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Biological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure in PC3 cell line

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TABLE OF CONTENTES

ABSTRACT

Dioxins are commonly known as highly toxic compounds that are persistent organic pollutants. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent dioxin congener, and dioxin-like products are formed during the incomplete combustion of organic compounds in the presence of chlorine (waste incineration, burning of various fuels and poorly controlled combustion sources). In 1997, the International Agency for Research on Cancer (IARC) classified TCDD as carcinogen.

Prostate cancer (PC) is an extremely serious disease in dogs. PC also represents the most commonly diagnosed cancer in males in the Western world. In general, animal models of human cancer have evolved in attempts to capture the complexity of the human disease. In particular, as humans, the dogs are the only other mammals that develop PC. The canine prostate gland shares many morphological and functional similarities with the human prostate, so this specie represents thus an attractive model for the study of the prostatic disease in dogs. Consequently, to evaluate the biological effects of TCDD on prostate cancer, in this study we used human prostate cancer cell line, PC3. Until now, very few data are present in literature about PC3 and TCDD exposure. It is known that in PC3 cell line, a hormone-independent prostate cancer cell line, TCDD induces cytochrome P450 (CYP) 1A1 and CYP1B1 via the aryl hydrocarbon receptor (AhR). This effect of TCDD could result in higher elimination rates of concomitant drugs metabolized by these particular CYP isoenzymes.

Autophagy is a tightly regulated process playing a normal part in cell growth, development, and homeostasis and helping to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products through the degradation via the lysosome. Defects of autophagy machinery are responsible for pathogenesis of different diseases, including cancer. The role of autophagy in cancer is controversial. There is evidence that autophagy may play a critical role in cancer progression at later stages, such as dissemination and metastasis, which account for most cancer-associated deaths, whereas in other cases it clearly contributes to tumor suppression by inducing tumor cell death. So, the aim of this study is to evaluate the biological effects of TCDD exposure to a highly metastatic prostate cancer cell line, PC3.

The prostate cancer cell line PC3 was exposed to different concentrations of TCDD (0.1, 1 and 100 pg/ml) and after 24, 48, and 72 hours of exposure cell proliferation and viability, cell morphology and cell cycle analysis were performed. Also, autophagy was evaluated using the following: (a) detection of acidic vesicular organelles (AVOs) by acridine orange staining; (b) immunofluorescence analysis (IF) of LC3 (microtubule

associated light chain protein 3), one of the well-known autophagy markers; (c) LC3 gene expression by real-time PCR; (d) study of autophagic flux by chloroquine (CQ) (10μM), autophagy inhibitor, by western blot; (e) study the expression of multiple genes involved in autophagy machinery by real-time PCR.

When compared with their relative controls, TCDD (100 pg/ml) exposure at 24, 48, and 72 hours caused the following: (1) significant increase of cell proliferation (CP) (CP24h =20.3%, CP48h =54.5% and CP72h =52.4%); (2) significant increase of cell population at S phase; (3) significant increase of autophagy demonstrated by (a) detection of AVOs; (b) LC3 IF positivity; (c) LC3 over expression (expression ratio24h =1.4, expression ratio48h =6.2 and expression ratio72h =11.4); (d) CQ increased LC3-II accumulation; (e) moreover, we demonstrated that the induction of autophagy by TCDD in PC3 cells was accompanied by an increase in the mRNA levels of certain genes that are present in different subnetworks composing the autophagy interactive network, such as: PIK3C3, BECN-1, AMBRA1, MAP1LC3B, ATG4A, ATG4C, ATG5, ATG7, ATG10, ATG16L1, GABARAPL1, PRKAA, WIPI-1. Also, we showed that TCDD negatively influenced genes that are autophagy inhibitor, such as: AKT1 and BCL2. These data suggested a multiple effect of TCDD on autophagy machinery. Furthermore, we revealed that TCDD exposure upregulated TNF whose high levels have been related to prostate cancer progression via stimulation of proliferation, survival of malignant cells and increased resistance to chemotherapeutic agents.

From the 1980s, several illegal and uncontrolled sites of urban, toxic, and industrial waste disposal have been active in Campania region, Southern Italy, with the highest concentration reached in Naples and Caserta provinces. In 1994, Campania region is under a declared State of Emergency, because of the saturation of regional waste treatment facilities. There are growing national and international evidences that the accumulation of waste, illegal and legal, urban and industrial, has contaminated soil, water, and the air with a range of toxic pollutants including dioxins. A high correlation between incidences of cancer, respiratory illnesses, and genetic malformations and the presence of industrial and toxic waste landfills was also found. In Spring 2002, a dioxin emergency emerged in this region, as result of National Control Program of Residues in Foodstuff carried out by Health Minister in 2001, indeed, levels of TCDD exceeding the European Union tolerance were detected in dairy products and milk from cow and water buffalo, raised on in some areas of Campania Region. Cancer mortality descriptive studies demonstrated an increase of incidents rates of the prostate cancer in the same

areas where dioxin levels were elevated. Furthermore, studies performed in animal model suggested that TCDD exposure is associated with abnormal prostate development, altered prostate pathology and increase susceptibility to prostate cancer. The administration of TCDD to a variety of cultured cells may alter their ability to proliferate and die. In a previous study we demonstrated that TCDD induced proliferation in Madin-Darby Bovine Kidney (MDBK), an epithelial cell line, in which analysis of MDBK cell morphology revealed some alterations in a large number of exposed cells, where neither signs of apoptosis nor necrosis were detected, but we found that TCDD activated cell death with autophagy.

Herein, TCDD exposure in PC3, a cancer cell line, induced no signs of cell death. So, taken together, our results support the idea that TCDD, may induce the progression of prostate cancer enhancing cell proliferation, inducing autophagy, deregulating the expression of genes related to the autophagy machinery, and upregulating TNF resulting in an increased risk for both animal and human health.

INTRODUCTION

TCDD

Dioxins are commonly known as highly toxic compounds that are persistent organic pollutants (POPs) for the environment. POPs include polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), non-ortho and mono-ortho polychlorinated biphenyls (PCBs).

PCDDs or simply dioxins (but inaccurately), are derivatives of dibenzo-p-dioxin. There are 75 different congeners of PCDDs, and seven of them are specifically toxic (Pohjanvirta and Tuomisto, 1994).

PCDDs consist of 12 carbon atoms, forming two aromatic phenyl rings attached to one another through two oxygen bridges, and 8 other atoms that are attached to the molecule can be either hydrogens or chlorines (**Fig. 1**).

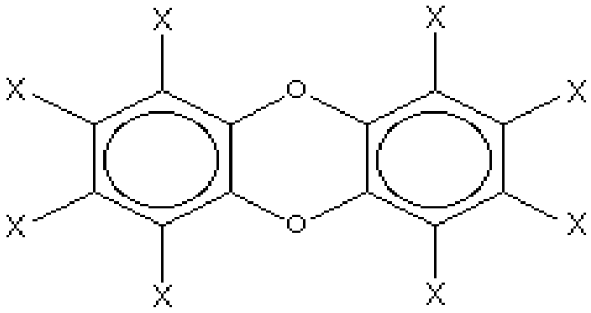

Fig. 1. Chemical structure of PCDD

Theoretically, 75 various combinations of hydrogen and chlorine are possible, and the resulting dibenzo-p-dioxin derivatives are called congeners. Chlorine increases the stability of these compounds, and chlorines in positions 2,3,7, and 8 (lateral chlorines) are especially important, because they are essential for toxicity and also prevention of enzymatic destruction of PCDDs (**Fig.2**).

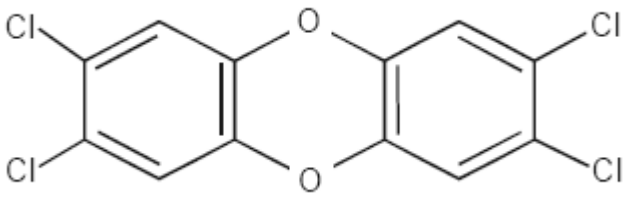

Fig. 2. Chemical structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

Therefore, the 7 congeners with 2,3,7,8-structure are toxicologically the most relevant. Each additional chlorine to 2,3,7,8-structure decreases toxicity, but the spectrum of adverse effects remains similar. Due to the fact that dioxin-like compounds normally exist in environmental and biological samples as complex mixtures of congeners, in mid-1980 the concept of toxic equivalents (TEQs) has been introduced to simplify risk assessment and regulatory control. In applying this concept, relative toxicities of dioxin-like compounds in relation to 2,3,7,8-TCDD (i.e. toxic equivalency factors, TEFs) are determined based on in vitro and in vivo studies. At 2,3,7,8-TCDD was assigned a TEF of 1.0 and all other congeners have lower TEF values ranging from 0.5 to 0.00001. Calculating the toxic equivalency (TEQ) of a mixture involves multiplying the concentration of individual congeners by their respective TEF. The sum of the TEQ concentrations for the individual congeners is the TEQ concentration for the mixture. This approach is based on the evidence that there is a common, receptor-mediated mechanism of action for these compounds, but it has its limitations due to a number of simplifications. The most important limitation is that the combined toxic effects of the components of a given mixture would be additive, neglecting possible synergism or antagonism. It should be recognized that revisions to the TEFs are periodically considered as new scientific information becomes available. If revisions in the TEFs are adopted, then it would be appropriate to adjust the TEQ release estimates calculated in the inventory (Birnbaum and DeVito, 1995).

Physical Properties of TCDD

TCDD is colorless, solid with no distinguishable odor. This compound is insoluble in water and soluble in organic solvents and lipids. It is resistant to acids and alkalis, and chemically degradable in few days by UV rays, in the presence of hydrogen ions donors (for example, in contact with the green foliage of plants). While, if present on a ground, it binds to the organic materials and it is degraded over months or years (Calkosinski et al., 2006).

Sources of TCDD

Microparticles of dioxins are found everywhere in the environment including the Arctic. TCDD and dioxin-like products are formed during the incomplete combustion of organic compounds (C, H, O), in the presence of chlorine. These can include waste

incineration (such as municipal solid waste, sewage sludge, medical waste and hazardous wastes), burning of various fuels (such as coal, wood, and petroleum products), other high temperature sources (such as cement kilns), and poorly controlled combustion sources (such as building fires). Dioxins can be formed during various types of primary and secondary metals operations including iron ore sintering, steel production, scrap metal recovery and formed as by-products from the manufacture of chlorine bleached wood pulp, chlorinated phenols (e.g., pentachlorophenol - PCP), PCBs, phenoxy herbicides (e.g., 2,4,5-T), and chlorinated aliphatic compounds (e.g., ethylene dichloride) (Clark et al., 1992) (**Fig. 3**). Since the TCDD is formed during the process of combustion of materials containing chlorine at temperatures lower than 800° C, the plants incineration must use combustion systems at temperatures not lower than 850° C for a sufficient time to complete destruction of products such as dioxins (Gribble, 1994).

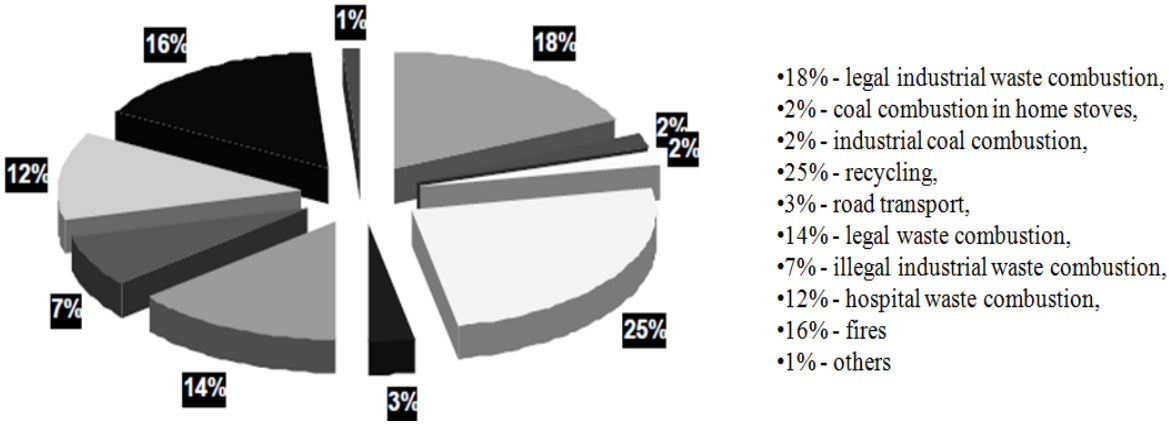

Fig. 3. Dioxin emission sources in percentage (Dobrzyński, Całkosiński et al. 2009)

Dioxins penetrate the human body mainly by ingestion of contaminated food with a high concentration of animal fat, by inhalation of contaminated air or by penetration through the skin (Mandal, 2005). Dioxin infiltration is facilitated by their liposolubility. More then 80% of accumulated dioxins are derived from food. A study showed that dioxins accumulated in human and animal fat tissues, and their elimination depends on life expectancy and the rate of metabolic processes. In humans, this period is about 9 years. Elimination of these compounds depends on the number of chlorine atoms present in dioxin's congeners. A significant amount of dioxin is removed from the body with milk during lactation. As presented by some authors, this may result in an overdose

(even 50-70 times higher) of dioxin concentration in mother's milk. Dioxin concentrations in human tissue depend on diet and lifestyle. Dioxin in the human body is transported by lipids in blood serum to the liver and fat tissue, where it accumulates (Dobrzyński et al., 2009) (**Fig.4**).

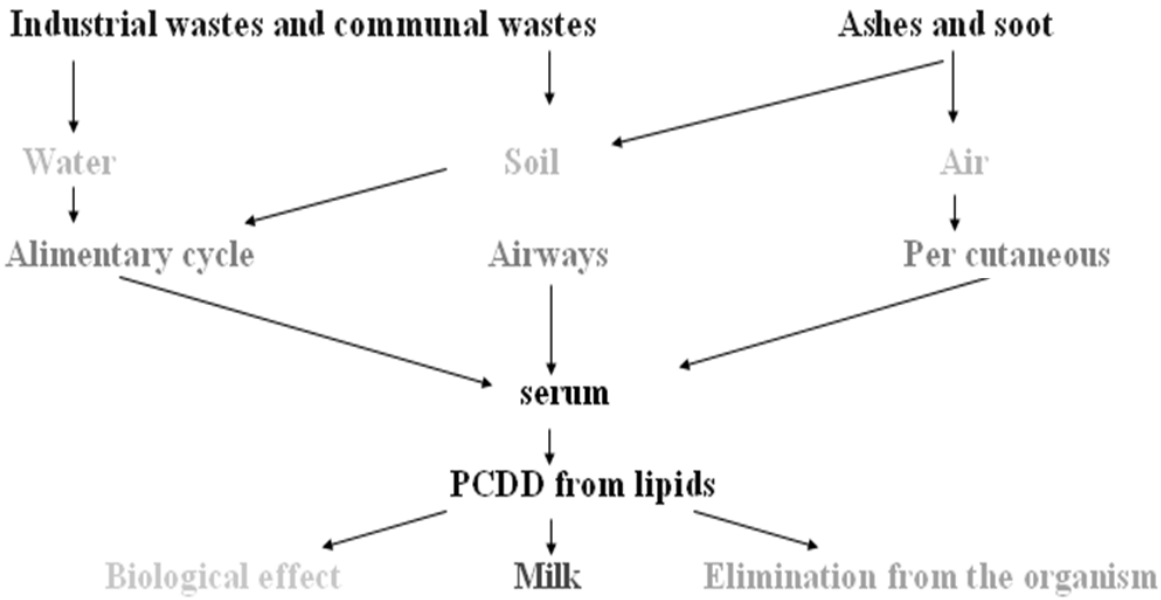

Fig. 4. Sources of natural environmental contamination (Dobrzyński, Całkosiński et al. 2009).

Mechanism of action of TCDD

The mechanism of action by which TCDD exerts the biochemical effects on vertebrate species is through the activation of the aryl hydrocarbon receptor (AhR)-mediated signal transduction pathway. AhR is a ligand-activated basic helix–loop–helix transcription factor, and a member of the PER-ARNT-SIM (PAS) superfamily of transcription factors. After the binding of TCDD to the AhR in the cytosol, the complex heterodimerizes with the aryl hydrocarbon nuclear translocator (ARNT). The TCDD-AhR-ARNT binding complex recognizes specific dioxin response elements (DRE) in the nucleus, leading to the expression of genes such as CYP1A1, CYP1A2, and CYP1B1 (Whitlock, 1999; Nebert et al., 2000) (**Fig.5**).

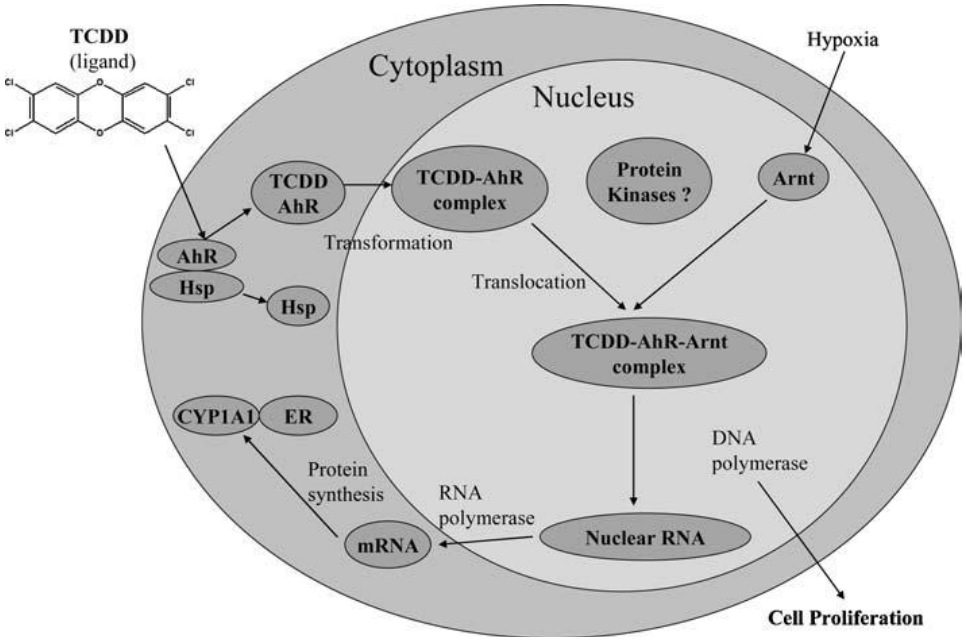

Figure 5: TCDD mechansm's of action inside the cell (Mandal 2005)

Persistent activation of the AhR is likely responsible for toxic responses of TCDD in experimental animals and humans. In rodents a wasting syndrome, immunosuppression, teratogenicity, chloracne, and carcinogenicity/tumor promotion have been well studied. There is good evidence for an involvement for the AhR in these responses. However, the chain of events from receptor activation to the diverse toxic endpoints is largely unknown.

Alteration of growth and differentiation of epithelial tissues may underlie most of the toxic responses.

Furthermore, AhR regulates the transcription of CYP1A1, -1A2 and -1B1 that induce the drug metabolism. These enzymes may metabolize the drug to an inactive compound, in which case the drug would not evoke the desired effect. Alternatively, the drug may be activated to a harmful intermediate and cause adverse reactions and therapy may be compromised. (Bock, 1994; Ramadoss et al., 2005).

TCDD CONTAMINATION INCIDENTS

The first episodes of accidental contamination can be found in the early '30s, when Monsanto chemical industry in Alabama, started producing PCBs as byproducts. The

workers of Monsanto were exposed to TCDD and developed chloracne, associated with other symptoms such as loss of appetite, loss of libido and a general sense of fatigue.

Documented accidents involving TCDD, date back to the 1960's, during the Vietnam War, during which TCDD was produced as a byproduct of the Agent Orange, a herbicide defoliant that was sprayed extensively by US military forces. The results seen when several of the soldiers were exposed to the herbicide, through the skin or ingestion of water contaminated with it present, were tumors, malformations in progeny and significant increase in the incidence of diabetes (Michalek et al., 1998).

The first Italian accident took place in Seveso. On July 10th 1976, there was an explosion of a safety valve of a reactor in the factory ICMESA Meda. This accident released massive amount of a toxic cloud containing TCDD, contaminating an area of 15 square kilometers where 37,000 people lived. Extensive studies in the affected population are still continuing to determine the long-term human health effects from this incident. However, these investigations are hampered by the lack of appropriate exposure assessments. Chloracne was the only effect linked with sufficient certainty to dioxin exposure but increased risks of different types of cancer, cardiovascular/endocrine effects, as well as, reproductive defects were reported in the Seveso population (Pesatori et al., 2003).

A case of TCDD contamination in food occurred in the United States of America in 1997. Chickens, eggs, and catfish were contaminated with dioxins when a tainted ingredient (bentonite/ball clay) was used in the manufacture of animal feed.

In 1999, high levels of dioxins were found in poultry and eggs from Belgium. Subsequently, dioxin-contaminated animal-based food (poultry, eggs, pork), were detected in several other countries. The cause was traced to animal feed contaminated with illegally disposed PCB-based waste industrial oil (Source: WHO registry).

One of the most recent known incidents of human exposure and a good example of acute dioxin toxicity happened in late 2004. The former president of Ukraine Viktor Andriyovych Yushchenko was victim of an assassination attempt during his election campaign. Following an ingestion of hazardous amounts of TCDD, he developed chloracne (Sorg et al., 2009) **(Fig. 6**).

Figure 6: President Andriyovych Yushchenko before (left) and after (right) TCDD exposure.

Since the 1980s, several illegal and uncontrolled sites of urban, toxic, and industrial waste disposal have been active in Campania region, Southern Italy, with the highest concentration reached in Naples and Caserta provinces (Barba et al., 2011).

Since 1994, the region of Campania in south-west Italy is under a declared State of Emergency, because of the saturation of regional waste treatment facilities. There are growing national and international evidences that the accumulation of waste, illegal and legal, urban and industrial, has contaminated soil, water, and the air with a range of toxic pollutants including dioxins. A high correlation between incidences of cancer, respiratory illnesses, and genetic malformations and the presence of industrial and toxic waste landfills was also found (Barba et al., 2011). In Spring 2002, a dioxin emergency emerged in this region, as result of National Control Program of Residues in Foodstuff carried out by Health Minister in 2001 (Reg.CE 2375/01). It was demonstrated that levels of TCDD exceeding the European Union tolerance were detected in dairy products and milk from cow and water buffalo, in some areas of Campania Region (South Italy) (Diletti et al., 2003; Santelli et al., 2006).

In July 2007, the European Commission issued a health warning to its Member States after high levels of TCDD were detected in a food additive - guar gum - used as thickener in small quantities in meat, dairy, dessert or delicatessen products. The source was traced to guar gum from India that was contaminated with pentachlorophenol (PCP), a pesticide no longer in use. PCP contains dioxins as contamination.

Between the month of October and the month of February of 2008 it was an alarm for levels of TCDD exceeding the European Union tolerance found in some sample of milk and mozzarella in dairies of different areas of Campania region. Following, in late 2008, Ireland recalled many tons of pork meat/products containing over 200 times more TCDD over the safe limits. This finding led to one of the largest food recalls related to a

chemical contamination. Risk assessments performed by Ireland indicated no public health concern (Source: WHO registry).

TOXIC EFFECTS OF DIOXINS

Animal Model Health Effects

TCDD showed a wide range of severe effects in animal experiments, including immunotoxicity, developmental and reproductive toxicity, teratogenicity, and carcinogenicity (DeVito et al. 1995). In particular, TCDD toxicity affects the immune system, increasing susceptibility to infectious diseases (viruses, various bacteria and parasites) (Burleson et al., 1996).

Exposure to TCDD during development has been extensively studied in mice, and can result in a number of different types of toxic effects, including: idronephrosis of the kidney (Abbott et al., 1987); cleft palate (Birnbaumn and DeVito, 1985); cardiac hypertrophy (Thackaberry et al., 2005); reduced cardiocyte proliferation (Thackaberry et al., 2005); suppressed immune response (Vorderstrasse et al., 2006); thymic atrophy (Staples et al., 1998); inihibition of prostate devolopment (Fritz et al., 2005); decresed epididymal sperm numbers (Theobald and Peterson, 1997); impaired learning ability (Powers et al., 2005); abnormal mammary gland formation (Vorderstrasse et al., 2004); altered keratinocy terminal differentation (Loertscher et al., 2002); increased relative liver weight (Lin et al., 2001).

It is well known that TCDD induces cancer in rats, mice, and hamsters (Huff et al., 1994)(International Agency for Research on Cancer, IARC 1997). Oncogenic response in laboratory animals has been shown to depend on age, sex, species, dose, and route of administration (National Toxicology, 1982).

The association between TCDD exposure and breast cancer risk in animal models have also revealed contradictory findings. Some studies (Brown et al., 1998) have demonstrated that TCDD increases the number of DMBA-or MNU-induced mammary tumour in rats. In the other hand, others claim that TCDD is chemopreventive against spontaneous breast cancer (Safe et al., 1991, Greenlee et al., 2001).

Animal Health Effects

In the last years several farms of cattles, river buffalos and sheeps in some areas of Campania Region (South Italy), have been unable to sell milk due to the presence of high levels of dioxins (Diletti et al., 2003; Santelli et al., 2006).

From a study of Iannuzzi et al. 2004, several cases of abortion (around 25% of total births), abnormal fetus (2.5% of total births) and increased frequencies of chromosome abnormalities and fragments and sister chromatid exchange were recorded in two flocks of sheeps raised in the province of Naples where a higher level of dioxins were found in the milk mass (Iannuzzi et al., 2004).

Human Health Effects

Exposure to TCDD can result in a number of toxic effects in several species. In humans, one of the most commonly reported toxic effect is chloracne. Dioxin is also classified as carcinogen by the IARC, based on limited evidence of carcinogenicity in humans, but sufficient evidence in animals and mechanistic data (IARC, 1997).

Descriptive studies on cancer mortality and congenital anomalies confirmed the presence of marked excesses mostly in the same areas where dioxin levels were elevated. In details, it has been demonstrated increase of incidents rates of the following: lung cancer in men and in women (Griffith et al., 1989; Goldberg et al., 1995), bladder cancer (Budnick et al., 1984; Griffith et al., 1989; Mallin, 1990), leukemia (Lotz et al., 1991), childhood leukemia (Cutler et al., 1986), liver cancer in men (Goldberg et al., 1995), prostate cancer (Goldberg et al., 1995), gastric cancer (Griffith et al., 1989, Goldberg et al., 1995), uterine cancer (Goldberg et al., 1995), rectum and breast cancer (Griffith et al., 1989), perinatal mortality and birth defects (Comba et al., 2006). The association between TCDD exposure and breast cancer risk has been inconclusive among different epidemiological studies (Manz et al., 1991; Warner et al., 2002), and animal models have also revealed contradictory findings (Safe et al., 1991; Brown et al., 1998).

More effects are summarized in the U.S. Environmental Protection Agency (US EPA's) draft exposure and human health reassessment of TCDD, and include peripheral nerve dysfunction, hepatic toxicity (increased liver size, hepatic enzyme changes), altered thyroid function in infants, altered hormone levels, developmental toxicity (decreased immune function), possible effects on organs (heart, lung, kidney) (US EPA, 2003). Other effects are spontaneous abortions (Bisanti et al., 1980), cytogenetic

abnormalities (Tenchini et al., 1983), congenital malformations (Rehder et al., 1978) and immunological (Knutsen, 1984) and neurological impairments (Barbieri et al. 1988).

In Vitro Effects

The mechanism of action by which TCDD exerts the biochemical effects on vertebrate species is through the activation of the aryl hydrocarbon receptor (AhR) (Dobrzyński et al., 2009) and a number of studies suggest that AhR itself is directly involved in the regulation of carcinogenesis. The constitutively active AhR mutant has been shown to produce tumors of stomach and liver (Andersson et al., 2002; Moennikes et al., 2004), while the unliganded AhR has been suggested to function as a tumor suppressor (Fan et al., 2010). Thus, the activation of AhR might play a much wider role in carcinogenesis than a simple transcriptional control of CYP enzymes.

One of TCDD effects in certain species is tumor promotion which is associated with alteration of differentiation and proliferation, TCDD-induced dysregulation of cellular growth and differentiation may be a possible mechanism of carcinogenesis. TCDD may activate the transcription of genes associated with cell proliferation through the TCDD responsive receptor/ enhancer system. Alternatively, this system may play a role in inhibiting the expression of genes normally suppressing cell division (Whitlock, 1990).

TCDD-dependent alteration of growth factors appears specific by species, tissues and developing stage. TCDD-induced toxicity and the subsequent compensatory cell proliferation may contribute to the induction of neoplasia.

In colon cancer cell lines, H508 and SNU-C4, TCDD stimulated a dose-dependent increase in cell proliferation and induced dose- and time-dependent phosphorylation of EGFR and ERK1/2 (Xie et al., 2012).

In breast cancer cell line, MCF-7, TCDD stimulated cell proliferation and inactivated Akt, increased cdc25C/cdc2 activity, decreased P21/P27 activity, and enriched G2/M phase (Chen et al., 2012).

The PI3K/PDK1/Akt signaling cascade mediates a variety of survival and proliferation responses. More recently, TCDD was found to activate and initiate plasmacytic differentiation of B lymphocytes through interfering Akt phosphorylation (North et al., 2010). Furthemore, it was demostarted that TCDD on PI3K/Akt signaling pathway in MCF-7 cells, dramatically decreased the phosphorylation of Akt (Thr308 and Ser473) (Chen et al., 2012).

PROSTATE CANCER

The prostate is a lobed gland that in male located at the junction of the bladder and the urethra. It contributes a liquid component to seminal fluid. The dog is the only species than man that spontaneously develops PC and many of the features of the disease in the dog are similar to its human counterpart. It has therefore been considered as a model for studing pathogenesis (Winkler et al., 2005; Winkler et al., 2006), treatment (Huang et al., 2005) and prevention (Waters et al., 2003). However a number of aspects of the pathogenesis, diagnosis and management of the disease are different between dogs and people (Starkey et al., 2005).

PC is an uncommon disease in the dogs, with prevalence between 0.2 to 0.6% (Bell et al., 1991) and it is an extremely serious disease that can affect male dogs of all breeds and ages, whether intact or neutered, although it occurs more frequently in older dogs (9-10 years)(Cooley and Waters, 2001).

Neoplastic prostate glands are often significantly and asymmetrically enlarged and may impinge on abdominal organs causing faecal tenesmus and dysuria. Haematuria, anorexia and weight loss may also occur. Some dogs may present signs of myelopathy or lameness that are manifestations of skeletal metastases. Indeed, PC is highly invasive, space-occupying masses that typically have metastasized by the time they are diagnosed. Commonly, PC given to metastasis to lumbar lymph-nodes, both inner and outer, to the vertebral body and lungs. The other targets for metastasis are the bladder neck, ureters, colon and pelvic muscle (Bell et al., 1991; Cornell et al., 2000).

In cases of prostate cancer, abdominal and rectal palpation can showed a large, asymmetrical, irregular and painful prostate gland. Haemogram, serum biochemistry, urinalysis and urine culture, as the symptoms of prostate cancer can mimic those of a urinary tract infection or kidney disease. Neoplastic epithelial cells may be seen cytologically on evaluation of urine or prostatic fluid. Another common test is an ultrasound of the prostate gland. This is the preferred method for assessing the general health of the prostate, as it can help to distinguish between prostatic cysts, abscesses and tumors. A fine needle aspirate of cells from the prostate gland can be done with ultrasound guidance, which also can help distinguish between benign prostatic hypertrophy (BPH), prostatitis, abscesses and cancer. Thoracic (chest) and abdominal radiographs (X-rays), as well as abdominal ultrasound, can be useful to look for signs of metastasis. Regional lymph nodes can be sampled by fine needle aspiration, or removed

entirely, and examined microscopically for the same purpose. Biopsy of the prostate, with subsequent microscopic (histopathologic) evaluation of the tissue samples, is the only definitive way to diagnose prostate cancer. Biopsy of the prostate will enable the veterinary pathologist not only to diagnose the existence (or absence) of cancer, but also to identify what type of cancer, if any, is involved. The lack of markers for PC makes early diagnosis difficult.

There is no known way to prevent PC because prostatic tumors are not influenced by testosterone, castration does not reduce the risk of this disease or slow its progression but it was proved that in castrated dogs the risk factor of developing PC is 2,38 fold higher than in intact ones (Teske et al., 2002).

Surgical resection is the only viable "cure" for prostate cancer. However, since the cancer usually has metastasized by the time it is diagnosed, surgical rarely is a curative option and tratment with curative intent is rendered difficult by the fact that radical prostatectomy in dogs with prostatic disease is associated with a very high incidence of postoperative urinary incontinence (Basinger et al., 1987).

Radiation and chemotherapy can be used to help minimize the effects of prostate disease and to help affected dogs live as comfortably as possible for the duration of their lives (Bell et al., 1991).

Dogs with naturally-occurring prostate cancer are relevant models for the disease in humans and pre-clinical studies of new diagnostics and therapies in dogs may benefit both humans and dogs with prostate cancer (Leroy and Northrup, 2009).

Prostate cancer is the most commonly diagnosed cancer in men. It is estimated that 1 in 6 men will be diagnosed with prostate cancer within their lifetime and 1 in 36 will die from the disease. Prostate cancer is also the second leading cause of all cancer related deaths in men. The five year survival rate for men diagnosed with local or regional prostate cancer is 100%. However, men diagnosed with a distant metastasis have a five year survival rate of just 29%. Most men who die of prostate cancer present with hormone refractory prostate cancer (HRPC) (Siegel et al., 2012). Androgen deprivation therapy (ADT) is the predominant form of treatment for men diagnosed with regional prostate cancer. ADT suppresses testicular androgen synthesis and inhibits androgen receptor activation. However, most patients acquire resistance to ADT and develop hormone refractory prostate cancer (HRPC) following therapy (Messing et al., 1999). This more advanced form of prostate cancer that is hormone refractory does not

rely on the presence of androgens for growth (Saraon et al., 2011). To significantly improve survival in men with PC, new therapeutic strategies to inhibit the appearance of this phenotype must be developed. PI3K/Akt/mTOR pathway plays a central role in various cellular processes including protein synthesis, cell cycle, cell survival, cell growth, motility and angiogenesis (Markman et al., 2010) and contributes to prostate cancer progression and transition to androgen-independent disease. Recent studies also demonstrated that this pathway is often deregulated during prostate cancer progression. Thus, targeting PTEN/PI3K/Akt and mTOR signaling pathway could be an effective strategy for the treatment of hormone refractory CP (Burgio et al. 2012). Many small molecule inhibitors targeting PI3K, Akt and/or mTOR are currently at various stages of clinical development. Despite promising results in certain preclinical tumor models (Markman et al., 2010), clinical responses to small molecule inhibitors of this pathway have been limited, since PI3K, Akt or mTOR inhibition typically promotes growth arrest rather than cell death in solid tumors (Cheng et al., 2009).

PROSTATE CANCER AND TCDD

In vivo studies, in mice and rats, demonstrated that TCDD exposure, early in development, can cause abnormal prostate development and may increase susceptibility to prostate cancer (Theobald and Peterson, 1997).

In fact, a single maternal dose (GD: gestation day 13) inhibits prostate growth, reducing ventral, dorsolateral, and anterior prostate weight , inhibits and delays differentiation of prostatic luminal epithelial cells and pericordial smooth muscle cells (Roman et al., 1998) decreases the number of main ducts and inhibits branching morphogenesis of all prostate lobes in rats, inhibiting the ductal morphogenesis and branching (Roman et al., 1998; Timms et al., 2002).

The effects of TCDD on prostate development in mice has focused on the ontogeny of these abnormalities, in large part because prostatic vulnerability to TCDD starts before birth (Lin et al., 2002). Morphologically, prostate development begins when urogenital sinus (UGS) epithelium begins to develop buds in response to inductive signals from the surrounding mesenchyme. It is demostrated that maternal TCDD treatment delays the formation of anterior and dorsolateral prostatic epithelial buds in mice by about a day, reduces the number of dorsolateral buds by about 25%, and

prevents ventral buds from forming (Lin et al., 2003). Effects on prostatic budding can account for many of the prostatic effects of TCDD seen in adult mice.

It is well known that perturbations of early development can have lifelong consequences for the prostate and that most prostatic diseases occur later in life depend from this perturbation (Schulman, 2000).

More importantly, in aged rats, exposed to TCDD, greater epithelial proliferation was accompanied by morphological alterations manifested as lobe-specific hyperplasia. The cribriform structures it was observed in the mouse are closely reminiscent of lobe-specific hyperplasia observed in the aging rat. Cribriform structures are commonly seen in the early stages of benign prostatic hyperplasia, and have also been reported in aged rats that develop spontaneous prostate adenocarcinoma and in transgenic mice that develop prostate cancer. While the presence of cribriform structures does not necessarily mean that overt prostatic disease will develop, some investigators consider these structures in rodents to be precancerous lesions (Fritz et al., 2005).

Descriptive studies on cancer mortality and congenital anomalies confirmed the presence of marked excesses of prostate cancer mostly in the same areas where dioxin levels were elevated (Goldberg et al., 1995).

Different studies evaluated the relation between Agent Orange exposure and prostate cancer in Vietnam veterans yielded conflicting results and were limited by small sample sizes and the lack of quantification of Agent Orange exposure (Zafar and Terris, 2001; Giri et al., 2004; Everly et al., 2009).

Furthermore, it was demonstrated in vitro that TCDD induces cytochrome P450 (CYP) 1A1 and CYP1B1 via the aryl hydrocarbon receptor (AhR) in PC3 and DU145 hormone-independent human prostate cancer cell lines. The induction of CYP1A1 and CYP1B1 by TCCD in hormone-independent prostate cancer cell lines suggests that CYP induction should be considered in patients with advanced prostate cancer. This effect of TCDD could result in higher elimination rates of concomitant drugs metabolized by these particular CYP isoenzymes (Schaufler et al., 2002).

AUTOPHAGY

Autophagy is a tightly regulated process playing a normal part in cell growth, development, and homeostasis and helping to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products through the degradation via

the lysosome. The initiation of autophagy begins with the formation of the autophagosome, which consists of cytoplasmic material sequestered inside a double membrane vescicle. Then, the autophagosome fuses with the lysosome and the cytoplasmic material is digested by hydrolases (Demarchi et al., 2006).

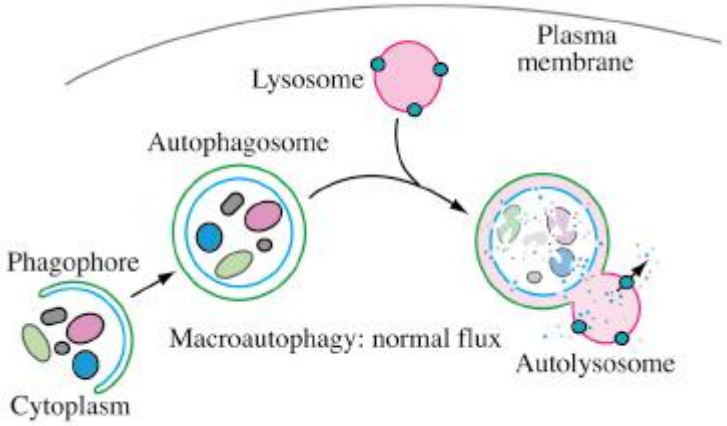

Figure 7: Autophagy (Klionsky et al. 2012).

Defects in this system are linked with pathogenesis of numerous diseases, including neurodegenerative disease (Rubinsztein et al., 2007), liver disease (Perlmutter, 2006), muscle disease (Nishino, 2006), cardiac disease (Terman and Brunk, 2005) microbial infection and cancer (White and DiPaola, 2009). The abnormal expression of Beclin-1 and LC3 has been found in human melanoma (Pirtoli et al., 2009), colon (Li et al., 2009), ovarian (Shen et al., 2008) and brain cancer (Miracco et al., 2007).

The autophagic pathway can be stimulated by multiple forms of cellular stress, including nutrient or growth factor deprivation, hypoxia, reactive oxygen species, DNA damage, protein aggregates, damaged organelles, or intracellular pathogens (Kroemer et al., 2010). Autophagy stress-induced most often exerts cytoprotective functions and favors the re-establishment of homeostasis and survival (Chen and Karantza, 2011).

Autophagy is regulated by a cellular network that involves upstream signaling pathways and this network is composed of 751 interactions with extensive connectivity among sub-networks: ULK kinase, PlKC3-BECN1, ATG2-WIPI, UBL conjuction system, human ATG8's and AMP kinase (**Fig. 8A**) (Behrends et al., 2010).

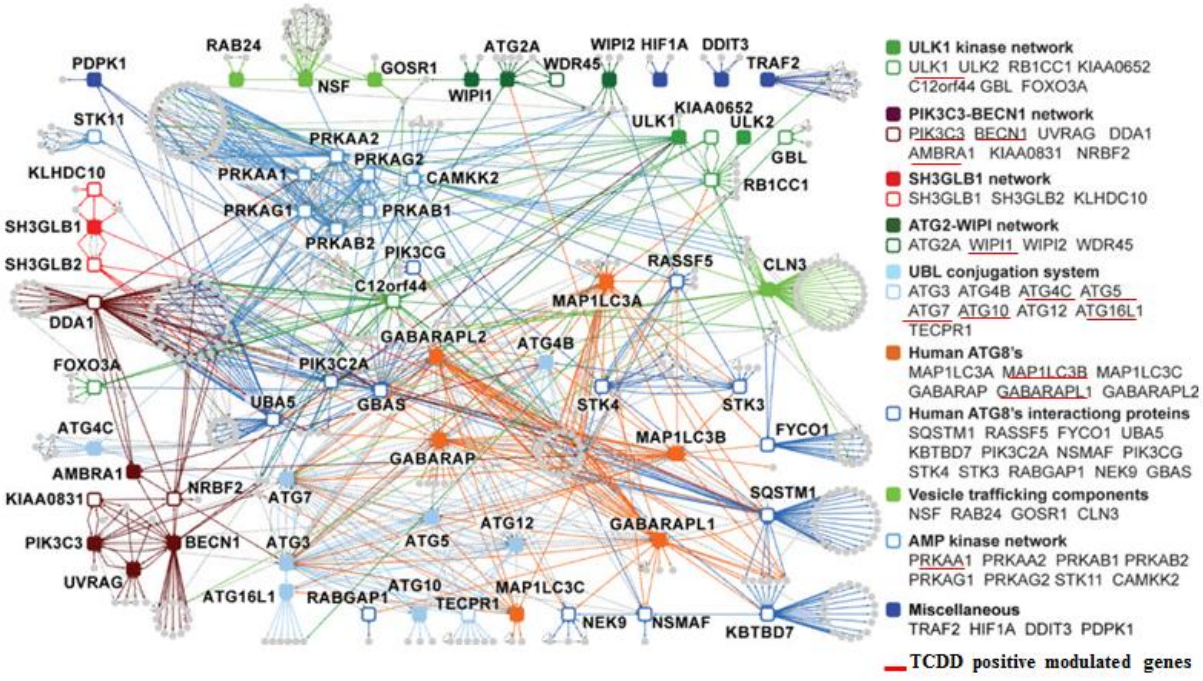

Fig 8A: Autophagy interactive network. Modifed by Behrends et al., 2010.

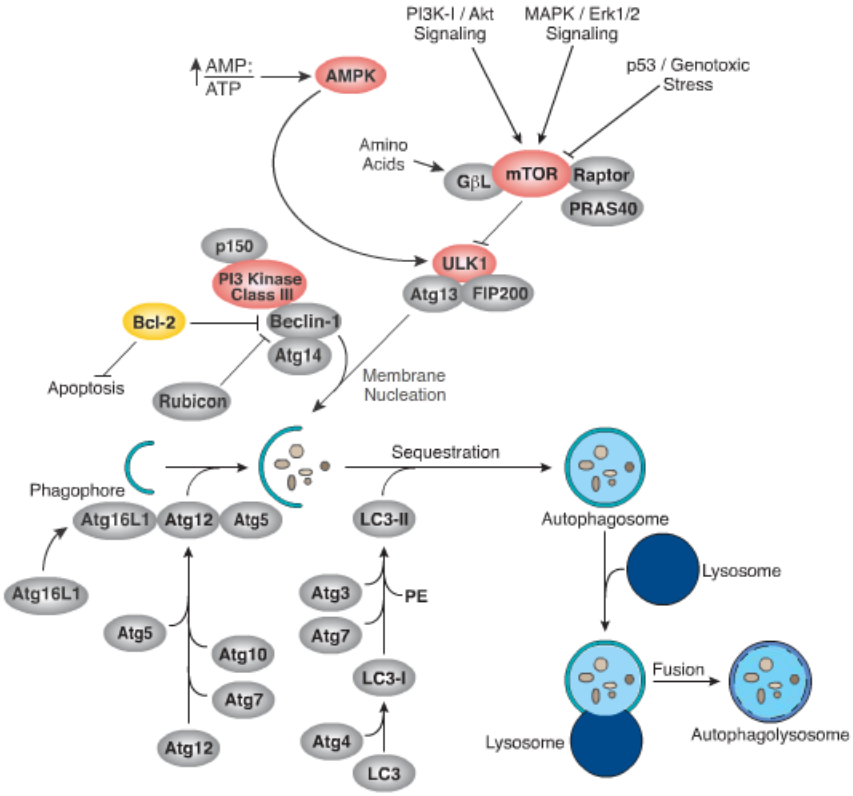

Figure 8B: Autophagy **(**From http://www.cellsignal.com/reference/pathway/Autophagy.html**).**

Class III PI3K complex, containing hVps34, Beclin 1, p150 (a mammalian homolog of yeast Vps15), and Atg14-like protein, is required for the induction of autophagy. The formation of autophagosomes is initiated by class III phophoinositide 3-kinase and Beclin-1, this complex play a key role in mammalian autophagy (Eskelinen and Saftig, 2009). Then, autophagosomes fuse with the lysosome, forming autophagolysosomes promoting the intracellular degradation. LC3-I (microtubule-associated protein chain 3 protein) is processed and activated by an ubiquitination-like reaction regulated by Atg7 and Atg3. The abundant cytoplasmic LC3-I recruited during the formation of autophagosomes, is cleaved into a soluble form known as LC3-II, special marker of autophagy (Tanida et al., 2004).

Nutrient and/or growth factor availability activates the PI3K/AKT/mTOR axis, which inhibits autophagy thus stimulating cell growth and proliferation (Li et al., 2009; Pirtoli et al., 2009; White and DiPaola, 2009).

The kinase mTOR is a regulator of autophagy induction, negative regulation of mTOR promoting it. Three related serine/threonine kinases, UNC-51-like kinase -1, -2, and -3 (ULK1, ULK2, UKL3), act downstream of the mTOR complex. ULK1 and ULK2 form a large complex with the mammalian homolog of an autophagy-related (Atg) gene product (mAtg13) and the scaffold protein FIP200 (an ortholog of yeast Atg17).

The Atg genes control the autophagosome formation through Atg12-Atg5 and LC3-II (Atg8-II) complexes. Atg12 is conjugated to Atg5 in a ubiquitin-like reaction that requires Atg7 and Atg10 (E1 and E2-like enzymes, respectively). The Atg12–Atg5 conjugate then interacts noncovalently with Atg16 to form a large complex. LC3/Atg8 is cleaved at its C terminus by Atg4 protease to generate the cytosolic LC3-I. LC3-I is conjugated to phosphatidylethanolamine (PE) also in a ubiquitin-like reaction that requires Atg7 and Atg3 (E1 and E2-like enzymes, respectively).

Autophagy and apoptosis are connected both positively and negatively, and extensive crosstalk exists between the two. During nutrient deficiency, autophagy functions as a pro-survival mechanism; however, excessive autophagy may lead to cell death, a process morphologically distinct from apoptosis (Tanida et al., 2001).

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AUTOPHAGY AND CANCER

The role of autophagy in cancer is controversial, as the factors that determine whether autophagy induces tumor cell death or protects tumor cells from unfavorable conditions have not been clearly elucidated (Rosenfeldt and Ryan, 2011). Autophagy is reported to play an anti-tumoral role by reducing the chromosomic instability, thus avoiding DNA damage (Mathew et al., 2007) and inhibiting cell proliferation (Levine and Klionsky, 2004; Pua et al., 2007). On the other hand, several studies demonstrated that autophagy-mediated support of tumor cell survival may play a critical role in cancer progression at later stages, such as dissemination and metastasis, which account for most cancer-associated deaths. Autophagy acts as an anti-apoptotic mechanism for tumor cells under stress, maintaining metabolism and energy needed for survival (Mathew et al., 2007) and could promote angiogenesis and tumor progression (Degenhardt et al., 2006; Mathew et al., 2007).

In favor of this hypothesis, starvation-induced autophagy is accompanied by suppression of protein synthesis, cell division and motility in an energy conservation effort that sustains cells in a dormant state with the capacity to resume cell growth and proliferation upon regular growth condition restoration. For example, autophagy induction in mammary epithelial cells upon their detachment from extraceullular matrix (ECM) sustains cell viability in an anoikis-resistant manner, whereas autophagy upregulation in ovarian cancer cells by the tumor suppressor ARHI (aplasia Ras homolog member I) promotes dormant cell survival in vivo (Chen and Karantza, 2011).

It was demostrated in some prostate cancer cell lines (LNCaP and PC346-Flu1) are very sensitive to monotherapy with the AKT inhibitor AZD5363, where the compound induces significant apoptosis at concentrations achievable in preclinical models, other prostate cancer cell lines, such as DU-145 and PC3, are relatively resistant. In fact, it was reported that AZD5363 monotherapy induced growth arrest and autophagy and failed to induce significant apoptosis in PC-3 and DU145 PCa cells. In light of these reasons, autophagy seems to be a good candidate for prostate cancer research because its deregulation in this disease (Lamoureux et al., 2013). Finally, many studies have clearly documented that in cancer cells, autophagy is upregulated in response to metabolic and genotoxic stress induced by hormonal deprivation, chemotherapy and radiation as a cell survival mechanism, likely contributing to treatment resistance (Chen and Karantza, 2011).

Furthermore, autophagy became a novel therapeutic target in cancer because inhibition or induction of autophagy promotes cancer cell death and potentiates various anticancer therapies (White and DiPaola, 2009). The impact of autophagy inhibition on anticancer therapy has been evaluated in multiple tumor models, including glioma (Ito et al., 2005), myeloma (Shanmugam et al., 2009), breast (Vazquez-Martin et al., 2009), colon (Apel et al., 2008), and prostate cancers (Kim et al., 2009).

AIMS

TCDD represents a persistent organic pollutant and exposure to TCDD can result in a number of toxic effects in several species (Pohjanvirta and Tuomisto, 1994). In 1997, the International Agency for Research on Cancer (IARC) classified TCDD as carcinogen (IARC, 1997) but the molecular mechanisms of action of TCDD have not been elucidated so far (Mandal, 2005).

Prostate cancer (PC) is an extremely serious disease in dogs (LeRoy and Northrup, 2009) and PC represents the most commonly diagnosed cancer in males in the Western world (Siegel et al., 2012). In general, animal models of human cancer have evolved in attempts to capture the complexity of the human disease because the canine prostate gland shares many morphological and functional similarities with the human prostate (LeRoy and Northrup, 2009). Consequently, to evaluate the biological effects of TCDD on prostate cancer in this study we used human prostate cancer cell line, PC3. Until now, very few data are present in literature about PC3 and TCDD exposure. It is known that in PC3 cell line, a hormone-independent prostate cancer cell line, TCDD induces CYP1A1 and CYP1B1 via AhR. This effect of TCDD could result in higher elimination rates of concomitant drugs metabolized by these particular CYP isoenzymes (Schaufler et al., 2002). Moreover, several studies suggest that TCDD exposure is associated with abnormal prostate development, altered prostate pathology and increase susceptibility to prostate cancer (Theobald and Peterson, 1997; Fritz et al., 2005; Everly et al., 2009) and cancer mortality descriptive studies demonstrated an increase of incidents rates of the prostate cancer in the same areas where dioxin levels were elevated (Goldberg et al., 1995).

The administration of TCDD to a variety of cultured cells may alter their ability to proliferate and die. In a previous study we demonstrated that TCDD induced proliferation in Madin-Darby Bovine Kidney (MDBK), an epithelial cell line, in which analysis of MDBK cell morphology revealed some alterations in a large number of exposed cells, where neither signs of apoptosis nor necrosis were detected, but we found that TCDD activated cell death with autophagy (Fiorito et al., 2011).

Autophagy is a tightly regulated process playing a normal part in cell growth, development, and homeostasis and helping to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products through the degradation via the lysosome (Demarchi et al., 2006). Defects of autophagy machinery are responsible for pathogenesis of different diseases, including cancer. The role of autophagy in cancer is controversial. There is evidence that autophagy may play a critical role in cancer

progression at later stages, such as dissemination and metastasis, which account for most cancer-associated deaths, whereas in other cases it clearly contributes to tumor suppression by inducing tumor cell death (Chen and Karantza, 2011).

Taking in account all the above, **the aim of this study was to evaluate the biological effects of TCDD exposure to a highly metastatic prostate cancer cell line, PC3.**

Indeed, a better understanding of inter-species mechanism of action of TCDD action is necessary to enhance the knowledge of TCDD role on prostate cancer that is the most commonly diagnosed cancer in males in the Western world and is an extremely serious disease in our dogs.

MATERIALS AND METHODS

Cell culture

Prostate carcinoma cells PC3 were purchased from ATCC (American Type Culture Collection, Manassaa, Va, USA). Cells were grown to 70–80% confluence in ATCC-formulated F-12K Medium (Catalog No. 30-2004), containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in a 37°C humidified incubator supplied with 5% of carbon dioxide (CO2)

TCDD

TCDD (Supelco, 48599) was diluited at 0.01, 1 and 100 pg/ml in F-12K cell culture medium according to Fiorito et al. 2008a,b, 2010, 2011 and Santamaria et al. (2011).

Cell Proliferation and Viability

Cell proliferation and viability was assessed by Trypan blue (TB) exclusion test. PC3 cells (5×10^4 cells/well) were grown in 24-well plates, at 70–80% confluence, exposed to TCDD (0.01, 1, or 100 pg/ml) and incubated for 24, 48 and 72 h. Cells were collected by trypsinization, at the indicated times after TCDD exposure, and an aliquot of the cell suspension was mixed with an equal volume of 0.2% Trypan-blue (Sigma) in 1 × phosphate-buffered saline (PBS). After 10 min, the cells were evaluated in a Burker chamber at a light microscope. Stained blue cells with damaged cell membrane represented non-viable cells. Cell proliferation was evaluated by count of total number of cells. Cell viability was calculated as percentage of live cells over the total cells number, and results are the mean ± S.E.M. of four independent experiments performed in duplicate.

Cell cycle analysis

PC3 cells (1×10^5 cells/ml), were grown in tissue culture dishes and exposed at different concentrations of TCDD (0.01, 1 or 100 pg/ml). After 24, 48 and 72 h of exposure, adherent cells were washed twice with PBS and removed from the dish by treatment with trypsin–EDTA solution. Then, 1×10^6 PC3 cells were harvested and fixed in 70% ethanol after washes with cold phosphate-buffered saline (PBS), and stored at 4°C until the analysis. After centrifugation, the resulting cell pellet was incubated in the dark

for 30 min, in 0.3 ml of freshly prepared PBS containing 0.02 mg/ml propidium iodide (PI) and 0.25 mg/ml ribonuclease A (Sigma). The samples were then analyzed for DNA content using a BD Accuri™ C6 Flow Cytometer (30,000 events/sample).

Cell morphology analysis

Cell morphology was performed by phase-contrast microscopy. PC3 cells (5×10^4 cells/well) were grown in 24-well plates, at 70–80% confluence, exposed to TCDD (0.01, 1, or 100 pg/ml) and incubated for 24, 48 and 72 h. Cells were washed twice with PBS, fixed with 95% ethanol, drained and dried. The slides were mounted in Entellan (Merck, 100869), coverslipped and phase-contrast microscopy studies and photomicrographs observations were carried out.

Acridine orange staining

The staining with acridine orange was performed to detect AVOs. PC3 cells (5×10^4 cells/well) were grown in 24-well plates, at 70–80% confluence, exposed to TCDD (100 pg/ml) or, autophagy inductor, thapsigargin (TG) (0.5 µM) (Grotemeier et al., 2010) and incubated for 48 h. After exposure to TCDD or TG, cells were washed with PBS and stained with acridine orange (Sigma, A6014) at a final concentration of 1 µg/ml for 15 min. Stained cells were placed on a microscope slide and observed under UV with a fluorescence microscope (Olympus).

Immunofluorescence microscope analysis

PC3 cells (1×10^4 cells/well) were grown in chambers slide, exposed to TCDD (100 pg/ml) or TG (0.5 µM) and incubated for 48 h. Cells were washed with PBS and then fixed with paraformaldehyde (4%, w:v). After rinsing in PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 1 min and blocked in 4% BSA in PBS for 30 min. This was followed by incubation in rabbit polyclonal microtubule associated protein light chain 3A/B (LC3A/B) antibody (1:50) (ABCAM, ab58610), for 24 h at 4°C in a humidified chamber. Cells were washed with PBS and then incubated in Goat Anti-Rabbit IgG (1:1000) (Alexa Fluor® 594) for 1 h at room temperature. Finally, cells were

rinsed in PBS, coverslipped and observed under UV with a fluorescence microscope (Nikon).

Gene expression analysis

PC3 cells were grown in tissue culture dishes and exposed to TCDD (100 pg/ml), autophagy inhibitor chloroquine (CQ, 10µM) and TG (0.5 µM) in the following: PC3 exposed to TCDD (PC3_TCDD); PC3 exposed to TCDD and CQ (PC3_TCDD+CQ); PC3 exposed to TG (PC3_TG). After 24, 48 and 72 h of exposure, PC3_TCDD, PC3_TCDD+CQ and PC3_TG cells were collected and total RNA extraction was performed using RNeasy Mini Kit (Qiagen, Catalog no. 74104) according to the manufacturer's instruction. Reverse transcription of 1 µg of total RNA as a template was used to produce cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystem) according to the manufacturer's instructions. Real-time PCR was performed using the LightCycler® 480 Real-Time PCR System (Roche Applied Science) in reactions of 25 µl containing 100 ng of cDNA, 10 µl of TaqMan® Universal PCR Master Mix (2x), No AmpErase®, UNG (Applied Biosystems®, Catalog Number: 4366072), and 1 µl of TaqMan® Gene Expression Assay (20x)(Applied Biosystem). The target gene analyzed was LC3 (LC3, Hs00917683) and the reference used was hypoxanthine-guanine phosphoribosyltransferase (HPRT, 4333768)

Protein extraction and Western blot analysis

PC3 cells were grown in tissue culture dishes and exposed to TCDD (100 pg/ml) and autophagy inhibitor chloroquine (CQ, 10µM). After 48 h of exposure adherent cells were washed twice with PBS and removed from the flask by treatment with trypsin–EDTA solution. Then cells were collected by centrifugation and resuspended at an adequate concentration in PBS. The pellets, obtained by centrifugation, were stored at -20° C. Later it, cells were homogenized into lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 0.5 mM sodium orthovanadate, and 20 mM sodium pyrophosphate).

The lysates were clarified by centrifugation at 14,000 rpm × 10 min. Protein concentrations were estimated by an assay (Bio-Rad) and boiled in Laemmli buffer

[0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2- mercaptoethanol, and 0.002% bromophenol blue] for 5 min before electrophoresis.

Proteins were subjected to SDS-PAGE (12.5% polyacrylamide). After electrophoresis, proteins were transferred to nitrocellulose membranes (Millipore, Immobilon); complete transfer was assessed using pre-stained protein standards (Bio-Rad). After blocking with Tris–buffered saline–BSA [25 mM Tris (pH 7.4), 200 mM NaCl, and 5% BSA], the membrane was incubated with the primary antibodies. The following antibodies, dissolved in 5% bovine serum albumin-TBST, were used: rabbit polyclonal microtubule-associated protein light chain 3A/B (LC3A/B) antibody (1:800) (ABCAM, ab58610), for 24 h at 4° C in a humidified chamber. After membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:1000) (at room temperature), and the reaction was detected with an enhanced chemiluminescence system (Amersham Life Science). The images of Western immunoblot specific bands on X-ray films were imported into a computer by a scanner and captured as digital TIFF images. The results were plotted in a graph after densitometry analysis of the blots obtained.

Autophagy Related Gene Expression Profiling

The analysis of the autophagy pathway was performed using RT^2 Profiler™ PCR Array (Quiagen, PAHS-084ZF).

PC3 cells were cultered in tissue culture dishes and exposed to TCDD (100 pg/ml) or to TG (0.5 µM). After 48 h of exposure, PC3 cells were collected and total RNA extraction was performed using RNeasy Mini Kit (Qiagen, Catalog no. 74104) according to the manufacturer's instruction. Reverse transcription of 2 µg of total RNA was performed to produce cDNA using RT^2 First strand kit (Quiagen 330401) according to the manufacturer's instructions.

RT^2 Profiler™ PCR Array profiles the expression of 84 genes involved in autophagy. Controls for genomic DNA contamination and for the efficiency of the RT-PCR and PCR reactions were also included in the array. Each cDNA samples was mixed with RT^2 SYBR Green qPCR Master Mix (330500) and then added to the 96-well plate. The PCR was performed in LightCycler® 480 Real-Time PCR System (Roche Applied Science) and the results were calculated using the comparative CT method for separate tubes with GAPDH and RPLP0 as housekeeping genes. The fold change of gene

expression was calculated using the Excel-based PCR Array data analysis program (Qiagen).

DATA ANALYSIS

Data are presented as mean ± S.E.M. One-way ANOVA with Tukey's post-test was performed using GraphPad InStat Version 3.00 for Windows (GraphPad Software, San Diego, CA). A p value <0.05 was considered statistically significant.

RESULTS

TCDD enhances PC3 cell proliferation and viability

We analyzed the effect of TCDD exposure on PC3 cell proliferation and viability by Trypan blue (TB) exclusion test. PC3 cells were incubated in the presence of 0.01, 1 and 100 pg/ml of TCDD and then the TB exclusion test was performed at different times of exposure, 24, 48 and 72h. As shown in Fig. 9A, the exposure of various doses of TCDD on PC3 cells caused a significant increase in the total cells number of cells at all concentrations of TCDD studied from 24 to 72 h of exposure ($p < 0.001$ and $p < 0.05$). TCDD exposure determined an increase in cells proliferation (**Fig. 9**), in dose-dependent manner at 24 and 48 h. Whereas, examining treated cells at 0.01 and 100 pg/ml of TCDD compared to 1 pg/ml of TCDD at 72 h, we observerd an U-shaped does responsiveness cell proliferation (see also Discussion).

As shown in Fig. 9B, the exposure of various doses of TCDD on PC3 cells caused a significant increase in the cell viability in dose-dependent manner at all concentrations of TCDD studied from 24 to 72 h of exposure ($p < 0.001$, $p < 0.01$ and $p < 0.05$).

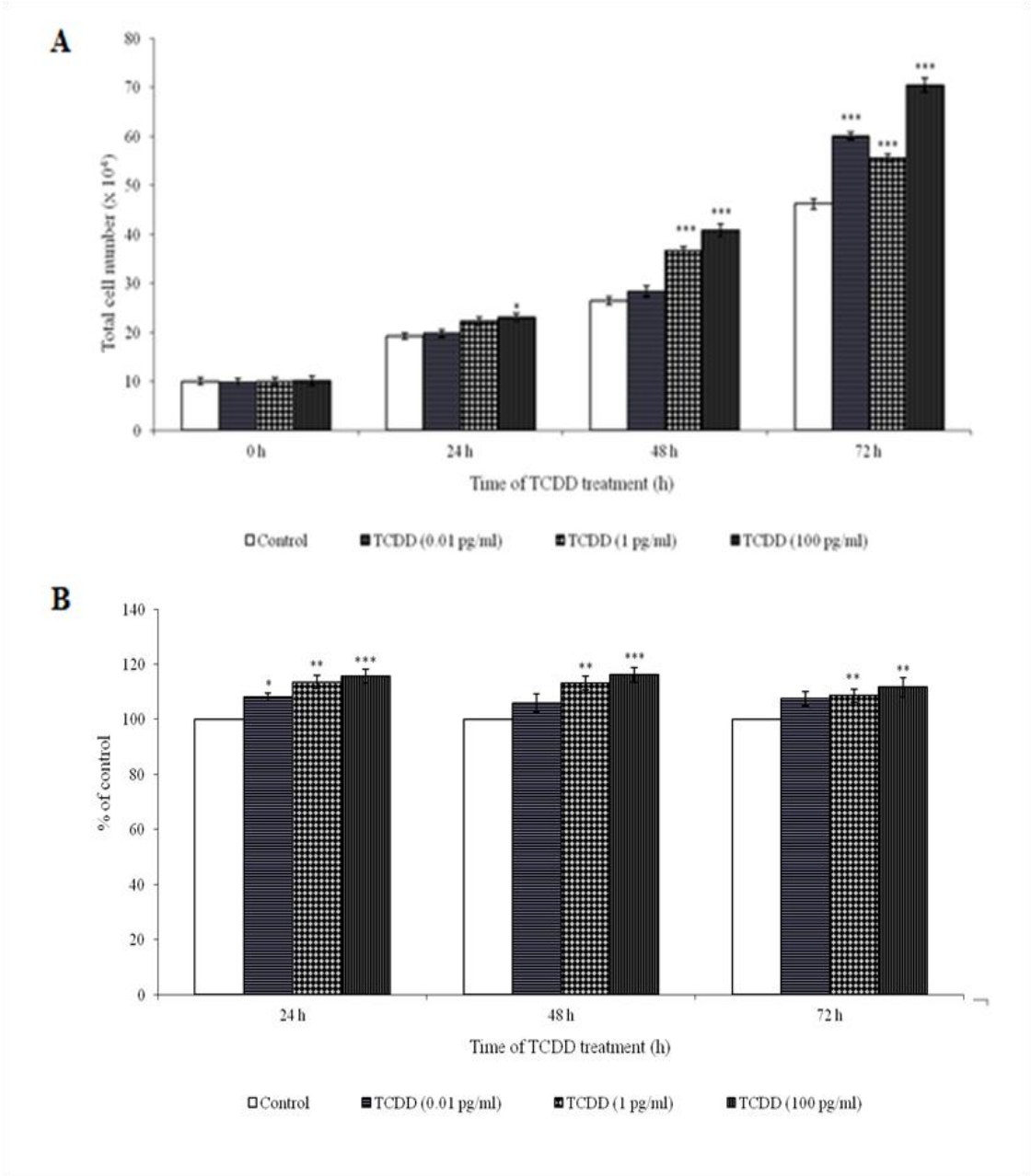


Fig.9: Effects of exposure to different concentrations of TCDD (0.01, 1, or 100 pg/ml) and incubated for 24, 48 and 72 h on PC3 cell proliferation (A) and viability (B). Data are presented as a percentage of the control (B) and results are expressed as the mean ± SE of four independent experiments performed in duplicate (A and B). Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability p. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Moreover, Trypan-blue exclusion test also indicated that no significant differences were observed between control and TCDD-treated groups in time dependent alteration of cell membrane permeability (TB permeable cells), at all tested doses tested (**Fig.10**).

TCDD does not induce cell death in PC3 but TCDD enhances PC3 cells viability and proliferation.

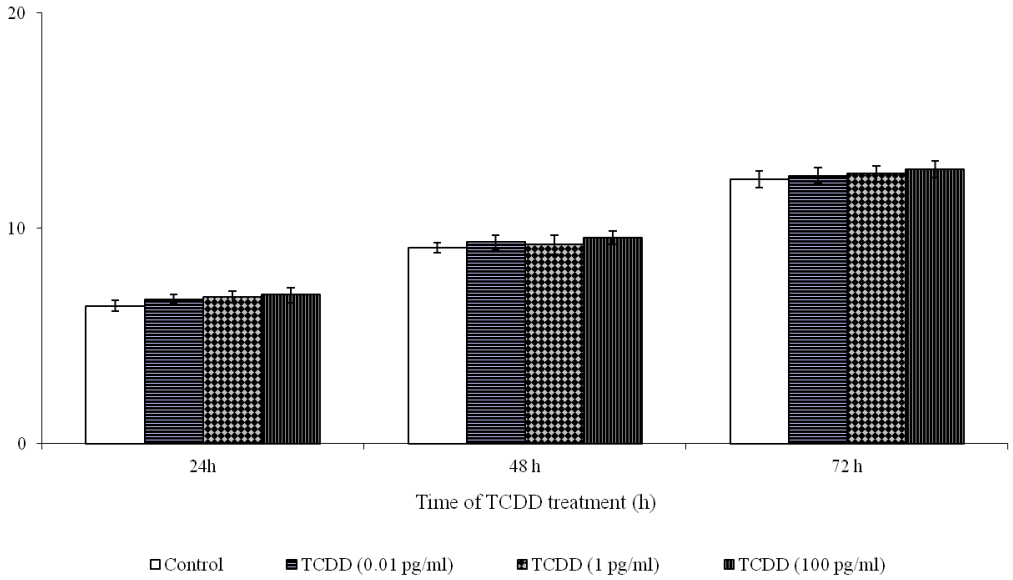
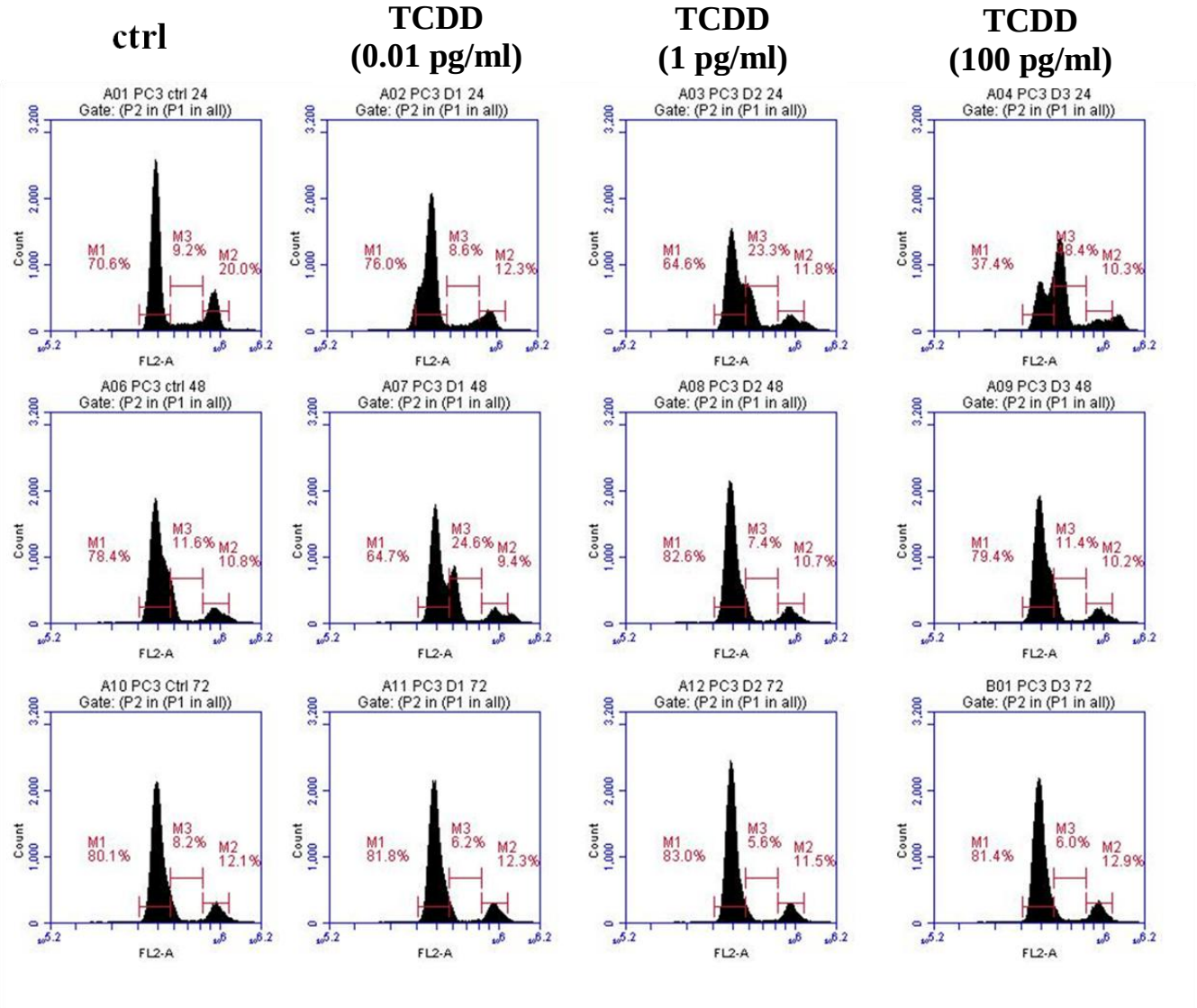


Fig. 10. Effects of exposure of different concentrations of TCDD (0.01, 1, or 100 pg/ml) incubated for 24, 48 and 72 h on cell membrane permeability/cell death. Values were percentage of non-viable cells, as determined by assessment of cells permeable to Trypan-blue. Data are presented as percentage of TB permeable cells. Data are presented as a percentage of the control and results are expressed as the mean ± S.D. of four independent experiments performed in duplicate.

TCDD induces cell cycle progression

TCDD significantly increased the cell population at S phase at 24 with 1 and 100 pg/ml and at 48h with 0.01 pg/ml compared with contol (**Fig. 11**). So, TCDD induces cell cycle progression.

A


ctrl
TCDD (0.01 pg/ml)
TCDD (1 pg/ml)
TCDD (100 pg/ml)
24 h
48h
72 h
A01 PC3 ctrl 24
Gate: (P2 in (P1 in all))
M1 70.6%
M3 9.2%
M2 20.0%
A02 PC3 D1 24
M1 76.0%
M3 8.6%
M2 12.3%
A03 PC3 D2 24
M1 64.6%
M3 23.3%
M2 11.8%
A04 PC3 D3 24
M1 37.4%
M3 48.4%
M2 10.3%
A06 PC3 ctrl 48
M1 78.4%
M3 11.6%
M2 10.8%
A07 PC3 D1 48
M1 64.7%
M3 24.6%
M2 9.4%
A08 PC3 D2 48
M1 82.6%
M3 7.4%
M2 10.7%
A09 PC3 D3 48
M1 79.4%
M3 11.4%
M2 10.2%
A10 PC3 Ctrl 72
M1 80.1%
M3 8.2%
M2 12.1%
A11 PC3 D1 72
M1 81.8%
M3 6.2%
M2 12.3%
A12 PC3 D2 72
M1 83.0%
M3 5.6%
M2 11.5%
B01 PC3 D3 72
M1 81.4%
M3 6.0%
M2 12.9%
Count
FL2-A

B

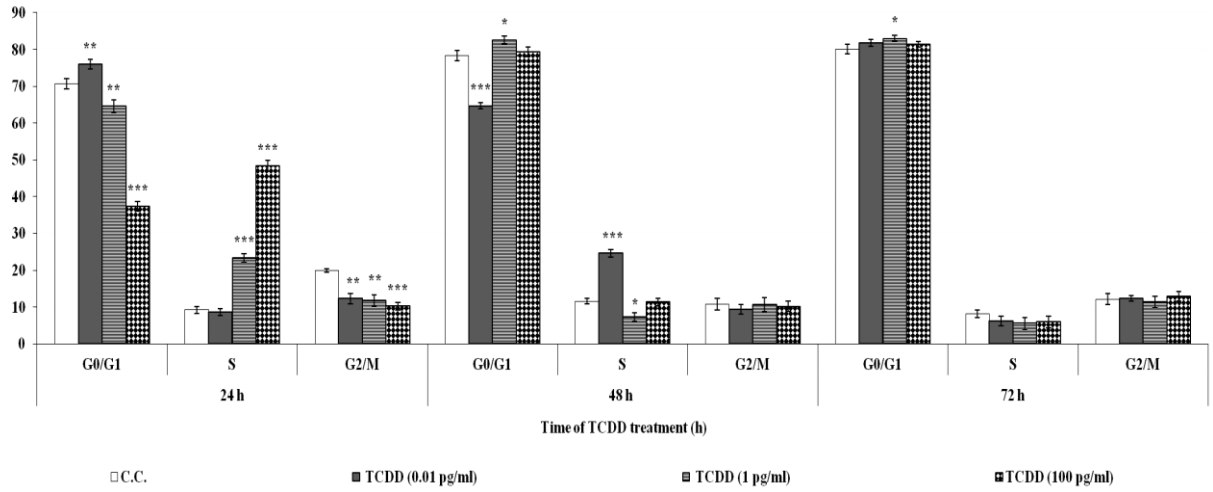
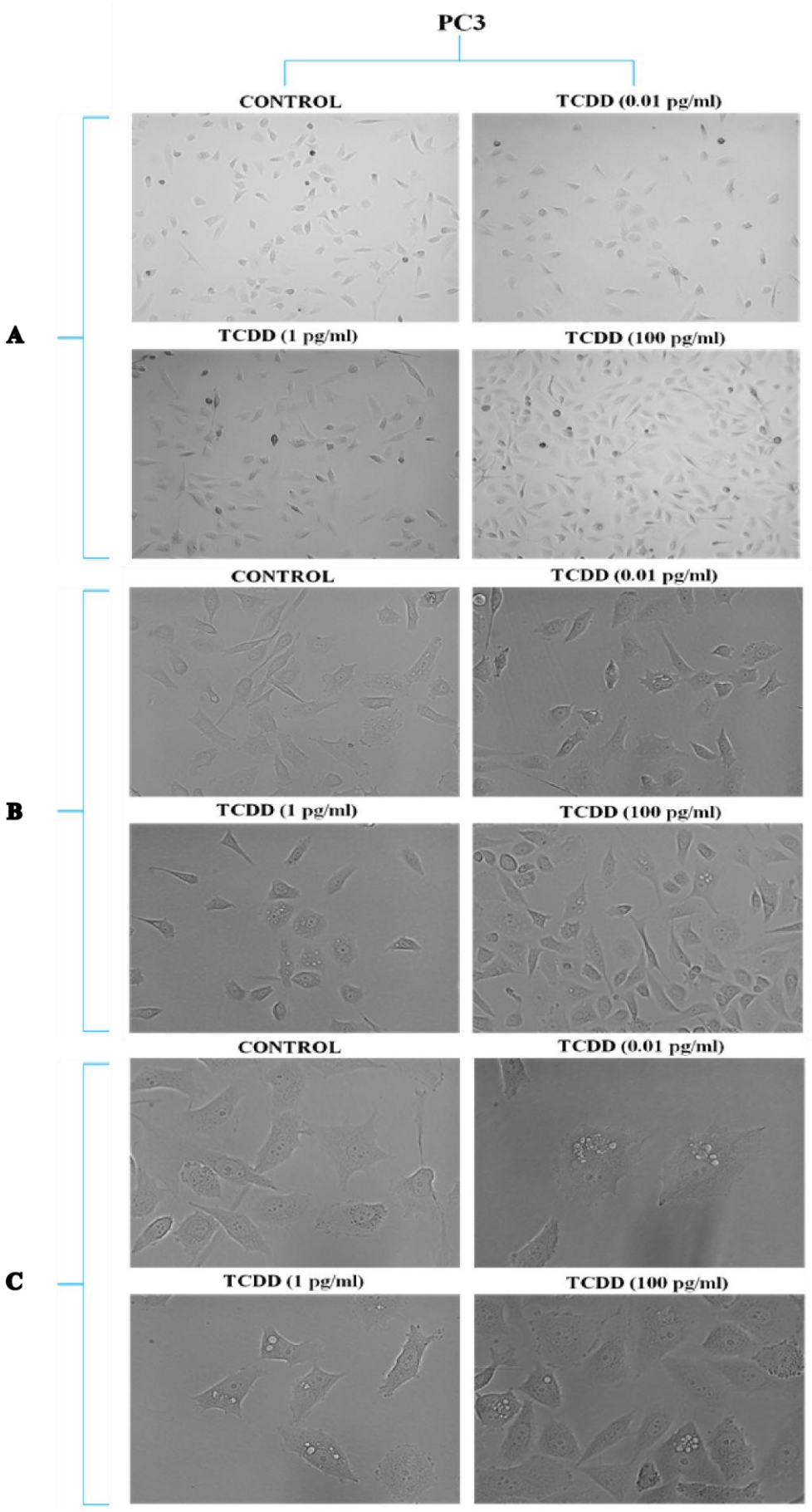

G0/G1
S
G2/M
24 h
48 h
72 h
Time of TCDD treatment (h)
C.C.
TCDD (0.01 pg/ml)
TCDD (1 pg/ml)
TCDD (100 pg/ml)

Fig.11: Effects of TCDD exposure to PC3 cell proliferation**.** PC3 cells exposed to different concentrations of TCDD (0.01, 1, or 100 pg/ml) were collected at 24, 48 and 72 h and the cell cycle analysis was performed by flow cytometry. Results of flow cytometry are shown as cell cycle analys (Fig. 3A) and as representative histograms of cell cycle distribution (Fig. 3B). Results are representative of 3 independent experiments.

TCDD induces morphological alterations in PC3 cells

PC3 cells were incubated in the presence of 0.01, 1 and 100 pg/ml of TCDD and cell morphology analysis was performed at different times of TCDD exposure, 24, 48 and 72h, by phase-contrast microscopy. At 48 h, a large number of cells showed the following morphological charateristic signs of autophagy: expanded cytoplasm (magnified cells) and an elevated degree of vacuolization, suggesting autophagy **(Fig. 12).** As positive control, we used TG (0.5 μmol/L), an inductor of autophagy. Cells exposed to TG showed an elevated degree of vacuolization similar to TCDD **(Fig 12**).


PC3
CONTROL
TCDD (0.01 pg/ml)
A
TCDD (1 pg/ml)
TCDD (100 pg/ml)
CONTROL
TCDD (0.01 pg/ml)
B
TCDD (1 pg/ml)
TCDD (100 pg/ml)
CONTROL
TCDD (0.01 pg/ml)
C
TCDD (1 pg/ml)
TCDD (100 pg/ml)

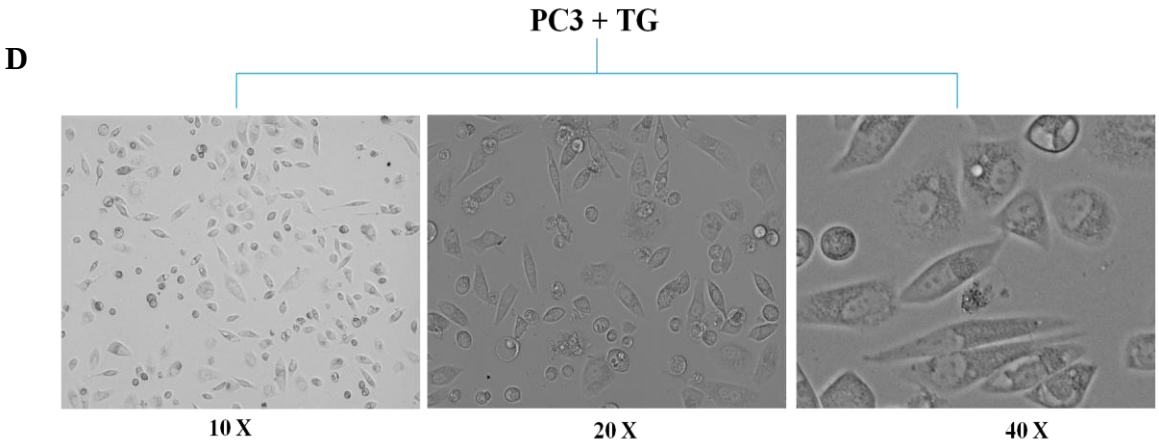
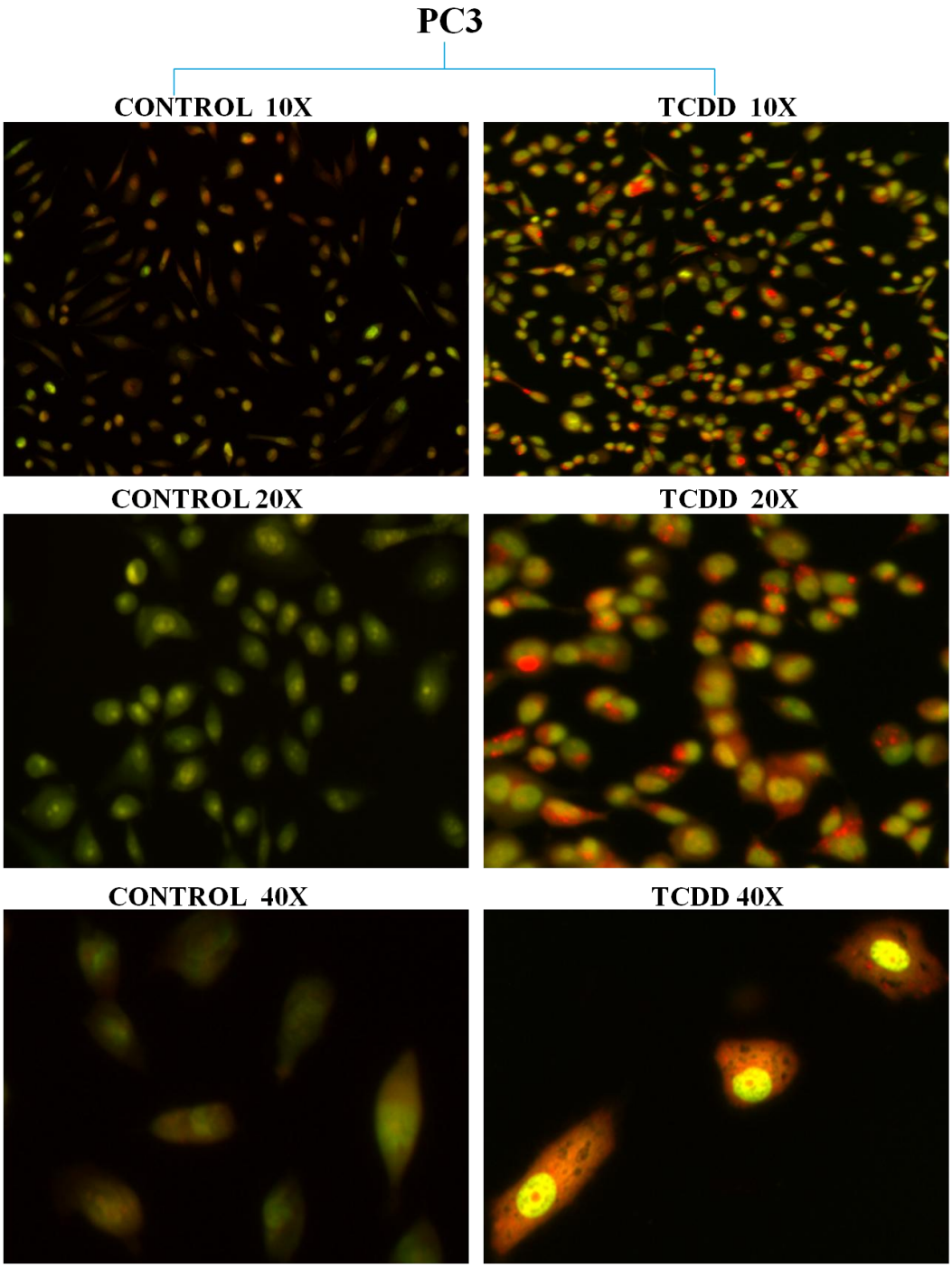


Fig 12. Effects of different concentrations of TCDD (A, B, C) or TG (D) exposure on PC3 cell morphology. PC3 cells were treated with different concentrations of TCDD (0.01, 1 and 100 pg/ml) or with TG (0.5 µmol/L) and then observed under light microscope. After 48 h of exposure, TCDD exposed PC3 cells compared to the control groups, displayed significant vacuole formation (A, magnification 10X; B, magnification 20X; C magnification 40X). After 48 h of exposure, TG exposed cells compared to the control group, displayed a similar elevated degree of vacuolization (D, magnification 10X, 20X and 40X).

TCDD induces AVOs formation in PC3 cells

The effects of TCDD treatment on PC-3 cells were evaluated by fluorescence microscopy following staining with the lysosomotropic agent acridine orange (**Fig. 13**). In acridinge orange-stained autophagic cells, the cytoplasm and nucleolus fluorescence bright green and dim red, whereas acidic compartments fluorescence bright red (Traganos and Darzynkiewicz, 1994; Chiu et al., 2009). The intensity of the red fluorescence is proportional to the degree of acidity of the cellular acidic compartment. Untreated PC-3 cells displayed primarily green fluorescence with minimal red fluorescence (minimal presence of AVOs) while PC-3 cells treated with TCDD (100 pg/mL) displayed red fluorescence (increased presence of AVOs) (**Fig. 13A**). The presence of AVOs and expanded cytoplasm (magnified cells), represent characteristic signs of autophagy (Kroemer and Levine, 2008; Zakeri et al., 2008; Lamparska-Przybysz et al., 2005). Similar morphological alterations were displayed in PC3 exposed to the autophagy inductor TG (**Fig. 13B**).

A


PC3
CONTROL 10X
TCDD 10X
CONTROL 20X
TCDD 20X
CONTROL 40X
TCDD 40X

B

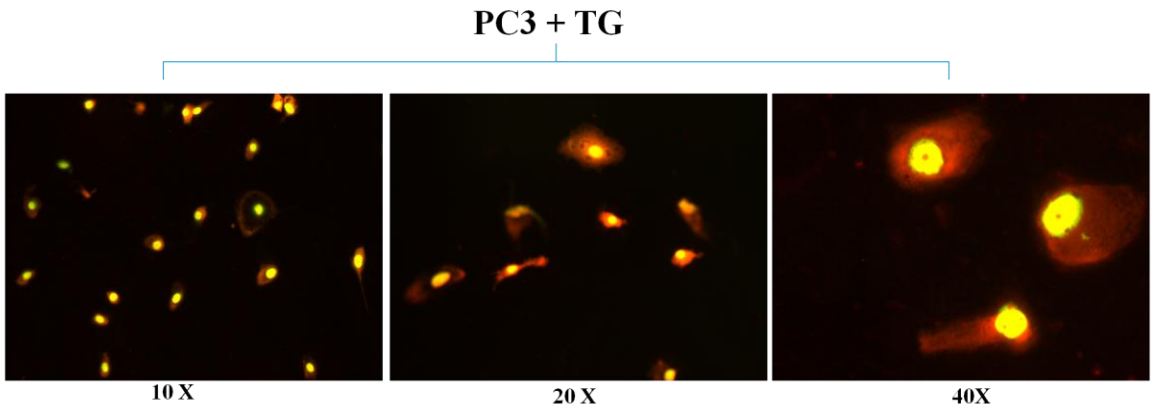


Fig. 13. Morphological effects of TCDD (A) or TG exposure (B) on PC3 by acridine orange staining. PC3 cells were treated with TCDD or with TG, stained with acridine orange and then observed under light microscope. After 48 h of exposure, untreated PC-3 cells displayed minimal presence of AVOs (green fluorescence with minimal red fluorescence), while PC3 cells treated with TCDD (100 pg/mL) displayed increased presence of AVOs (red fluorescence) (A, magnification 10X; 20X; 40X). Similar morphological alterations were displayed in PC3 exposed to TG (0.5 µmol/L) (B, magnification 10X; 20X; 40X).

TCDD induces alteration of LC3 expression in PC3 cells

The effects of TCDD (100 pg/ml), TCDD (100 pg/ml) and CQ (10µM) or TG (0.5 µmol/L) on relative mRNA levels of the autophagic marker LC3 (Kabeya et al., 2000; Wu et al., 2006), were evaluated in PC3 cells at 24, 48 and 72 h by Real Time PCR. TCDD induced a significant increase of mRNA levels of LC3 at 48 and 72 h ($p < 0.001$), indicating a high rate of autophagy for PC3 cells exposed to TCDD. In details, PC3 cells showed the following LC3 expression ratios: 1.4 (after 24 h), 6.2 (after 48 h) and 11.4 (after 72 h). Treatment of PC3 cells with TCDD in absence or presence of CQ was used to assess the autophagic flux. **Fig 14A** shows a significant downregulation of LC3 mRNA levels ($p < 0.001$) after exposure to TCDD and CQ with the following expression ratios: -12.8 (after 24 h), -28.1 (after 48 h), and -2.8 (after 72 h). As positive control, we use the autophagy inductor TG. TG induced a significant increase of LC3 mRNA levels at 48h (expression ratio= 11.7, $p < 0.001$) **(Fig. 14A**).

Furthermore, to assess the autophagic flux, we used the western blot analysis of LC3 protein in PC3 cells exposed to TCDD in absence or presence of CQ. **Fig. 14B** shows that CQ increased LC3-II accumulation in presence of TCDD at 48 h post exposure, when significant TCDD-induced autophagy started **(Fig. 14A).** These results confirmed that TCDD activated autophagic flux, indeed autophagy can increase as a compensatory means of protein degradation when the autophagic pathway is blocked (Pandey et al., 2007). **(Fig. 14B**).

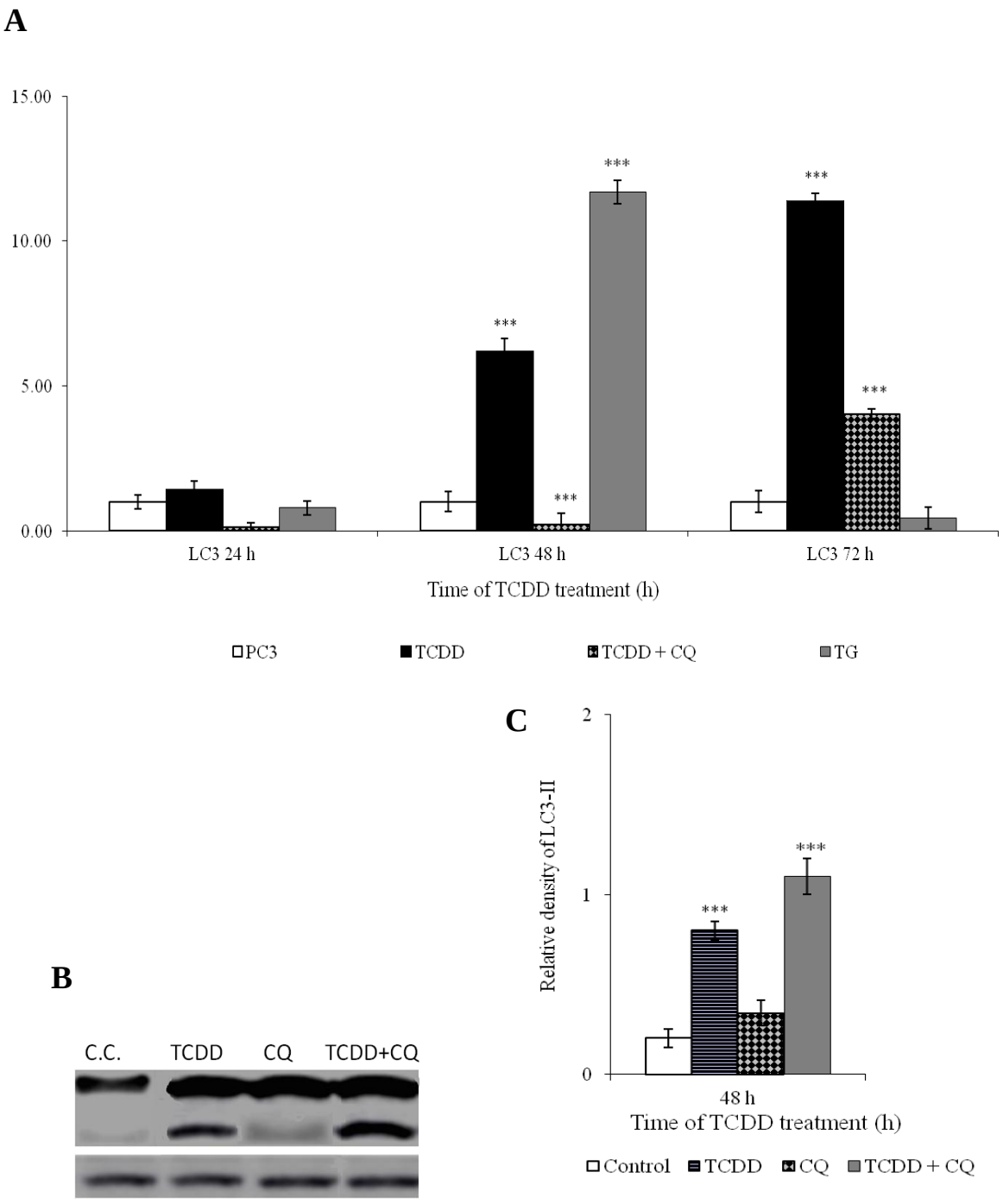
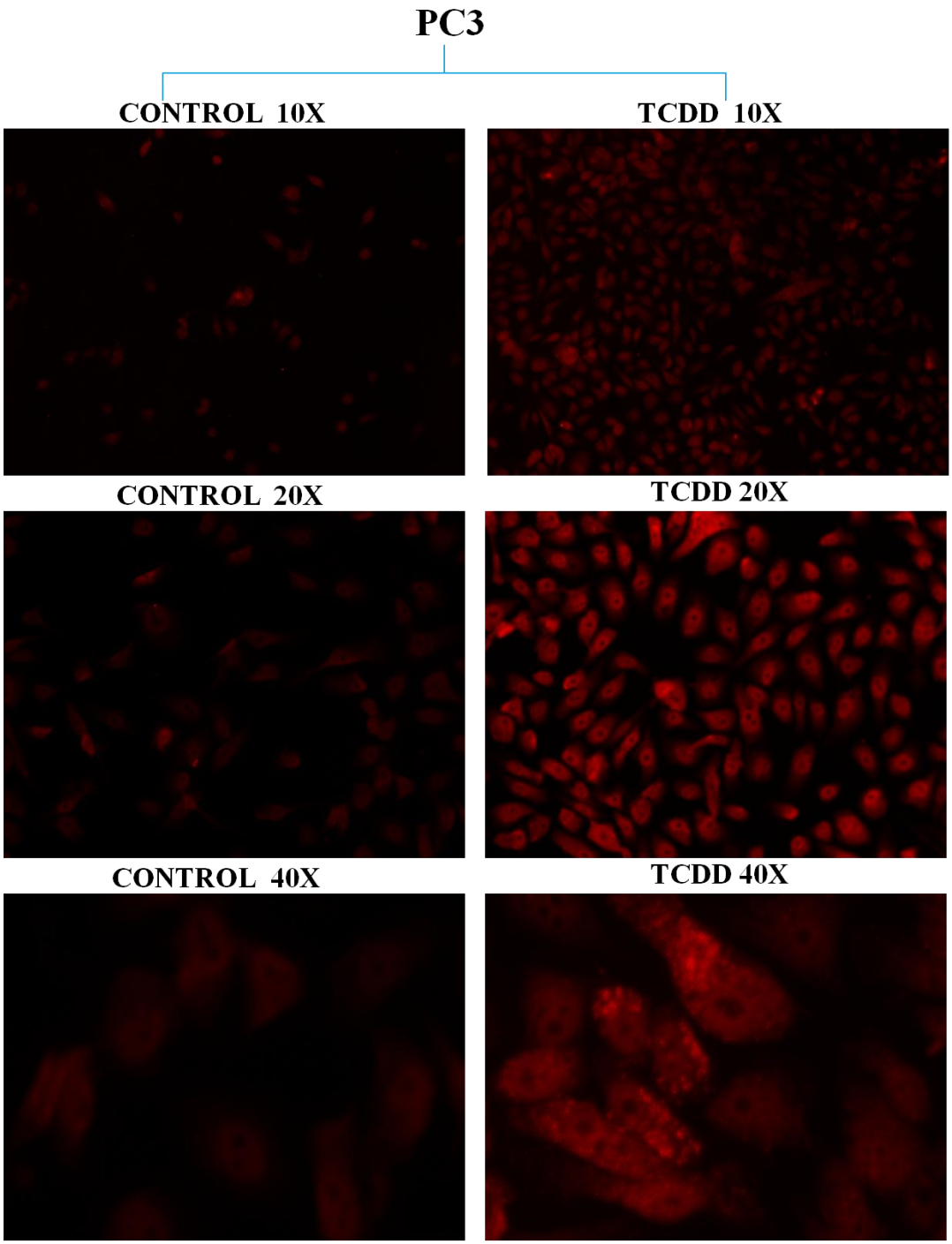


Fig. 14 TCDD exposure induces alteration of LC3 gene expression (A) and activated autophagic flux increased the levels of LC3-II protein (B and C). Expression of relative mRNA levels of LC3 in PC3 cells exposed to TCDD (100 pg/ml), or TCDD and CQ (10μM) or TG (0.5 μmol/L) at 24, 48 and 72 h by Real time PCR (A). TCDD exposure induces alteration of levels of LC3 protein at 48h of exposure. Western blot analysis was performed with an antibody which specifically recognized LC3, Actin was used as an internal loading control (B) and representative histograms of relative density of LC3-II (C).

Since, we analyzed the effects of TCDD (100 pg/ml), or TG (0.5 μmol/L) exposure on LC3 protein expression, after 48 h, by immunofluorescence. Immunofluorescence is considered reliable methods for monitoring autophagy (Tanida et al., 2008). Both TCDD and TG singles treatments showed an increased LC3 punctate staining when compared to its controls (**Fig. 15A/B**).

A


PC3
CONTROL 10X
TCDD 10X
CONTROL 20X
TCDD 20X
CONTROL 40X
TCDD 40X

B

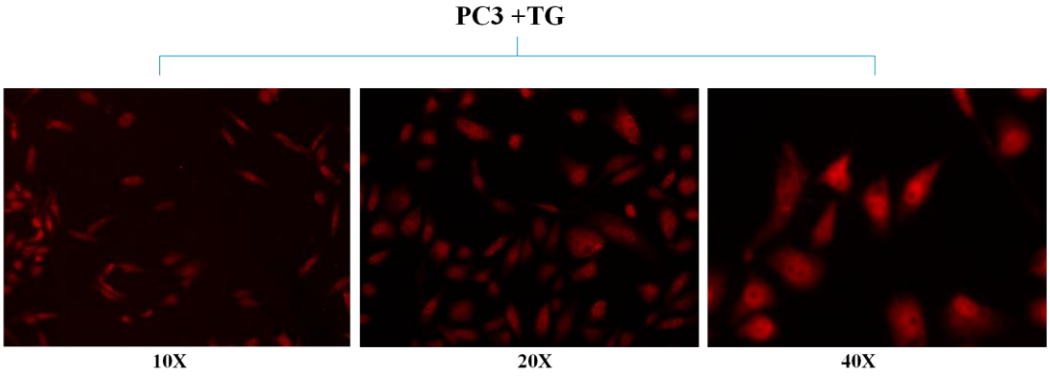
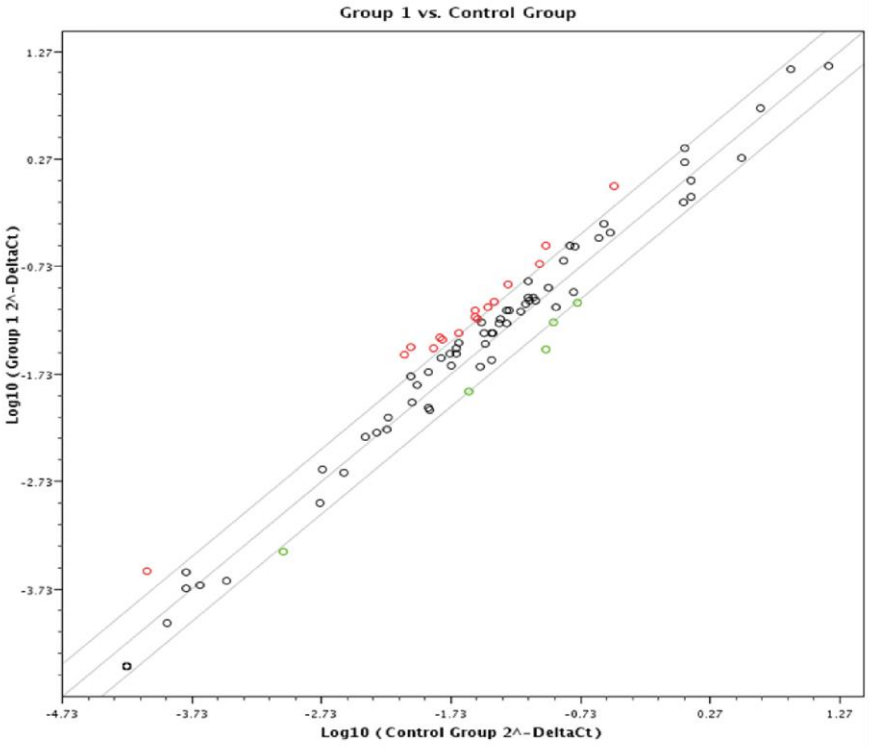


Fig. 15. LC3 detection of TCDD (A) or TG (B) exposure on Pc3 cells by immunofluorescence. TCDD (100 pg/ml), or TG (0.5 µmol/L) exposure on levels of LC3 showed an increased LC3 punctate staining when compared to its controls after 48 h of treatment confirming that TCDD exposure induced autophagy in PC3 cells. (A, magnification 10X; 20X; 40X). Similar morphological alterations were displayed in PC3 exposed to TG (0.5 µmol/L) (B, magnification 10X; 20X; 40X).

TCDD modulates Autophagy related Transcriptomics

In order to analyze the effects of TCDD exposure on PC3 cells at transcriptional levels, we evaluated the gene expression profiles of some genes involved in the following pathways: autophagic vacuole formation**;** protein targeting to membrane/vacuole**;** protein transport autolysosome formation; protein ubiquination; protease activity; co-regulation of autophagy and apoptosis; and cell cycle. The gene expression profiles of PC3 cells after 48h of TCDD or TG exposures are shown the results illustrated in **Fig 16** and **Tab 1**.

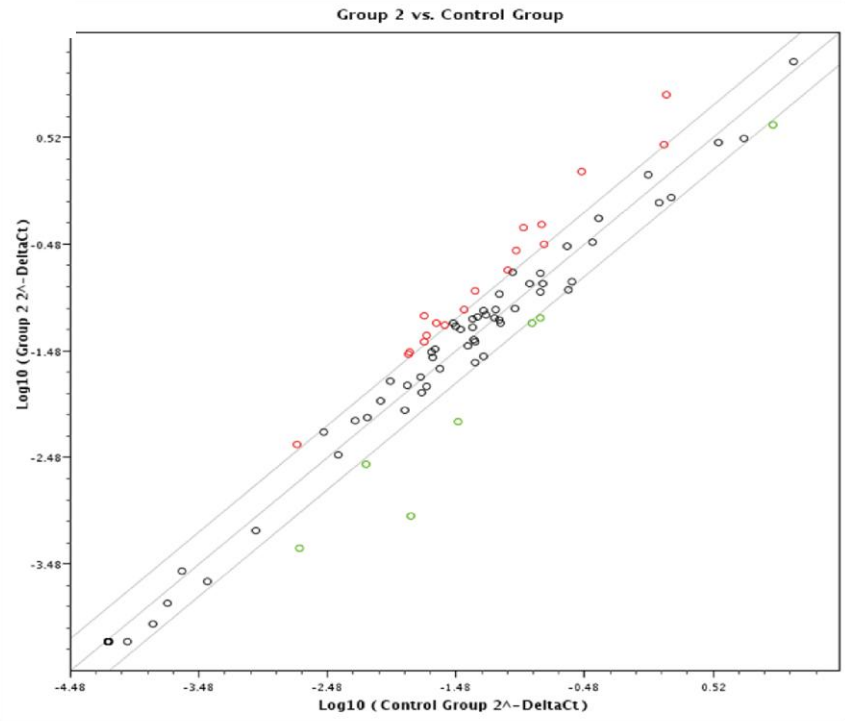
A



Positive modulation of gene expression PC3 + TCDD (TCDD vs. Control Group)	
Gene Symbol	Fold Regulation
AMBRA1	3.4224
ATG10	2.5403
ATG16L1	2.4538
ATG4A	2.0922
ATG4C	2.4033
ATG5	2.181
ATG7	2.0634
BECN1	2.5228
GABARAPL1	2.6851
HGS	3.6175
MAP1LC3B	3.0844
MAPK14	2.1962
MAPK8	2.4033
PIK3C3	2.2125
PRKAA1	2.181
TNF	3.283
WIPI1	2.918

Negative modulation of gene expression PC3 + TCDD (TCDD vs. Control Group)	
Gene Symbol	Fold Regulation
AKT1	-3.1711
BCL2	-2.2894
BCL2L1	-2.0491
CDKN2A	-2.0069
RB1	-2.035

B



Genes Over-Expressed in PC3 + TG (TG vs. Control Group)	
Gene Symbol	Fold Regulation
ATG10	2.3054
ATG12	2.035
ATG3	3.3519
ATG4C	2.2268
ATG5	2.0777
BNIP3	2.0491
CASP3	2.3867
CASP8	2.1962
CDKN2A	2.9588
EIF2AK3	2.5758
GABARAPL1	3.7974
MAP1LC3B	5.0455
MAPK8	2.166
RB1	2.166
RPS6KB1	2.2423
SQSTM1	5.8767
ULK1	2.5403
WIPI1	4.3319

Genes Under-Expressed in PC3 + TG (TG vs. Control Group)	
Gene Symbol	Fold Regulation
AKT1	-2.2115
ATG16L2	-2.3054
CXCR4	-15.6165
GAA	-2.166
TGM2	-4.7733
TNFSF10	-4.302

Fig. 16. Gene expression profile analysis of PC3 cells exposed toTCDD **(A)**. Gene expression profile analysis of PC3 cells exposed to TG **(B)**. mRNA levels of genes involved in autophagy were analyzed by quantitative RT-CR. Left: scatter plot data analysis. mRNA levels of genes involved in autophagy were analyzed by quantitative RT-PCR. Right: positive and negative regulations of gene expression. It was used a cut-off ratio ≥2.

Gene Symbol	Modulation by TCDD	Modulation by TG
AKT1	-3.17	-2.21
ATG10	2.54	2.31
ATG4C	2.4	2.23
ATG5	2.18	2.08
BECN1	2.5228	1.99
GABARAPL1	2.69	3.8
MAP1LC3B	3.08	5.05
MAPK8	2.4	2.17
PRKAA1	2.18	1.98
WIPI1	2.92	4.33

Table 1. Modulation of gene expression by TCDD and TG exposure with a cut-off ratio ≥2. Red: positive modulation; blue: negative modulation. mRNA levels of genes involved in autophagy were analyzed by quantitative RT-PCR.

DISCUSSION

Autophagy is a tightly regulated process playing a normal part in cell growth, development, and homeostasis and helping to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products through the degradation via the lysosome (Demarchi et al., 2006). Defects of autophagy machinery are responsible for pathogenesis of different diseases, including cancer. The role of autophagy in cancer is controversial. There is evidence that autophagy may play a critical role in cancer progression at later stages, such as dissemination and metastasis, which account for most cancer-associated deaths, whereas in other cases it clearly contributes to tumor suppression by inducing tumor cell death (Chen and Karantza).

We have demonstrated that the exposure of TCDD enhances PC3 cell viability and proliferation (**Fig. 9**) and the alteration of differentiation and proliferation is associated with tumor promotion and TCDD-induced dysregulation of cellular growth and differentiation suggesting a possible involvement of TCDD in prostate cancer progression.

These results took place in a dose-dependent manner at 24 and 48 h, in accordance with other studies on normal cells (Fiorito et al., 2008; Santamaria et al., 2011), as well as in cancer cells, as colon cancer cells (Xie et al., 2012) and breast cancer cells (Moore et al., 1993; Chen et al., 2012). After 72 h of exposure with 0.01 and 100 pg/ml of TCDD compared to 1 pg/ml of TCDD, PC3 showed an U-shaped dose responsiveness. This modulation was too in accordance with literature (Murayama et al., 2002; Ahn et al., 2005; Fiorito et al., 2008; Fiorito et al., 2011).

Then, we study cell cycle analysis of PC3 cells exposed to TCDD. Our data showed an increase of cell population at S phase in cell cycle. This result was in accordance with a study where TCDD enriched the cell population in G2/M phase in breast cancer cells (Chen et al., 2012) and TCDD-induced modulation of cell cycle and cell proliferation in hepatoma and WB-F344 cells (Proikas-Cezanne et al., 2007). In the present study we demonstrated for the first time that TCDD-promoted S enrichment and proliferation of prostate cancer cells.

Herein, analysis of cell morphology, by phase-contrast microscopy, demonstrated that PC3 cells exposed to TCDD, compared to controls, displayed a large number of cells with expanded cytoplasm (magnified cells) and an elevated degree of vacuolization, characteristic signs of autophagy, as reported above. These results were in accordance with previously described in HT-29 colon cancer cells exposed to hyperthermia in combination with oxidative stress (Chen et al. 2008). Furthermore, we used, as positive

control, thapsigargin (TG), an inductor of autophagy. Cells exposed to TG, at 0.5 μmol/L, showed an elevated degree of vacuolization similar to TCDD. Our result was confirmed by acridne orange, where PC3 cells exposed to TCDD showed formation of AVOs that were relatively more control. Formation of AVOs is another characteristic feature of cells engaged in autophagy after treatment with different stimuli, including radiation or ceramide (Paglin et al. 2001). Finally, our results confirmed AVOs detection, as we previously reported after TCDD exposure in MDBK cells (Fiorito et al. 2011).

The induction of autophagy is accompanied by an increase in the mRNA levels of certain autophagy genes, such as Atg8/LC3 and assessing the levels of LC3 mRNA by qRT-PCR may provide correlative data relating to the induction of autophagy (Kabeya et al., 2000; Wu et al., 2006). TCDD exposure on PC3 cells increased the relative mRNA levels of the autophagic marker LC3 and this up-regulation suggests that PC3 cells evidenced a high rate of autophagy. This result was confirmed by western blot and immunofluorescence for detection of LC3 protein, a reliable methods for monitoring autophagy (Tanida et al., 2008).

Furthermore, to assess the autophagic flux, we used the western blot analysis of LC3 protein in PC3 cells exposed to TCDD in absence or presence of CQ and our samples shows that CQ increased LC3-II accumulation in presence of TCDD at 48 h post exposure, when significant TCDD-induced autophagy started. These results confirmed that TCDD activated autophagic flux, indeed autophagy can increase as a compensatory means of protein degradation when the autophagic pathway is blocked (Pandey et al., 2007).

Our understanding of the global organization the autophagy system is still limited. Recently, it was revealed the autophagy interaction network in human cells undergoing conditions of ongoing basal autophagy (Behrends et al., 2010). This network is composed of 751 interactions with extensive connectivity among sub-networks (**Fig. 8A**).

Autophagy is constitutively active on a basal level. As a consequence, the constant turnover of cytoplasmic material maintains cellular homeostasis. Above this basal level, autophagy is further induced upon activation of the PIK3C3/BECN-1 complex, that generates phosphatidylinositol 3-phosphate (PI(3)P, the product of both the class II and III phosphoinositide 3-kinases (PI 3-kinases) activity on phosphatidylinositol (Maiuri et al., 2007). The generation of PI(3)P is considered to be required for canonical induction of autophagy. In fact, BECN1 (BECN1; coiled-coil, myosin-like BCL2-interacting

protein), that is also known as autophagy-related gene Atg6, binding partner PI3K, for the initiation of the formation of the autophagosome in autophagy (Maiuri et al., 2007).

One of the cofactors interacting with BECN1 is AMBRA1 (activating molecule in Beclin1-regulated autophagy). This positive regulator of BECN1 is known to activate the lipid kinase PIK3C3 (Jaber et al., 2012). From our results, TCDD upregulates PlK3C3-BECN1 network by modulating PIK3C3 (Class III PI3K, Vps34), BECN-1 and AMBRA1 (**Fig 17**). This upregulation was-novel, indeed, until now, there is no literature information available about this mechanism induced by TCDD.

Autophagy induction is followed by an increase in the mRNA levels of Atg8/LC3/ MAP1LC3 (microtubule-associated protein 1 light chain 3). LC3 synthesis starts with the production of an unprocessed form, proLC3 that is then converted into a proteolytically processed form lacking amino acids from the C terminus, LC3-I and ultimately modified into the PE-conjugated form, LC3-II. Atg8—PE/LC3-II/ MAP1LC3B is the only protein marker that is reliably associated with completed autophagosomes, but is also localized to phagophores, the precursors to autophagosomes (Huang et al., 2000). MAP1LC3B, is significativly upregulated by TCDD (**Fig17** and **Table 1**). Autophay induction by TG exposure, confirmed the same upregulation (**Fig17** and **Table 1**). These results are in agreement with our previous work (Fiorito et al. 2011) where we demonstrated that TCDD induced autophagy in MDBK cells, by increasing the amount of LC3-II protein.

LC3 is a member of ATG8 family that is composed of 6 members: MAP1LC3A, MAP1LC3B, MAP1LC3C, GABA(A) receptor-associated protein (GABARAP), GABARAPL1, and GABARAPL2. GABARAPL1 is not only involved in autophagy, partially colocalizes with LC3 in intracellular vesicles and was associates with autophagic vesicles (Chakrama et al., 2010), but is also implicated in various mechanisms such as cell proliferation and tumor progression. Our results indicate a positive modulation of GABARAPL1 in PC3 cells exposed to TCDD or TG.

It is well known that Atg genes control the autophagosome formation. LC3 is initially synthesized with a C-terminal and then is cleaved at its C terminus by Atg4 protease to generate the cytosolic LC3-I. There are four Atg4 homologs in mammals, and they have different activities with regard to the LC3/Atg8 subfamiles of proteins. ATG4A is able to cleave the GABARAP subfamily, but has very limited activity toward the LC3 subfamily whereas, ATG4B is active against most or all of these proteins and ATG4C and ATG4D isoforms have minimal activity for any of the Atg8 homologs (Klionsky,

2011). Here, both ATG4A and ATG4C were upregulated after TCDD exposure, while TG exposure upregulated only ATG4C (**Fig. 17, Table 1**).

Other Atg genes controlling the autophagosome formation/maturation and cargo recruitment are also the genes involved in the ubiquitin-like protein (UBL) conjugation system (**Fig 18**) - composed of the Atg3, Atg5, Atg7, Atg10, Atg12 and ATG16L1 (Behrends et al., 2010). Also, Atg10 is essential for normal cell cycle progression and for responses to various stress conditions that perturb the cell cycle (Flanagan et al., 2013). Our results indicate a novel positive modulation of 5 genes involved in the UBL conjugation system: ATG4C, ATG5, ATG7, ATG10, ATG16L1. ATG4C, ATG5 and ATG10 were modulated in PC3 exposed to both TCDD and TG.

PRKAA (Protein kinase, AMP-activated, alpha 1 catalytic subunit), also called AMPK, is generally antagonistic toward MTOR function. AMPK-mediated mTORC inhibition and autophagy stimulation, for example, occur during glucose starvation. Aminoacid starvation, on the other hand, can strongly induce autophagy even in cells completely lacking AMPK catalytic activity (Klionsky et al., 2012). Our results showed a significant upregulation of this gene in PC3 cells exposed to TCDD, while in cells exosed to TG the upregulation is not significant.

Human WIPI-1 (WD-repeat protein interacting with phosphoinositides-1) specifically binds PI(3)P and localizes at autophagosomal membranes upon PI(3)P-mediated induction of autophagy (Proikas-Cezanne et al., 2007). The exposure with TCDD or TG resulted in a positive regulation of WIPI-1 in our in vitro system.

PC3 cells exposed to TCDD also showed upregulation of two member of the MAP kinase family: MAPK14 (Mitogen-activated protein kinase 14) and MAPK8 (Mitogen-activated protein kinase 8). MAP kinases act as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development. These kinases are activated by various environmental stresses and proinflammatory cytokines. The roles of this kinase in stress are related transcription and cell cycle regulation, as well as in genotoxic stress response (Wei et al., 2008). In particular, it is known that autophagy is induced by the release of BECN1 from BCL2 by phosphorylation of BCL2 by MAPK8/JNK1 (at Thr69, Ser70 and Ser87).

Other autophagy related genes significantly up-regulated only by TG exposure are ULK1, BNIP3, p62/SQSTM1 and EIF2AK3. ULK family is composed by three related serine/threonine kinases: UNC-51-like kinase -1, -2, and -3 (ULK1, ULK2, UKL3).

ULK1 and ULK2 form a large complex, Atg1/ULK, that plays a role in the initiation of autophagy: receiving signals of cellular nutrient status, recruiting downstream Atg proteins to the autophagosome formation site, and governing autophagosome formation (Mizushima, 2010).

BNIP3 (BCL2/adenovirus E1B 19kDa interacting protein 3) is an autophagy receptor that signals autophagic degradation of mitochondria (mitophagy) via interaction of its LC3-interacting region (LIR) with Atg8 proteins (Zhu et al., 2013). Mammalian sequestosome 1 (p62/SQSTM1) is a multifunctional ubiquitin-binding scaffolding protein that serves multiple cellular functions ranging from cell signaling to autophagy. p62 appears to be important for the clearance of proteins through autophagy. It was reported that self-oligomerization of p62 is important for its localization to the autophagosome formation site that is associated to the endoplasmic reticulum and it is recruited to early autophagic structures independententtly of LC3 (Nezis and Stenmark, 2012).

EIF2AK3 phosphorylates the alpha subunit of eukaryotic translation-initiation factor 2 (EIF2), leading to its inactivation, and thus to a rapid reduction of translational initiation and repression of global protein synthesis. It is a type I membrane protein located in the endoplasmic reticulum (ER), where it is induced by ER stress caused by misfolded proteins. It is gene invoved in induction of autophagy (Zhao et al., 2013).

TCDD exposure induced the downregulation of AKT1 (V-akt murine thymoma viral oncogene homolog 1) gene expression. Akt is a serine-threonine protein kinase known to inhibit autophagy (Yang et al., 2013). Our result was in accordance with literature (Chen et al., 2012). In fact, Chen and collegues observed that TCDD promoted breast cancer cell proliferation through the inactivation of Akt–FoxO3a signaling.

We demonstrated for the first time that TCDD exposure positively regulated autophagy in PC3 cells by modulating the following autophagy-related genes: PIK3C3, BECN-1, AMBRA1, MAP1LC3B, ATG4A, ATG4C, ATG5, ATG7, ATG10, ATG16L1, GABARAPL1, PRKAA, WIPI-1,MAPK8, MAPK14, and AKT1. Interestengly, these genes are present in different subnetworks composing the autophagy interactive network (**Fig. 18**) suggesting a multiple effect of TCDD on autophagy machinery. In particular, TCDD exposure positively influence autophagy system by upregulating autophagy genes having roles in the following networks: PlKC3-BECN1, ATG2-WIPI, UBL conjuction system, human ATG8's and AMP kinase (**Fig. 17 and Fig. 8A**). Also, for the first time, we demonstrated that TCDD negatively influenced genes that are autophagy inhibitors, such as, AKT1 and BCL2. Therefore, our data

suggested that autophagy induction by TCDD exposure positively modulated autophagy inductors and negatively modulated autophagy inhibitors (**Fig 17** and **Table 1**).

These results were in agreement with the results obtained by exposing PC3 to TG, a known inductor of autophagy. TG induced autophagy positively influences autophagy system by upregulating autophagy genes having roles in the following networks: ULK1 kinase, PlKC3-BECN1, ATG2-WIPI, UBL conjuction system and ATG8's (Fig. 17 and Fig 8A). Also, we demonstrated that TCDD negatively influenced a gene that is autophagy inhibitor, as AKT1(**Fig 17** and **Table 1**).

Future transcriptomics and pathways studies will further analyze the molecular influence of TCDD exposure after a larger window of hours on autophagy machinery.

TCDD exposure upregulated also the Tumor necrosis factor (TNF) gene. TNF is a pleiotropic cytokine involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation with dual roles in cancer biology. In fact, despite its name, discovery history and approval as anticancer agent but TNF has been also implicated in cancer development and progression (Mocellin, 2008). It was demostrated that high TNF levels have been related to prostate cancer progression via stimulation of proliferation, survival of malignant cells and increased resistance to chemotherapeutic agents (Srinivasan et al., 2010). Our result were in agreement with a previous study that demonstrated the property of TCDD to induce TNF (Massa et al., 1992). So we suggested a new role of plausible mechanism for TCDD-induced stimulation of proliferation and tumor progression.

Our results showed also that TCDD significativly decreased the expression of anti-apoptotic genes (Bcl-2, Bcl-2L1) but it does not significantly upregulate caspases. Our results are in agreement with the fact that, in normal conditions, BECN1 is inhibited by its binding to the anti-apoptotic protein BCL2 (Pattingre et al., 2005). This results suggested that TCDD might regulate autophagy in PC3 by targeting different pathways leading to the enhancement of the autophagy system and negatively modulating pathways leading to the inhibibition of autophay. On the other hand, TG exposure upregulated caspase 3 and 8 (Fig.17), in agreement with the known fact that TG is an inductor not only of autophay but also of apopotosis (Chandrika et al., 2010).

Interestingly, we noticed a shift of expression for tumor suppressor genes CDKN2A, RB1 in PC3 cells exposed either to TCDD or TG. In fact, while these two genes were significantly downregulated after TCDD exposure, they are significantly upregulated after TG treatment. A similar situation was encountered in another publication where it

was demonstrated that TCDD expsoure significantly increased the lung tumor incidence by diminishing CDKNA mRNA levels in female lung cancer mice (Wang et al., 2011).

CONCLUSIONS

Herein, we studied the influences of TCDD on PC3, a prostate cancer cell line. Our data showed, for the first time, that TCDD exerts its effects in PC3 by: enhancing cell proliferation, inducing cell cycle progression and cell morphology changes (a large number of cells with an elevated degree of vacuolization, characteristic sign of autophagy), increasing both gene expression and protein levels of LC3, that provided correlative data relating to the induction of autophagy.

Interestingly, we demonstrated that TCDD positively regulated genes that are present in different subnetworks composing the autophagy interactive network (**Fig. 8A**) suggesting a multiple effect of TCDD on autophagy machinery. In particular TCDD, in PC3 cells, modulated the following autophagy-related genes: PIK3C3, BECN-1, AMBRA1, MAP1LC3B, ATG4A, ATG4C, ATG7, ATG5, ATG10, ATG16L1, GABARAPL1, PRKAA, WIPI-1.

Generally, autophagy was classified for a long time as form of programmed cell death; however accumulating evidence revealed that, while apoptosis is an irreversible way to cell death, autophagic cells often survives since autophagy leads to energy production to sustain survival upon severe stress (Chen and Karantza, 2011).

Furthermore, we demostrated that TCDD exposure upregulated also TNF gene and high TNF levels have been related to prostate cancer progression via stimulation of proliferation, survival of malignant cells and increased resistance to chemotherapeutic agents (Srinivasan et al., 2010) suggesting a role of plausible mechanism for TCDD-induced tumor progression.

A correlation between incidences of cancer and the presence of industrial and toxic waste landfills was found in some areas of Campania Region (South Italy) (Barba et al., 2011). Cancer mortality descriptive studies demonstrated an increase of incidences rates of the prostate cancer in the same areas where dioxin levels were elevated (Goldberg et al., 1995). Furthermore, studies performed in animal model suggested that TCDD exposure is associated with abnormal prostate development, altered prostate pathology and increase susceptibility to prostate cancer (Theobald and Peterson, 1997; Fritz et al., 2005; Everly et al., 2009).

More studies are needed to further confirm our data and ultimately to better clarify the role of autophagy in prostate cancer but herein, from our results we can declare that TCDD, may induce prostate cancer progression by: enhancing cell proliferation, inducing autophagy, deregulating the expression of genes related to the autophagy

machinery, and upregulating TNF resulting in an increased risk for both animal and human health.

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CURRICULUM VITAE

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Granato G.E., Fiorito F., Ciarcia R., De Blasio E., Barretta A., Marfè G., Iovane V., Florio S. 2,3,7,8-Tetraclorodibenzo-p-diossina anticipa l'attivazione di Nf-Kb durante l'infezione da Bovine Herpesvirus Tipo 1. Giornate Scientifiche del Polo delle Scienze e delle Tecnologie per la Vita. Napoli, November 25-26, 2010.

Invited reviewer:

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Publications:

1. Montagnaro S., Ciarcia R., Pagnini F., De Martino L., Puzio M.V., **Granato G.E.**, Avino F., Pagnini U., Iovane G., Giordano A.. Bovine herpesvirus type 4 infection modulates autophagy in a permissive cell line. J Cell Biochem. (2013) [Epub ahead of print].
2. 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11. Fiorito F., Ciarcia R., **Granato G.E.**, Marfe' G., De Martino L., Florio S., Iovane G. 2,3,7,8-Tetrachlorodibenzo-p-dioxin modifies the levels of P21 and TERT proteins during Bovine

Herpesvirus 1 infection. Cellular Host-Pathogen Interaction Conference, Amsterdam-The Netherlands, September 5-7, 2010.

12. Fiorito F., De Blasio E., **Granato G.E.**, Cantiello A., Florio S., De Martino L. 2,3,7,8-Tetrachlorodibenzo-p-dioxin modifies telomerase activity in Bovine Herpesvirus 1 infection. The 8th International Cell Death Society Symposium "Cell Death in Infectious Diseases and Cancer". Muldersdrift, Johannesburg, South Africa, June 5-8, 2009.

13. Fiorito F., **Granato G.E.**, De Blasio E., Marfè G., Arpenti C., De Martino L., Pagnini U. 2,3,7,8-Tetrachlorodibenzo-p-dioxin anticipates the increase of p53 protein levels in Bovine Herpesvirus 1 induced apoptosis. The 8th International Cell Death Society Symposium "Cell Death in Infectious Diseases and Cancer". Muldersdrift, Johannesburg, South Africa, June 5-8, 2009.

14. De Blasio E., Fiorito F., **Granato G.E.**, Cantiello A., Iovane V., Ciarcia R., Florio S. L'esposizione a 2,3,7,8-tetraclorodibenzo-p-diossina modifica i livelli della proteina Bovine Herpesvirus 1 immediate early (bICP0). Giornate Scientifiche del Polo delle Scienze e delle Tecnologie per la Vita. Portici, December 10-12, 2008.

15. **Granato G.E.**, Fiorito F., De Blasio E., Cantiello A., Arpenti C., De Martino L., Iovane G. 2,3,7,8-Tetraclorodibenzo-p-diossina anticipa l'apoptosi indotta da Bovine Herpesvirus Tipo 1 up-regolando l'attivazione delle caspasi. Giornate Scientifiche del Polo delle Scienze e delle Tecnologie per la Vita. Portici, December 10-12, 2008.

16. Fiorito F., **Granato G.E.**, De Blasio E., Longo M., Ciarcia R, Florio S. 2,3,7,8-Tetraclorodibenzo-p-diossina anticipa l'apoptosi indotta da Bovine Herpesvirus tipo 1 attraverso i membri della famiglia bcl-2. IIIa Meeting of Campani Pharmacologists. Benevento, September 26, 2008.

17. Fiorito F., **Granato G.E.**, De Blasio E., Longo M., Ciarcia R., Iovane V., Marfè G., Sinibaldi- Salimei P., De Martino L. 2,3,7,8-Tetrachlorodibenzo-p-diossina anticipates Bovine Herpesvirus type 1 induced apoptosis up-regulating caspase-3. Apoptosis World 2008, From mechanism to applications. Luxembourg, January 23-26, 2008.

18. **Granato G.E.**, Fiorito F., De Blasio E., Ciarcia R., Damiano S., Arpenti C., Florio S. La 2,3,7,8 tetraclorodibenzo-p-diossina induce apoptosi in cellule Madin Darby Bovine Kidney infettate con Bovine Herpesvirus tipo-1. Giornate Scientifiche del Polo delle Scienze e delle Tecnologie per la Vita. Naples, September 20-21, 2007.

19. De Blasio E., Fiorito F., **Granato G.E.**, Ciarcia R., Damiano S., Florio S. La 2,3,7,8 tetraclorodibenzo-p-diossina modifica l'espressione della proteina immediate early bICP0. Giornate Scientifiche del Polo delle Scienze e delle Tecnologie per la Vita. Naples, September 20-21, 2007.

International and National Workshops and Courses:

1. Certificate of participation at "Giornate Scientifiche del Polo delle Scienze e delle Tecnologie per la Vita" - University of Naples "Federico II" - Faculty of Pharmacy, September 10-12, 2007.

2. Certificate of participation at "Giornate Scientifiche del Polo delle Scienze e delle Tecnologie per la Vita" - University of Naples "Federico II" - Portici, December 10-12, 2008.

3. Certificate of attendance at the conference "LE DIOSSINE: AMBIENTE E SALUTE", Centro Congressi "Federico II" - Aula Magna Partenope, Naples, April 29 - 30 2009.

4. Certificate of participation at the 11th International Congress of EAVPT, Leipzig (Germany), July 12 - 16, 2009.

5. Certificate of participation at the seminar "Application Blotting and Imaging Systems" held by Bio-Rad, CNR of Naples, Naples, June 10, 2008.

6. Certificate of participation at the "Giornata studio su Leishmaniosi canina: up-date sugli organi ed i tessuti coinvolti, vecchi e nuovi sintomi e nuove strategie terapeutiche" - Grand Hotel Salerno, December 14, 2008.

7. Certificate of participation at the training course "Ecografia del Cane e del Gatto", organized by Ordine dei Medici Veterinari Salerno - Castello Arechi, Salerno, September 26 - 27, 2009.

8. Certificate of participation at the "Aggiornamenti sulla TSE: Diagnosi ed epidemiologia" - IZSM, Portici, October 20, 2009.

9. Certificate of participation at the "Autocontrollo, perchè è necessario parlarne ancora", organized by Ordine dei Medici Veterinari Salerno - CREMOPAR, Eboli, October 22 - 23/29 - 30, 2009.

10. Certificate of participation at the "Paure e Fobie ed ansia da separazione del cane nuove normative legali", organized by Ordine dei Medici Veterinari Salerno, November 15, 2009.

11. Certificate of participation at the "Malattia Vescicolare Suina: Obiettivo Accreditamento" - Salone Conferenze Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, November 23, 2009.

12. Certificate of participation at the "Emergenze Epidemiche: il Ruolo della Medicina Preventiva" - Centro Congressi dell'Ateneo Federico II of Naples, July 7, 2010.

13. Certificate of participation in the XII International Congress of Toxicology, Barcelona - Spain, July 19 - 23 2010.

14. Certificate of poster presentation "2,3,7,8-Tetrachlorodibenzo-p-dioxin anticipates the activation of NF-kB during Bovine Herpesvirus 1 infection" - XII International Congress of Toxicology, Barcelona - Spain, July 19 - 23, 2010.

15. Certificate of participation at the Congress Cellular Host-Patogen Interaction - Amsterdam, The Netherlands, September 5-7, 2010.

16. Certificate of participation at the Giornate Scientifiche del Polo delle Scienze e delle Tecnologie per la Vita - Naples, November 25-26, 2010.

17. Certificate of participation at the 16th Congress of the European Hematology Association - London, United Kingdom, June 9 - 12, 2011.

18. Certificate of participation in the 47th Congress of the European Societies of Toxicology - Paris, France, August 28 - 31, 2011.

Curriculum/Development

1. Grant writing workshop, March 21th 2012, Temple University, Philadelphia, PA

Informatics:

1. ECDL: **E**uropean **C**omputer **D**riving **L**icence.(2003)

Other:

1. CERTIFICATE OF PERFORMER BLS-D - ASL SALERNO 1, Nocera Inferiore, July 14, 2010.

2. CERTIFICATE OF PERFORMER PBLS - ASL SALERNO 1 Nocera Inferiore, February 22, 2011.

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GRAZIE E' STATA
UN'ESPERIENZA FANTASTICA!