT2 protein showed lower IR score values compared to G1 carcinomas (Fig. 12f). Based on a semi-quantitative assessment of CPT2 immunoreactive cells, the CPT2 protein expression levels correlates inversely with the degree of tumour differentiation (Fig. 12g).

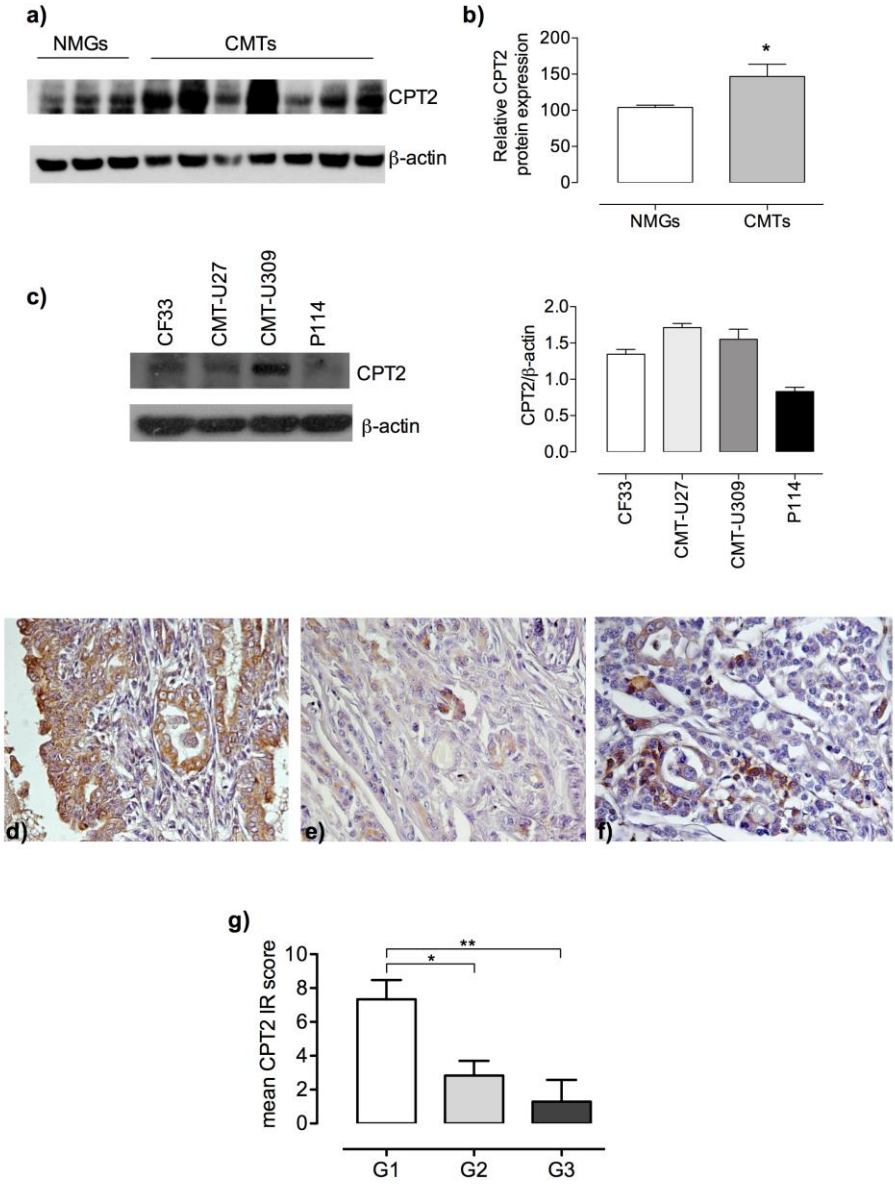


Fig. 12. Expression of CPT2 in CMT samples and CMT cell lines

a) Western blot analysis of CPT2 protein expression in three normal mammary glands (NMGs) versus seven canine mammary tumor tissues (CMTs). β-actin has been used as control to verify equal protein loading. b) Graph shows the Mean ± SEM of relative CPT2

*protein expression in CMTs vs NMGs (Student's-t-test). (b, left panel) Representative western blotting showing expression of CPT2 protein in four CMT cell lines of different tumor origin (CF33: mammary adenocarcinoma; CMT-U27: simple ductal carcinoma; CMT-U309: spindle-cell carcinoma and P114: anaplastic carcinoma). (b, right panel) Densitometric analysis of CPT2 protein expression levels expressed as CPT2/β-actin densitometric ratios for each cell line. β-actin has been used as loading control. c) Representative CPT2 immunohistochemical expression in CMT glands. Cytoplasmic expression of CPT2 in ductal epithelial cells of G1 carcinomas (**d**), G2 carcinomas (**e**), G3 carcinomas (**f**). (**g**) Graph shows the Mean ± SEM of Immunoreactive (IR) score for the expression of CPT2 in tumoral samples with different malignancy grade (ANOVA) * $p<0.05$ G2 vs G1 carcinomas; ** $p<0.01$ G3 vs G1 carcinomas; G1: grade 1 carcinomas; G2: grade 2 carcinomas; G3: grade 3 carcinomas.*

5.1.5 Expression of CrAT in CMTs

Antibody cross-reactivity has been assessed on three NMGs and seven CMTs with different histological grades by WB. Immunoreactive bands have been observed at the expected molecular weight range (around 71 kDa), with different signal intensities, both in normal and in mammary tumor tissues. (Fig. 13a). Furthermore, WB analysis also revealed a marked increase of CRAT protein in CMT samples compared to NMG ones (Fig. 13b). Different signal intensities were also observed in CMT cell line (CF33, CMT-U27, CMT-U309 and P114) used as *in vitro* model (Fig. 13c). Upon immunohistochemical analysis, all G1 carcinomas (6/7, 85%; mean IR score=6.1±1.56 range 3-12) have showed strong CrAT immunostaining diffused throught the cell cytoplasm (Fig. 13d) whereas all G2 (8/9 89%; mean IR score=3.7±0.99 range 1-9) (Fig. 13e) as well as four out of nine (44%) (mean IR score=1.1±0.48 range 2-4) are weakly

immunostained for CrAT protein showed lower IR score values compared to G1 carcinomas (Fig. 13f). Based on a semi-quantitative assessment of CRAT immunoreactive cells, the CRAT protein expression levels correlates inversely with the degree of tumour differentiation (Fig. 13g).

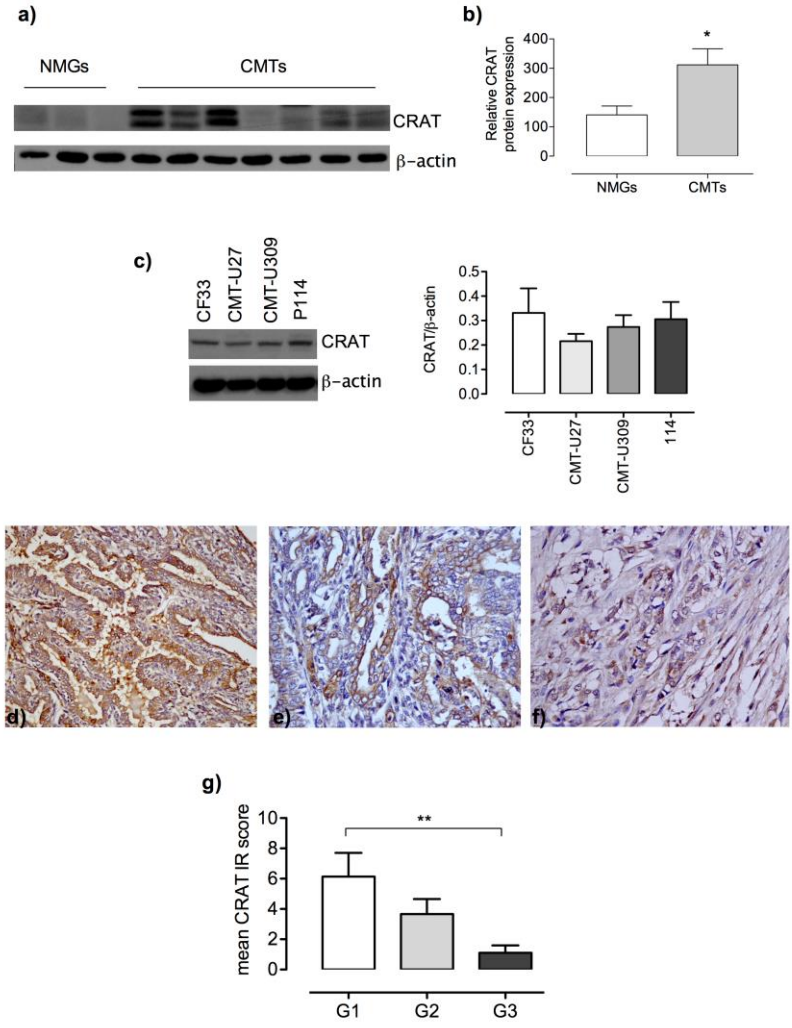


Fig. 13. Expression of CrAT in CMT samples and CMT cell lines. a) Western blot analysis of CrAT protein expression in three normal mammary glands (NMGs) versus

*seven canine mammary tumor tissues (CMTs). β-actin has been used as control to verify equal protein loading. b) Graph shows the Mean ± SEM of relative CrAT protein expression in CMTs vs NMGs (Student's-t-test). (b, left panel) Representative western blotting showing expression of CrAT protein in four CMT cell lines of different tumor origin (CF33: mammary adenocarcinoma; CMT-U27: simple ductal carcinoma; CMT-U309: spindle-cell carcinoma and P114: anaplastic carcinoma). (b, right panel) Densitometric analysis of CrAT protein expression levels expressed as CrAT/β-actin densitometric ratios for each cell line. β-actin has been used as loading control. c) Representative CrAT immunohistochemical expression in CMT glands. Cytoplasmic expression of CrAT in ductal epithelial cells of G1 carcinomas (**d**), G2 carcinomas (**e**), G3 carcinomas (**f**). (**g**) Graph shows the Mean ± SEM of Immunoreactive (IR) score for the expression of CRAT in tumoral samples with different malignancy grade (ANOVA) ** p<0.01 G3 vs G1 carcinomas; G1: grade 1 carcinomas; G2: grade 2 carcinomas; G3: grade 3 carcinomas.*

5.2 The CPT1A inhibitor ST1326 exerts cytotoxic effects on CMT cell lines

Cytotoxic effect of ST1326 has been assessed in five CMT cell lines (CMT-U309, P114, CMT-U27, CMT-U229 and CMT-U131). Cells were treated with increasing concentrations of ST1326 (1-20 μM) for 48 h and the cytotoxic effect on cell viability has been evaluated by using Trypan blue assay. Results showed that ST1326 possesses cytotoxic effect on all the five CMT cell lines used. In particular, ST1326 at concentrations of 5, 10, and 20 μM reduced the viability (IC_{50} = 14.9± 0.053) of CMT-U309 cells by 21, 32, and 67%, respectively (Fig. 14a). At concentrations of 10 and 20 μM, ST1326 diminished the viability (IC_{50} = 15.49 ± 0.123) of P114 cells by 51, and 69%, respectively (Fig. 14b). Furthermore, ST1326

at concentrations of 5, 10 and 20 μM also diminished the viability (IC_{50} = 15.42 ± 0.044) of CMT-U27 cells by 15, 41, and 62%, respectively (Fig. 14c). At the same concentrations, ST1326 reduced the viability (IC_{50} = 1.42± 0.127) of CMT-U229 cells by 65, 82, and 90%, respectively (Fig. 14d). Finally, ST1326, at the concentration ranging from 1 to 20 μM, reduced the viability (IC_{50} = 5.95 ± 0.220) of CMT-U131 cells by 24, 55, 67, and 84%, respectively (Fig. 14e). Representative WB analysis of CPT1A protein expression in the CMT cell lines used is also showed (Fig. 14f). According to the IC_{50} estimation, it emerges that CMT-U229 and CMT-U131 show a higher sensitivity to ST1326 compared to CMT-U309, P114 and CMT-U27 cells, respectively. Thus, we considered CMT-U229 and CMT-U131 to further investigate the mechanisms through which ST1326 decreased cell viability of CMT cells.

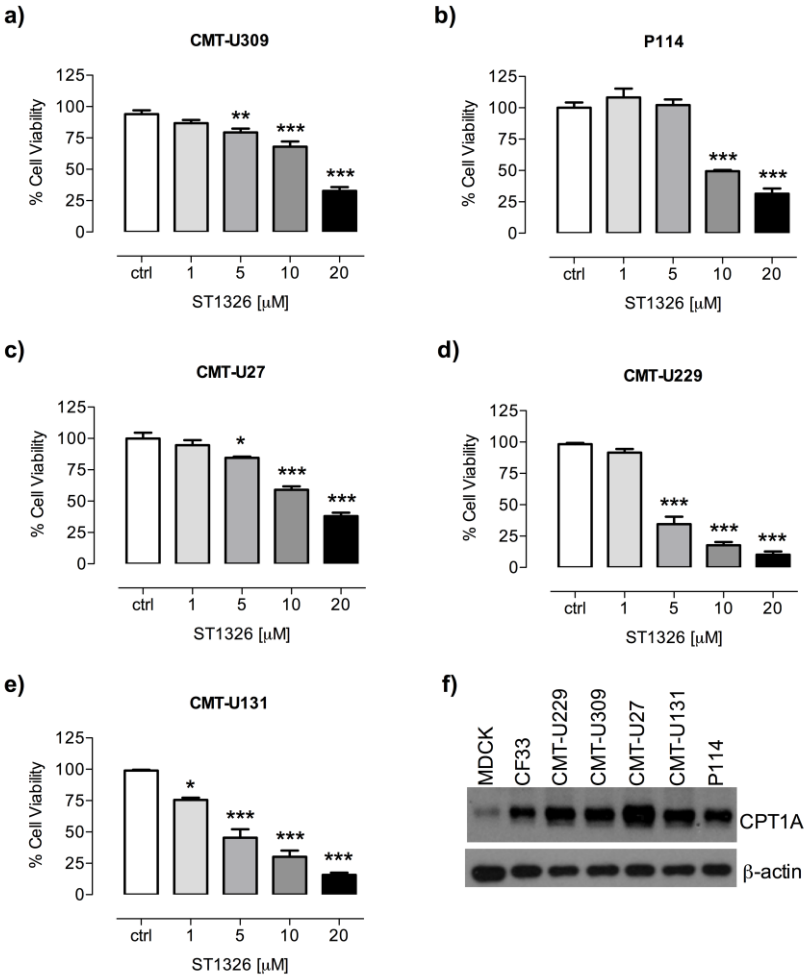


*Fig. 14. Effect of ST1326 on viability of CMT cell lines. Cytotoxic effects of ST 1326 (1–20 μM, 48 h exposure) in CMT-U309 (a), P114 (b), CMT-U27 (c), CMT-U229 (d), CMT-U131 (e) cells. Viability (expressed as percentage of cell viability) rate was investigated by using Trypan blue assay. Each bar represents the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control (untreated cells).*

5.3 ST1326 induces morphological changes in CMT cell lines

Microscopy observation of ST1326-treated CMT-U229 and CMT-U131 cells has revealed different morphological changes induced by this compound. As shown in Fig. 15, CMT-U229 or CMT-U131 cells treated with different concentrations of ST1326 (5, 10 and 20 µM) have undergone morphological changes visible after 48 h of treatment. A subpopulation of treated cells, at concentration of 5 µM exhibited a senescence-like phenotype characterized by elongated and flattening shape compared to control cells (Fig. 15 b, f). Moreover, CMT-U229 and CMT-U131 treated cells at concentration of 10 µM also reveal cytoplasmic vacuolation (Fig. 15 c, g). Finally, at concentration of 20 µM, CMT-U229 and CMT-U131 treated cells appear smaller and completely rounded, particular features of dying cells (Fig. 15 d,h).

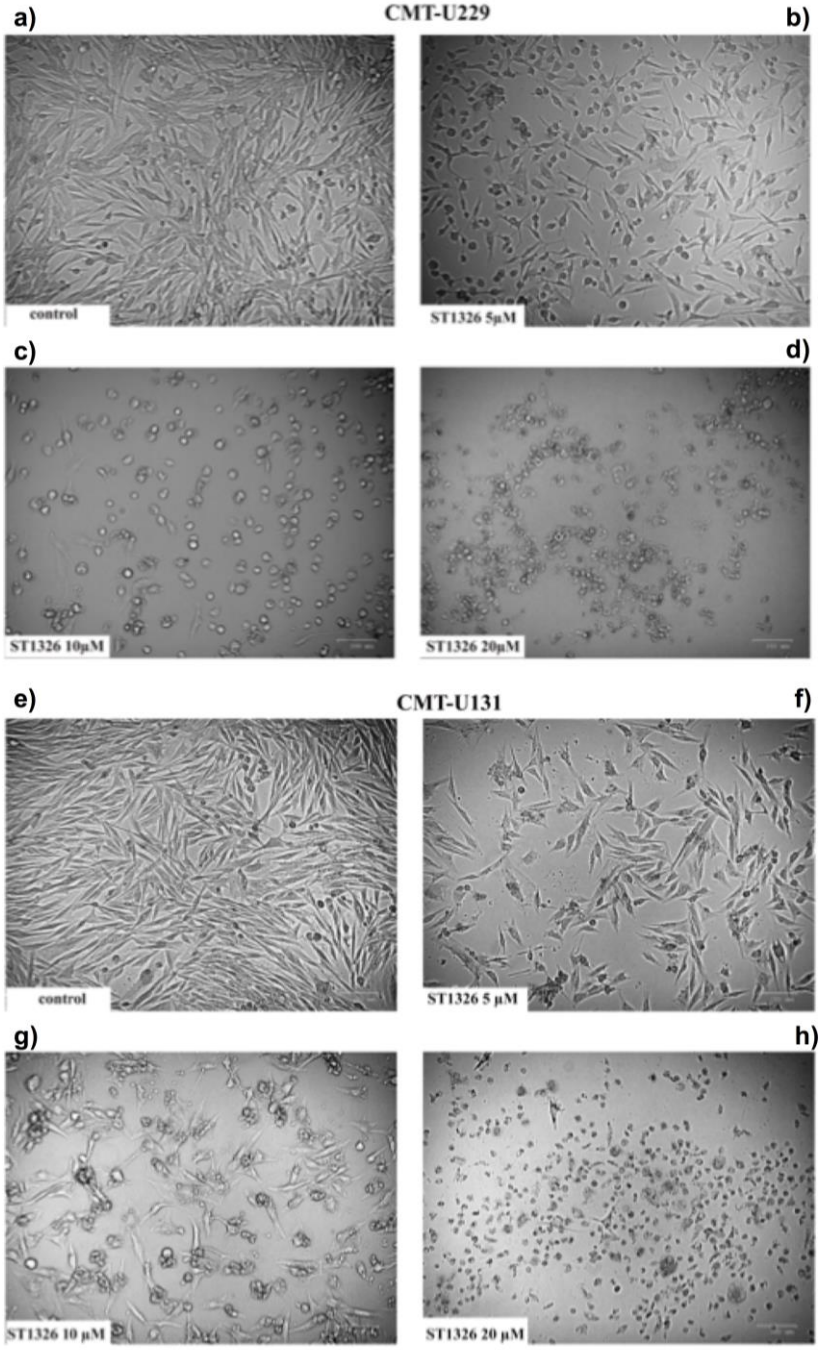
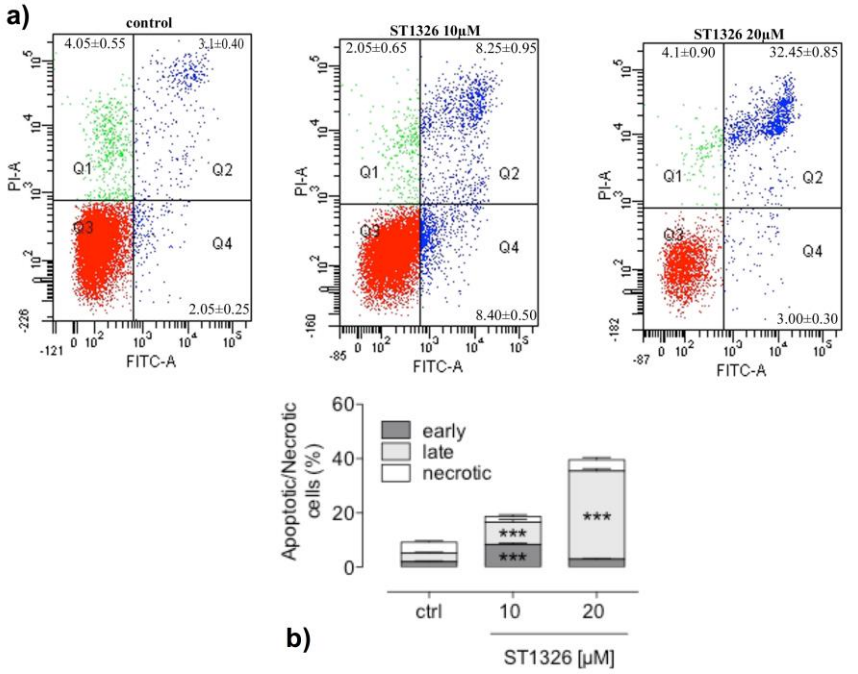


Fig.15 Morphological changes observed in CMT-U229 and CMT-U309 cells after 48 h of treatment with various concentration of ST1326.

5.4 ST1326 triggers apoptosis in CMTcell lines

Because cytotoxicity assay (Fig. 13) has shown that ST1326 induce marked cell death in CMT-U229 and CMT-U131 cells, we decided to investigate whether this is linked to the induction of apoptosis. To address this aim, Annexin V/PI staining has been used to determine the percentage of apoptotic cells induced by ST1326 after 48 h of treatment. The exposure of CMT-U229 to 10 μM of ST1326 led to a significant increase (8.40%) in early stage apoptotic population (Annexin V^+/PI^-) along with an increase (8.25%) in the late stage apoptotic/necrotic population (Annexin V^+/PI^+). The same cells treated with 20 μM of ST1326 also shown a more marked increase (32.45%) in the late apoptotic/necrotic phase (Annexin V^+/PI^+) (Fig 16 a,b). Similarly, CMT-U131 cells treated with 10 μM or 20 μM of ST1326 shown a significant increase (13.80%) and (40.85%) of the cells in the late apoptotic/necrotic phase (Annexin V^+/PI^+), respectively (Figure 16 c,d).


a)
control
4.05±0.55
3.1±0.40
2.05±0.25
ST1326 10µM
2.05±0.65
8.25±0.95
8.40±0.50
ST1326 20µM
4.1±0.90
32.45±0.85
3.00±0.30
Q1
Q2
Q3
Q4
PI-A
FITC-A
Apoptotic/Necrotic cells (%)
early
late
necrotic
***
ctrl
10
20
ST1326 [µM]
b)

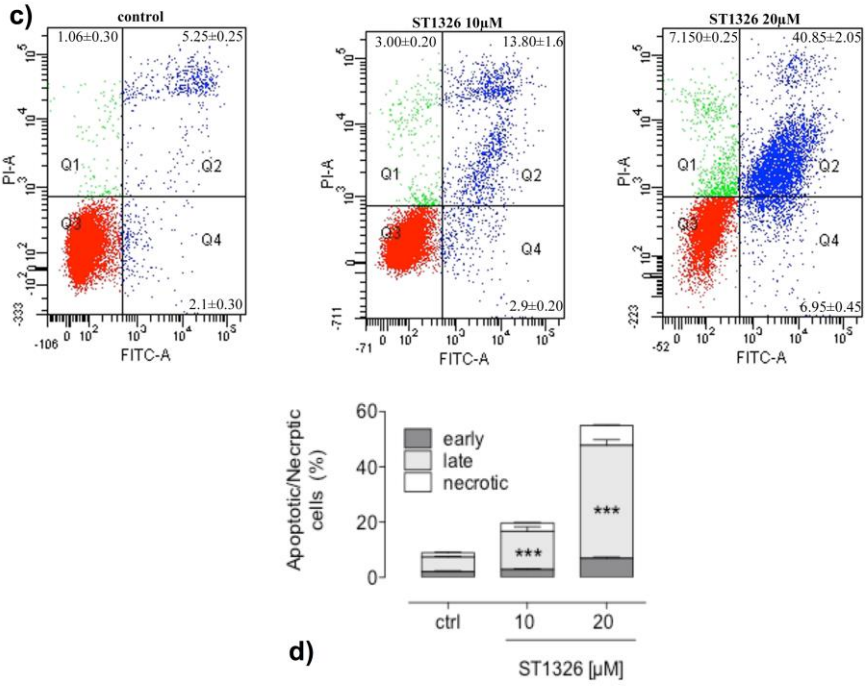

c)
control
1.06±0.30
5.25±0.25
2.1±0.30
ST1326 10µM
3.00±0.20
13.80±1.6
2.9±0.20
ST1326 20µM
7.150±0.25
40.85±2.05
6.95±0.45
Q1
Q2
Q3
Q4
PI-A
FITC-A
Apoptotic/Necrptic cells (%)
early
late
necrotic
***
ctrl
10
20
ST1326 [µM]
d)

*Figure 16. Determination of apoptosis in ST1326-treated cells. Induction of apoptosis in CMT-U229 cells (a–b) and in CMT-U131 (c-d). Representative dot plots showing cells in necrosis (Q1), late apoptosis (Q2), live cells (Q3), and early apoptosis (Q4). Annexin V binding was carried-out using Annexin V and PI apoptosis assay (Dojindo Molecular Technologies Inc., Munich, Germany). Cells were treated with or without increasing concentrations of ST1326 for 48 h. Detached and adherent cells were collected and stained with Annexin V/PI and then the events for early and late apoptotic cells were counted with the use of FACSCanto Cell Analyzer as described in Materials and Methods. Data represent the mean ± SEM of repeated independent experiments. Statistical analysis was performed using ANOVA followed by Tukey's Post-Hoc test to determine the significance (***$p < 0.001$).*

To further investigate the molecular mechanisms by which ST1326 induce apoptosis in CMT cells, CMT-U229 and CMT-U131 have been subjected to a time-course stimulation with 10 µM of ST1326 and the expression of Extracellular signal-Regulated Kinase (ERK), p-ERK, AKT and p-AKT has been analyzed using western blot analysis. AKT signaling pathway is one of the most important pathways that promote protein synthesis, cell metabolism, cell proliferation, apoptosis, and cell survival [234]. As shown in Fig. 17a, the levels of p-AKT markedely reduce in both CMT-U229 and CMT-U131 (Fig. 17c) after 3-, 6- and 12-h ST1326 treament. The expression levels of total AKT for both the cell lines, together with the internal control (vinculin) do not change at any time point (Fig. 17a,c). Mitogen-Activated Protein Kinase families play an important role in different cellular functions such as proliferation, differentiation, development, transformation, and also apoptosis [235]. Upon treatment with 10 µM of ST1326, the levels of p-ERK in CMT-U229 (Fig. 17b) and CMT-U131 (Fig. 17d) markedly increase upon 1 hr for up to 12-h ST1326

treatment, while the levels of total ERK decreased (Fig 17b,d) suggesting that ST1326 acts by inducing apoptosis.

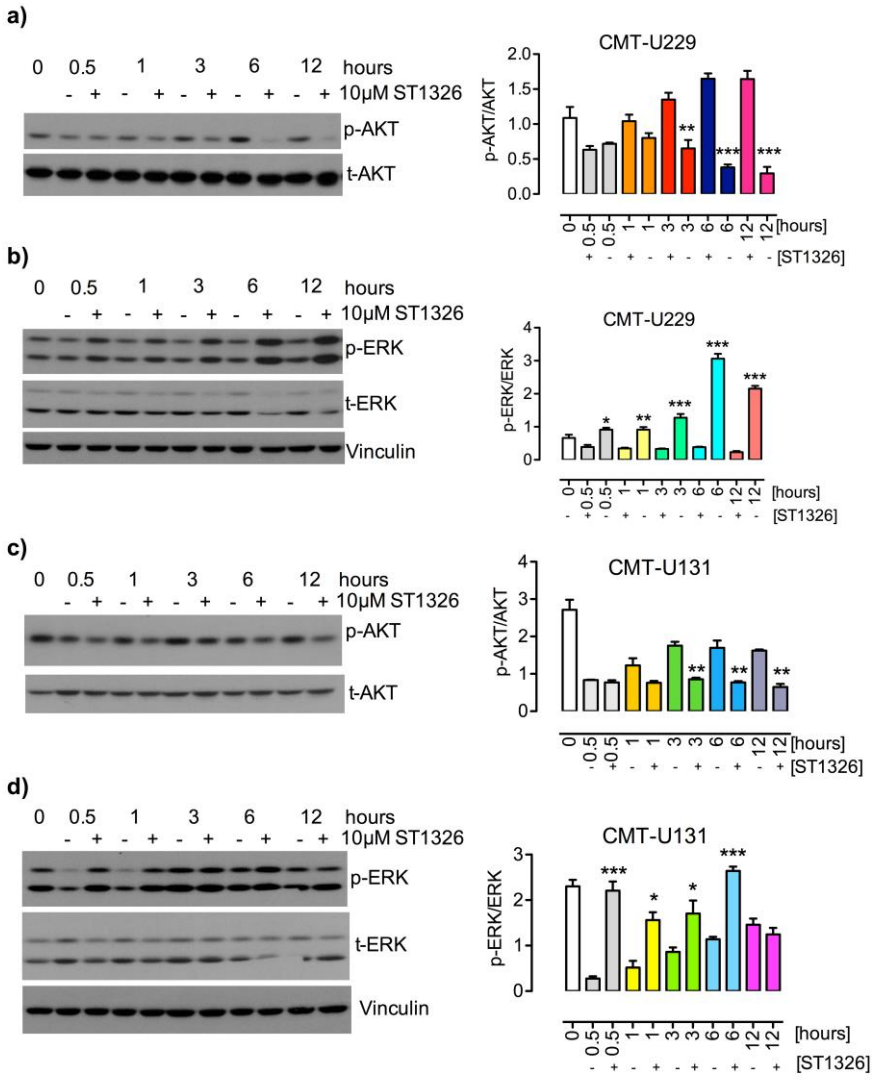


Fig. 17. Inhibition of the PI3K/Akt pathway and activation of MAPK pathways triggers apotosis in CMT cell lines. a-c) Western blotting analysis of p-AKT and AKT expression STq326-treated CMT-U229 and CMT-U131 cells; b-d)Western blotting analysis of p-ERK

and ERK expression in CMT-U229 and CMT-U131 cells. Cells were treated with or without 10 μM of ST1326 for 48 h, then whole cell lysates were extracted and subjected to western blot analysis for the abovementioned proteins. Vinculin was used to verify equal protein loading. The western blots shown are representative of at least three independent experiments.

Chapter 6

Discussion

6. Discussion

Within the tumor microenvironment cancer cells reprogram their metabolic patterns to fuel cell growth and proliferation [203]. Moreover, recent observations have demonstrated that the FAO pathway could represent an alternative energy source for anabolic processes in different tumors, therefore appearing promising for therapeutic purposes [226,227]. Our results demonstrate, for the first time, the presence and the increase expression of CS components (CPT1A, CACT, CPT2, and CRAT) in CMT cells and normal/tumor tissues. First, by using WB and qRT-PCR analyses we have found CPT1A protein and mRNA levels being significantly overexpressed in CMT cell lines (CMT-U309, P114 and CMT-U27) as compared to MDCK cells. This is consistent with previous works reporting an increase of CPT1A protein and/or mRNA expression levels in HBC cells [236,237]. It has been reported that CPT1A is overexpressed in several human malignancies originating from different tissues [201,210,211]. According to this, by WB analysis, after having confirmed the species cross-reactivity of CPT1A antibody used, we found that independently from tumor malignancy grade, CPT1A levels were higher in CMT tissue samples than in healthy control tissues. By IHC, CPT1A was found to be more expressed in well-differentiated G1 carcinomas than in NMGs. On the other hand, CPT1A protein expression correlated with the decrease of tumor differentiation, showing poorly differentiated G3 carcinomas lower CPT1A levels in comparison to NMGs, benign, G1 and moderately differentiated G2 tumors. Consistent with these results, we also found reduced CPT1A protein levels in highly aggressive P114 cells derived

from an anaplastic canine tumor [238]. These results share similarities with the findings of Louie & *coll.* (2013) which demonstrated that aggressive cancer cells possess lower levels of CPT1A expression compared with their non-aggressive counterparts suggesting that CPT1A and FAO were attenuated during cancer progression to shunt fatty acid from FAO pathway to generate more structural and oncogenic lipids [239]. Therefore, the strong CPT1A expression evidenced in some G3 carcinomas mostly by malignant cells infiltrating the surrounding tissue, may suggest that neoplastic cells use FAO as a source of energy for maintaining the mitochondrial membrane integrity [240]. On one hand, the decreased CPT1A protein expression found in less differentiated G3 tumors could be explained by the harsh and hypoxic microenvironment that may generate a stimulus towards a return to a glycolytic metabolic pathway, since aggressive breast cancer subtypes depend on increased glycolytic metabolism [241,242]. Moreover, it has been also reported that FAO could be mainly relevant to tumors that grow in adipocyte-rich environments, such as breast cancer [243]. Therefore, we hypothesize that CMT cells - in the same way as HBC cells - can use lipids stored in neighboring adipocytes as a source of energy for FAO and as building blocks for tumor cell growth [244]. To date, the correlation between CACT and cancer has received little attention, and only a few studies have reported a link between the altered expression of CACT and cancer. However, by using WB technique, we have also demonstrated that CACT is expressed at high levels in CMT samples with respect to NMGs and these results have been also confirmed in CMT cell lines (CF33, CMT-U27, CMT-U309 and P114) which express high levels of this protein. By IHC, CACT expression has been found to be

more expressed in G1 carcinomas than in NMGs. On the other hand, CACT protein expression correlate with the decrease of tumor differentiation, showing G3 carcinomas lower CPT1A levels in comparison to NMGs, G1, and G2 tumors. Our results are in line with Valentino et al.'s work (2017), which have demonstrated that in human prostate cancer (PC) specimens as well as androgen-dependent and -independent PC cells, the overexpression and the increased activity of CACT is a hallmark of PC [245]. However, our results also share similarities with Kim et al.'s work (2016), in which the authors have shown that in human bladder cancer patients the expression of carnitine enzymes such as CACT has significantly deregulated in tumor tissues compared to normal bladder tissues [246]. As far as CPT2 expression is concerned, our results have also demonstrated that CPT2 is slightly expressed in CMT samples in comparison to NMGs. These results have been also confirmed in CMT cell lines (CF33, CMT-U27, CMT-U309 and P114), which express different levels of this protein. By IHC, CPT2 expression has been found to be more expressed in G1 carcinomas than in NMGs. On the other hand, CPT2 protein expression correlate with the decrease of tumor differentiation, showing G3 carcinomas lower CPT1A levels in comparison to NMGs, G1, and G2 tumors. Compared to CPT1A isoform, less is known about CPT2 deregulation in cancer; nevertheless, a recent study has reported that this enzyme could be considered as an independent prognostic factor in colorectal cancer patients [247]. Another study has analyzed the expression of several enzymes involved in fatty acid metabolism in the leukemia cells compared to normal cells. Interestingly, the results have shown that leukemia cells present a higher expression of most CPT isoforms,

including CPT2, suggesting that chronic lymphocytic leukemia cells are highly active in fatty acid catabolism [222]. As far as CRAT expression is concerned, by using WB analysis, we have shown that CrAT is markedly expressed in CMT samples with respect to healthy mammary gland. These results have been confirmed by assessing CrAT protein expression in CMT cells, in which this protein is expressed at different levels. However, also in this case, by IHC, CRAT expression has been found to be more expressed in G1 carcinomas than in NMG. On the other hand, CRAT protein expression correlate with the decrease of tumor differentiation, showing G3 carcinomas lower CPT1A levels in comparison to NMGs, G1, and G2 tumors. Our results share similarities with Valentino et al.'s findings (2017), in which the authors found both in PC biopsy and PC cells (PC3 and LNCaP, androgen-dependent and androgen-independent, respectively) increased levels of this protein [245]. Intringuingly, studies in CrAT knockout mice have demonstrated that CrAT deficiency leads to abnormal fuel selection, which results in a perturbation of glucose homeostasis and suggests that deficits in CrAT activity might contribute to diet-induced metabolic inflexibility by exacerbating the Randle glucose-fatty acid cycle [248]. The higher CrAT exppression observed both in well differentiated CMT specimens and CMT cell lines (CF33, CMT-U309, CMT-U27 and P114) may highlight the importance of CrAT to contribute to maintaining a high metabolic plasticity of CMTs. The key enzymes or regulators of FAO have also emerged as promising targets for cancer therapy [211,226,227]. The focus on FAO in cancer cells has mostly been driven by experimental findings related to CPT1A [206]. The assumption has been that increased CPT1A expression and sensitivity to CPT1A inhibition

represents a demand for FAO. Therefore, in the last years, several efforts have been made by many researchers to develop inhibitors of CPT1A as anticancer agents. Among them, etomoxir, a non-selective irreversible CPT1A inhibitor is able to exert anti-proliferative effects against tumor cell lines [212,227]. It has been reported that etomoxir is able to reduce FAO in the human body and is clinically well tolerated [249]. However, etomoxir clinical use has been stopped due to its detrimental cardiac and hepatic side effects [250,251]. Therefore, etomoxir is no longer under clinical development, and there are no relevant clinical informations available from oncology clinical trials [252]. Following this, an aminocarnitine derivative named ST1326 has been developed to selectively inhibit CPT1A activity and it has been reported to be safer in comparison to etomoxir [253,254]. Several studies have shown that this novel CPT1A inhibitor possesses anti-tumor activity in hematologic malignancies, such as acute myeloid leukemia, Burkitt's lymphoma and chronic lymphocytic leukemia cells [226,227,255]. Our results also suggest that CPT1A could be considered as a potential pharmacological target even for CMTs, since the trypan blue exclusion assay has shown the reduction of CMT cells viability in a dose dependent manner after treatment with CPT1A inhibitor ST1326. Notewhorthy, the light microscopic observation has shown significant morphological changes such as shrinkage, elongated and flattened phenotype and reduced cell size or detachment of cells from the surface in CMT-U229 and CMT-U131 treated cells as compared to non-treated cells. Flow cytometry analysis has shown that treatment with ST1326 for 48 h induces apoptotic cell death on both CMT-U229 and CMT-U131 cells, respectively. Interestingly the CMT-U229 cells at 10 µM of ST1326 have shown a 2-

fold increase in both early and late stage apoptotic cell population. The same cells treated with 20 µM of ST1326 have shown a 4-fold increase in the late apoptotic/necrotic phase. The same results have also been observed in CMT-U131 treated cells at 10 µM ST1326 (2-fold increase) or 20 µM of ST1326 (7-fold increase) in the late apoptotic/necrotic phase, respectively. These results are in agreement with the work of Ricciardi and *coll.* (2015), in which the authors have documented that ST1326 inhibits leukemia cell growth in a dose- and time-dependent manner with a concomitant induction of apoptosis cell death [226]. Moreover, our results also have shown similarites with Gugiatti et al.'s study (2018), in which the authors have also found that CPT1A inhibitor ST1326 possesses cytotoxic activity and induces apoptosis in chronic lymphocytic leukemia cells [255]. We have demonstrated that the reduction of cell viability is at least in part due to the enhancement of apoptotic mechanisms as shown by the results obtained by FCM analysis. To corroborate signaling pathways of apoptosis induction, we analyzed AKT and ERK pathways by western blotting analysis. The induction of apoptosis observed in both cell lines could be ascribed to downregulation of p-AKT, a well-known pro-survival protein kinase that antagonizes apoptosis by affecting several cellular processes [234]. This result is also confirmed by the increased levels of p-ERK along with a decreased of total ERK that have been also reported to induce apoptotic process [235]. Even if mastectomy is still regarded as the most effective treatment for CMTs, our findings open the avenue for additional therapeutic modalities such as the use of cell metabolism-modifying therapies especially for less aggressive or low grade tumors [239]. From this point of view, molecules able to specifically inhibit CPT1A

function and/or activity may represent an alternative to currently available therapies for CMTs treatment. In closing, the similarity of CPT1A expression between HBCs and CMTs confirms the role of dog as animal model for spontaneous neoplastic disease and also for new therapy modalities employing CTP1A inhibitors in either species.

Chapter 7

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