UNIVERSITÀ DEGLI STUDI DEL MOLISE

CAMPOBASSO

Dipartimento di Bioscienze e Territorio

Dottorato in Scienze e Tecnologie biologiche e ambientali
(XXVIII Cycle)

S.S.D ***MED 46***

FROM HEPATOCARCINOMA TO BREAST CANCER: SELENOPROTEINS AS LINK BETWEEN TWO DIFFERENT REALITY

SUPERVISOR

ILLUSTRIOUS Professor

Antonella Angiolillo

COORDINATOR

ILLUSTRIOUS Professor

Claudio Cafrani

POSTGRADUATE

Fabiola Rusolo

Matr. 149329

2016/2017, Academic Year

CONTENTS

1

ABSTRACT

Selenium is an essential trace mineral of fundamental importance to human health. It is known primarily for its antioxidant activity, for its chemopreventive, anti-inflammatory and antiviral properties, *(Papp et al. 2007)* hence its deficiency has been recognized as a contributing factor to pathophysiological conditions, including heart disease, neuromuscular disorders, cancer, male infertility and inflammation. Much of its beneficial influence on human health is attributed to its presence within at least 25 proteins (*Selenoproteins*) *(Papp et al. 2007).* It is becoming more evident how the cancer and its the behaviour not dependent only on the genetics of tumor cells but also by surrounding milieu [stromal tissue (immune cells, fibroblasts, myofibroblasts, cytokines, and vascular tissue), as well as the surrounding extracellular matrix], necessary for tumor cells survival, growth, proliferation and metastasis. Inflammatory cells and mediators are present in the microenvironment of most, if not all, tumors, irrespective of the trigger for development *(Leonardi et al. 2012).* Hepatocarcinoma (HCC) and Breast Cancer (BC) are examples. The liver is a hormone-sensitive organ and a several lines of evidence suggest that sex hormones and their receptors play a role in liver carcinogenesis *(Wang et al. 2006).* Continuous oxidative stress, impaired synthesis of antioxidant enzymes, in HCC and in BC, un-regulated synthesis and secretion of sex hormones laid the foundation for research into selenoproteins a great help, not only to mitigate these mechanisms, but also to modulate the hormonal signaling exacerbating the hepatocarcinogenesis. The aim of thesis has been to identify selenoproteins, whose de-regulation was, potentially, associated to hepatocarcinogenesis and to breast cancer. These preliminary investigations direct future studies to understand how BC cells can influence the hepatocarcinogenesis through the secretion of hormones, cytokines, chemokines and growth factors and how the HCC cells can exercise control on breast cancer progression. Furthermore, it will be interesting to investigate how the modulation of selenoproteins, by treatment with selenium alone or in combination with chemotherapeutic molecules, might influence the key signaling of these two cancers.

2

INTRODUCTION

2.1 PREFACE

Initially identified as a new species of sulfur, the selenium (Se) was discovered as new element in 1817 by the Swedish chemist Berzelius *(Fig.1)*, establishing its properties, as well as the properties of the compounds it formed with metals, oxygen, hydrogen, sulfur, phosphorus, and different salts. In the appendix to the third volume of his *Textbook in Chemistry*, published in 1818, Berzelius gave the formulas of 90 different selenium compounds (58 selenias, 20 selenietum, and 12 hydroselenietum) together with the atomic weight of the element itself. A remarkably high number of compounds *(Jan Trofast, 2011).* Today, almost 200 years later, selenium is well established as an essential trace mineral of fundamental importance to human health. It is known primarily for its antioxidant activity and, in therapeutic aspects, for its chemopreventive, anti-inflammatory, and antiviral properties *(Papp et al. 2007)*, hence its deficiency has been recognized as a contributing factor to pathophysiological conditions, including heart disease, neuromuscular disorders, cancer, male infertility, and inflammation, as well as numerous other disorders. Selenium has been also implicated in mammalian development, immune function, inhibition of viral expression and delaying the progression of AIDS in HIV positive patients *(Labunsky et al. 2014).* Much of its beneficial influence on human health is attributed to its presence within at least 25 proteins. Unlike other metal elements that interact with proteins in form of cofactors, selenium becomes co-translationally incorporated into the polypeptide chain as part of the amino acid selenocysteine (SeCys) e through which carries out its functions *(Papp et al. 2007).* The group of proteins that contain Sec as an integral part of their polypeptide chain are defined as ***selenoproteins***. Selenoproteins are present in all lineages of life; the largest repertoire exists in fish with 30 individual selenoproteins, followed by humans and rodents with 25 and 24 selenoproteins, respectively. Their small size can be explained by a limited selenium supply in nature and a rather energy-expensive synthesis process (*Papp et al. 2007*). These include glutathione peroxidases (GPx) (five genes), thioredoxin reductases (TrxR) (three genes), iodothyronine deiodinases (DIO; three genes), and selenophosphate synthetases 2 (SPS2). The remaining selenoproteins have been annotated in alphabetic order and include the 15-kDa selenoprotein/ Sep15, SelH, SelI, SelK, SelM, SelN, SelO, SelP/SepP, SelR, SelS, SelT, SelV, and SelW. Only a few of these proteins have been functionally characterized. These

Figure 1. *Jacob Berzelius (1779-1848)*

include the GPxs, the TrxRs, SPS2, and DIOs, which all have oxidoreductase functions. Mapping the selenoproteomes in species across the domains has paved the way for the main challenge within the selenium field: the functional characterization of these proteins and their involvement in the etiology of disease (*Papp et al. 2007*).

2.2 *METABOLISM OF SELENIUM*

2.2.1 *Nutritional source of selenium*

The main route for Se intake is via the diet that varies widely depending on the food type and composition. The major contributors to Se intake is typically provided by bread and cereals, meat, fish, eggs, and milk/dairy products *(Roman et al. 2014).* An crucial aspect is that food types provide Se in distinct combinations of chemical forms which in turn entail a different bioavailability of the element, depending on the plant/animal species, the environment and the growth conditions (natural or supplemented) *(Roman et al. 2014)(Fig.2).* The forms of selenium more prevalent in plants are *selenomethionine* (SeMet) and inorganic *selenate* (SeO_4^{2}) or *selenite* (SeO_3^{2-}) which is then used to produce the organic compound, SeMet, through the sulfur assimilation pathway *(Terry et al. 2012).* Minor species are *selenocysteine* (SeCys), major prevalent in animal tissues, *Se-methyl-selenocysteine* (SeMCys) and *g-glutamyl-Se-methylselenocysteine* (GGSeMCys) *(Roman et al. 2014).*

FOOD	SELENIUM LEVEL RANGIE
Cereals	~10–550 μg kg^{-1}
Bread	~ 60–160 μg kg^{-1}
Allium Family (Garlic and Onion)	~ 68 and 96 μg g^{-1}
Brazil Nuts	~ 83 μg g^{-1}
Meat, Eggs, and Fish	~ 49–739 μg kg^{-1}
Specific Organs (Liver and Kidney)	~ 1500 μg kg^{-1}

Figure 2. *Selenium-rich foods*

2.2.2 *Selenium uptake and metabolic pathway*

Once it has been ingested, the distribution of selenium within the body of humans, as well as, of other animals, and also its absorption and excretion, depend on several factors, particularly the chemical form as well as on total amount of the element in the diet *(Terry et al. 2012).* In addition

intake can be affected by the presence of certain other components of food, including sulfur, heavy metals, and vitamins, as well as, on other factors, such as, sex, age, condition of health and nutritional status *(Terry et al. 2012)*. The absorption of Se-species occurs mainly across intestinal epithelial membrane, by different pathways *(Fig.3)*. Absorption of selenate (SeO_4^{2}) appears to be by a sodium-mediated carrier transport mechanism shared with sulfur, while selenite (SeO_3^{2-}) uses passive diffusion. Both forms of inorganic selenium compete with inorganic sulfur compounds for absorption *(Terry et al. 2012)*. In contrast, the Se-amino acids SeMet and SeCys are absorbed through transcellular pathways mediated by transporters which are basically shared with their sulphur-containing analogues *(Roman et al. 2014)*. Absorbed selenium is transported in the blood mainly bound to protein, following an initial reduction within the erythrocytes to *selenide* (Hse^{-}) by reduced glutathione and involving the enzyme glutathione reductase (*Terry et al. 2012*). In humans, almost all the protein-bound selenium in blood is reported to be in the very low-density β-lipoprotein fraction, with smaller amounts bound to other proteins (*Terry et al. 2012*). However, distribution between these proteins appears to depend on the composition of the diet, in fact Whanger et al. (1993) showed that nearly 50% of the selenium in plasma is associated with albumin in people who consume a diet in which selenomethionine is the main form of the element (*Terry et al. 2012*).

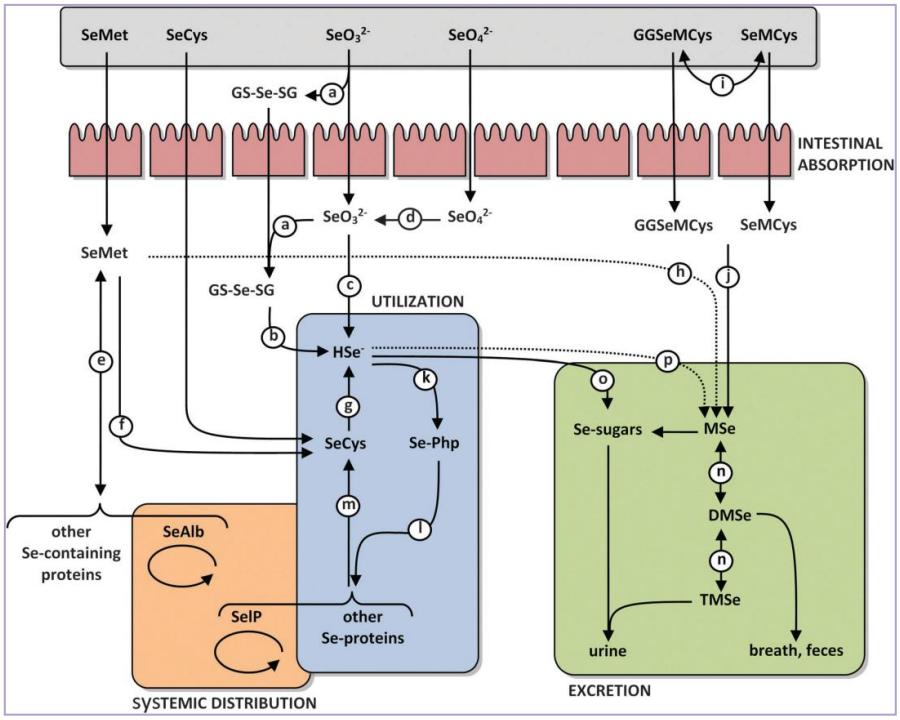

Figure 3. *Global view of Se metabolism in mammals*

2.2.3 *Systemic distribution*

The Se-species absorbed into the gastro-intestinal tract are firstly transported into the liver: SeMet is usually transported in the form of Se-albumin (SeAlb) while selenate and the other organic species may be transported intact or through mechanisms which are still not elucidated. The liver is the foremost organ in Se metabolism, since it synthesizes most of the Se-proteins and regulates the excretion of Se metabolites. The *Selenoprotein P* (SelP), produced into the liver, is released into the bloodstream and is responsible for the distribution of Se to the other organs, where other Se-proteins can be synthesized. The local uptake of Se from plasma has been shown to occur by endocytosis mediated by receptors of the *Apolipoprotein* family such as apoER2 in testis and brain and megalin (Lrp2) in kidney. Thus, the liver regulates the whole-body Se distribution by sorting the metabolically available Se between the two pathways of Se-proteins synthesis and the excretory metabolite synthesis. Such regulation might be passive, so that the fraction of Se that cannot be utilized for Se-proteins synthesis enters the excretory pathway. Active regulation of the excretory metabolites has been also hypothesized, but not yet investigated *(Roman et al. 2013) (Fig.4).*

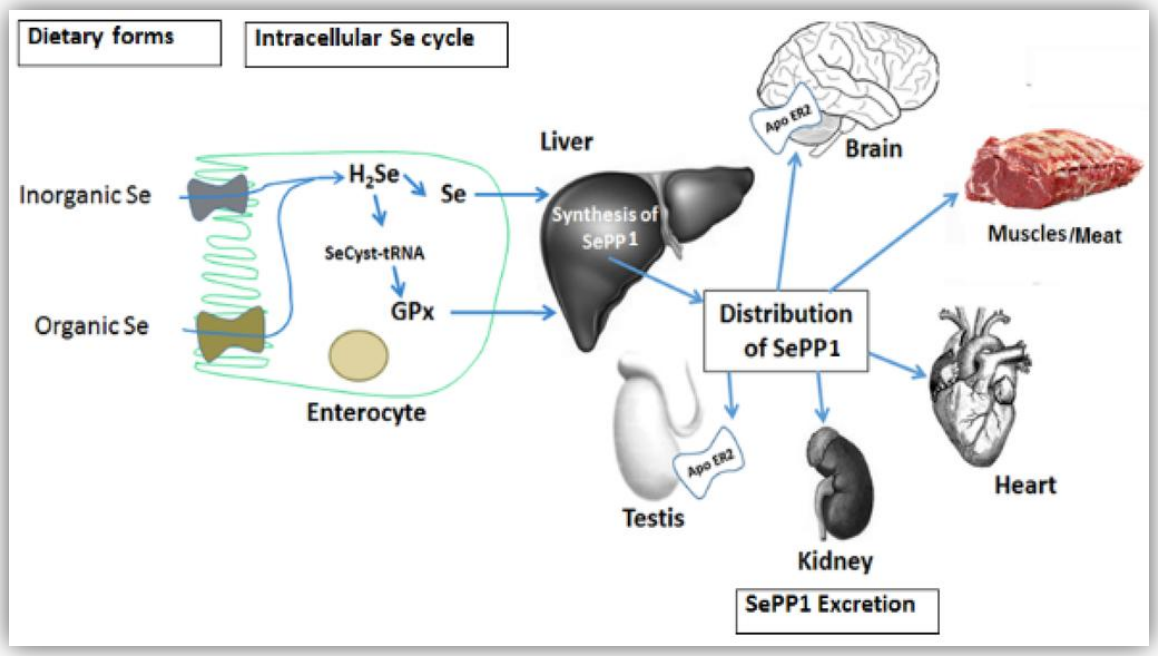

Figure 4. *General pathways of Se absorption and distribution to various organs*

2.2.4 *Selenium retention and excretion*

Actual retention times will depend on a number of factors, including present selenium status, the specific form in which the element is ingested, as well as the state of health of the subject. Selenium is excreted from the body by three distinct routes, in urine via the kidneys, in feces from the

gastrointestinal trace, and in expired air via the lungs. The amounts and proportions of each type of excretion depend on the level and form of the element in the diet (*Terry et al. 2012*). The urinary pathway is the dominant excretion route for selenium in humans *(Yang et al. 1989)*. The proportion of intake excreted in this way depends on the level of intake in the diet. When this is high, urinary excretion will also be high *(Thomson and Robinson, 1986)*. At low levels of intake, half or less of the dietary selenium will appear in urine *(Robinson et al., 1973)*. These findings point to the importance of renal regulation of selenium levels in the body that are not, apparently, homeostatically controlled by the gut *(Burk, 1976)*. Fecal selenium consists largely of unabsorbed dietary selenium, along with selenium contained in bilary, pancreatic, and intestinal secretions *(Levander and Baumann, 1966)*. It has been postulated that secretion of selenium in bile and its enterohepatic reabsorption may provide a mechanism, in addition to renal control, for conserving body stores. This could have major implications for populations with a low dietary intake *(Dreosti, 1986)*. Excretion of selenium via the pulmonary route in expired air and via the dermal route in sweat are of minor significance at normal levels of dietary intake. Excretion through the lungs occurs principally when intake is unusually high. Excess selenium is detoxified by successive methylation to form the volatile dimethyl selenide and other methylated species. The garlic-like odor of dimethyl selenide on the breath is characteristic of selenium intoxication *(Terry at al. 2012)*

2.2.5 *Selenoproteins biosynthesis pathway*

Selenium is the key component of the active site of several Se-proteins having essential biological functions *(Roman et al. 2014)*, which synthesis is an evolutionary conserved process and requires a several components. Co-translational incorporation of Sec into proteins is dictated by in-frame UGA codons present in selenoprotein mRNAs. Sec is introduced into selenoproteins by a complex mechanism that requires special *trans*-acting protein factors, Sec-tRNA$^{[Ser]Sec}$ *(Fig.5a)* and a *cis-acting Sec insertion sequence element* (SECIS). When a ribosome encounters the UGA codon, which normally signals translation termination, Sec machinery interacts with the canonical translation machinery to augment the coding potential of UGA codons and prevent premature termination. SECIS elements serve as the factors that dictate recoding of UGA as Sec. In response to the SECIS element in selenoprotein mRNA, Sec-tRNA$^{[Ser]Sec}$, which has an anticodon complimentary to the UGA, translates UGA as Sec. At least two *trans*-acting factors are required for efficient recoding of UGA as Sec in eukaryotes: *SECIS binding protein 2* (SBP2, itself is a selenoprotein in most organisms with selenoproteomes *[Papp et al. 2007]*) and *Sec-specific translation elongation factor* (eEFSec). SBP2 is stably associated with ribosomes and contains a distinct L7Ae *RNA-binding domain* that is known to bind SECIS elements with high affinity and

specificity. Aside from binding to ribosomes and SECIS elements, SBP2 also interacts with eEFSec, which recruits Sec-tRNA$^{[Ser]Sec}$ and facilitates incorporation of Sec into the nascent, growing polypeptide *(Fig.5b)*. Since the discovery of SBP2, additional SECIS-binding proteins were identified and their roles in selenoprotein synthesis were characterized, including ribosomal *protein L30*, *eukaryotic initiation factor 4a3* (eIF4a3) and *nucleolin* that together serve as regulatory proteins that modulate synthesis of selenoproteins and may contribute to the hierarchy of selenoprotein expression *(Labunsky et al. 2014)*.

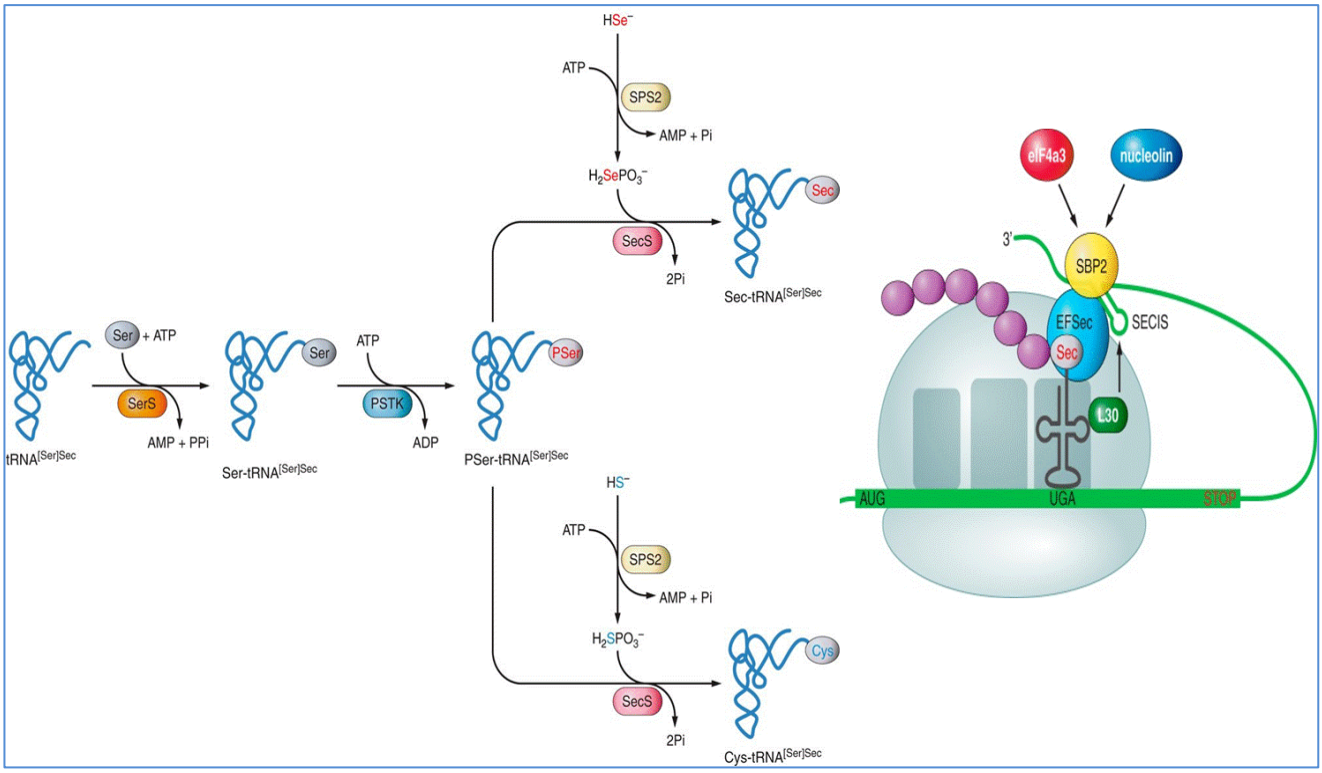

Figure 5a) Sec biosynthesis pathway in mammalian cells. *Sec biosynthesis initiates with the attachment of serine to the Sec tRNA[ser]sec by seryl tRNA synthetase to yield SeryltRNA[ser]sec. The phosphoseryl tRNA kinase phosphorylates the complex. The phosphate is then replaced by the selenium donor selenide (H2Se-P), activated by selenophosphate synthetase. The resulting molecule is selenocysteyl- tRNA[Ser]Sec, which delivers the Sec into the growing polypeptide chain (Papp et al. 2007)* ***Figure 5b)*** *Factors that are required for Sec incorporation into proteins in response to the UGA codon and the factors that may influence the efficiency of the Sec insertion* ***(Nebraska Redox Biology Center Educational Portal).***

2.2.6 *Selenoproteins in physiology and pathology*

Only a few of the 25 identified mammalian selenoproteins have so far been functionally characterized. The Sec localization in the enzyme active site is responsible of enzymatic redox function, which confers their catalytic or antioxidant activities but many others are cellular

processes in which the selenoproteins are involved: biosynthesis of *dNTPs* for DNA, removal of damaging or signaling peroxides, reduction of oxidized proteins and membranes, regulation of redox signaling, thyroid hormone metabolism, selenium transport and storage and potentially protein folding (*Papp et al. 2007*) (*Fig.6).*

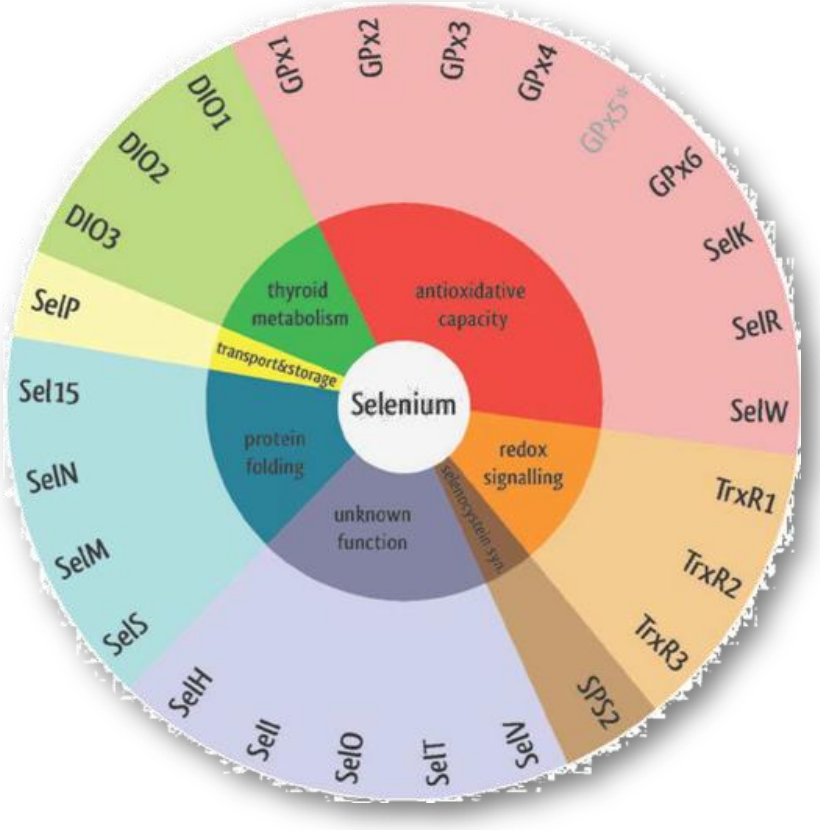

Figure 6. *The 25 human selenoproteins classified by their determined or potential function* (*Benstoem et al. 2015)*

Overall, *Thioredoxin Reductases* (TrxRs), *Glutathione Peroxidases* (GPxs) and *Deidinases* (DIOs) are the three best characterized selenoprotein families. TrxRs (TrxR1 in the cytosol/nucleus, TrxR2 in mitochondria and and thioredoxin glutathione reductase in the testis) are the enzyme able to reduce oxidized Trx. Trx provides electrons to ribonucleotide reductase, which is essential for DNA synthesis by converting ribonucleotide to deoxyribonucleotides. In addition, the Trx system participates in many cellular signaling pathways by controlling the activity of transcription factors containing critical cysteines in their DNA-binding domains, such as NF-κB, AP-1, p53 and the glucocorticoid receptor. It is also known that reduced Trx can bind to and inhibit apoptosis signal regulating kinase 1 (*ASK1)*, whereas the oxidization of Trx results in the activation of ASK1 and the induction of ASK1-dependent apoptosis. Therefore, TrxRs are involved in the control of cellular proliferation, viability, and apoptosis through the control of Trx activity and redox state *(Lu and*

Holmegren 2009). GPxs protect cells against peroxidative damage by reducing hydrogen peroxide, free fatty acid hydroperoxides, and phospholipid hydroperoxides *(Brenneisen et al. 2005)*. In humans, there are now five Sec-containing GPxs: the ubiquitous cytosolic GPx (GPx1), the gastrointestinal-specific GPx (GPx2), the plasma GPx (GPx3), the ubiquitous phospholipid hydroperoxide GPx (GPx4), and the olfactory epithelium- and embryonic tissue-specific GPx (GPx6). GPx1–3 catalyze the reduction of hydrogen peroxide and organic hydroperoxides, whereas GPx4 can directly reduce phospholipid and cholesterol hydroperoxides. GPx4 is also involved in sperm maturation and male fertility because it has been found to be a main structural component of the sperm mitochondrial capsule in mature spermatozoa as an enzymatically inactive, oxidatively cross-linked, insoluble protein *(Lu and Holmegren 2009)*. Conversely, GPx5, secreted in epididymis, have not the selenocysteine in the active site, and GPx7, recently described as new *phospholipid hydro-peroxide glutathione peroxidase* (PHGPX), incorporates cysteine instead of selenocysteine in the conserved catalytic motif *(Rusolo et al. 2015)*. Three DIOs (DIO1, DIO2 and DIO3) constitute a group of dimeric integral membrane thioredoxin fold–containing proteins that can activate or inactivate the thyroid hormone, depending on their action on the phenolic or the tyrosil ring of the iodothyronines *(Rusolo et al. 2015)*. DIO1 and DIO2 catalyze the deiodination of T4, the major thyroid hormone secreted by the thyroid gland, into the active hormone T3; DIO3 converts T4 into reverse T3 and also T3 into 3,3-diidothyronine. DIO1 and DIO2 can also convert reverse T3 into 3,3-diidothyronine *(Lu and Holmgren, 2009)*. The specific function of several other human selenoproteins are unknown although some details of their biology have been established. Thus, selenoproteins S, and K are *Endoplasmatic Reticulum* (ER) transmembrane proteins, involved in regulating ER-associated degradation of misfolded proteins and may have a role in immune function. The *selenoprotein 15* (Sep15/SEL15) and *selenoprotein M* (SelM) are also ER-localized and implicated in quality control of protein folding *(Brigelius and Sies, 2015)*. SelN (selenoprotein N) is found in the membrane of the ER and appears to be necessary for proper muscle development *(Bellinger et al. 2009)*. SelV was the least conserved mammalian selenoprotein that likely arose from a duplication of SelW in the placental stem. The functions of SelV and SelW are not known, but SelV is expressed exclusively in testes, whereas SelW *(Mariotti et al. 2012)*, expressed in a variety of organs, is similar to the GPxs family in that it shares the redox motif and binds glutathione. The ‘W’ stands for ‘white muscle disease’, a disorder among grazing livestock found in regions with low selenium soil levels. The completion of the human genome project led to the identification of many other selenoproteins though sequence homology and characteristic elements. SelH (selenoprotein H) is a nuclear-localized DNA-binding protein that may act as a transcription factor. SelH increases glutathione levels and GPx activity, and may up-regulate other selenoproteins

in response to stress *(Bellinger et al. 2009). Selenoprotein I* (SelI) is one of the least studied selenoproteins. It contains a highly conserved CDP-alcohol phosphatidyltransferase domain. This domain is typically encountered in *choline phosphotransferases* (CHPT1) and *choline/ethanolamine phosphotransferases* (CEPT1). CHPT1 catalyzes the transfer of choline to diacylglycerol from CDPcholine. CEPT1 catalyzes an analogous reaction but accepts both choline and ethanolamine *(Mariotti et al. 2012). Selenoprotein R* (SelR)/*selenoprotein* X (SelX) is a member of the methionine sulfoxide reductase family, important for reduction of sulfoxymethyl groups *(Bellinger et al. 2009).* The physiological and pathological effects of selenoproteins are closely related to selenium status. Selenium deficiency leads to a dramatic loss of activity of selenoproteins though the their expression exhibits a hierarchical style during selenium deprivation and repletion *(Lu and Holmgren 2009)*. Furthermore, genetic mutations in selenocodons have been identified like cause of different human deseases. One form of congenital muscular dystrophy, termed *multiminicore disease*, is characterized by a distinct loss of organization of muscle fibres. Mutations in ryanodine receptors, responsible for calcium-stimulated release of calcium from intracellular stores, and SelN have been identified as causing the disorder. There is evidence that other selenoproteins may also regulate calcium signalling and calcium stores. Overexpression of SelT, another ER localized selenoprotein, led to an increase in calcium levels, but inhibited calcium responses and endocrine release from *pituitary adenylate cyclase-activating polypeptide* (PACAP). Selenoprotein function in cardiovascular disease has been investigated primarily by analysis of oxidative stress under conditions of selenium supplementation and/or deficiency *(Bellinger et al. 2009).* Selenium supplementation elevates expression and activity of GPx1, GPx4 and TRxR1 in vascular endothelial or smooth muscle cells and thus inhibits oxidative stress, cell damage and apoptosis from oxidized *low-density lipoprotein* (LDL) or triol, a cytotoxic hydroxylated cholesterol derivative found in blood, cells, tissues and atherosclerotic plaques in humans *(Bellinger et al. 2009)*. Low blood Se concentrations have been associated with increased cardiovascular disease mortality. This may be a reflection of sub-optimal GPx4 activity in the prevention of LDL oxidation, with subsequent uptake by endothelial cells and macrophages in arterial blood vessels *(Brown and Arthur, 2001)*. Also other selenoproteins are involved in cardiovascular disease. GPx1 has been shown to inhibit ischaemia/ reperfusion-induced apoptosis of cardiac myocytes in mice and its deletion produces heart and vascular dysfunction and tissue irregularities; decreased GPx3 activity, generally abundant in plasma and modulatating redox-dependent aspects of vascular function, results in inadequate nitric oxide (NO) levels for excess ROS, which disrupts platelet inhibitory mechanisms and increases arterial thrombosis *(Bellinger et al. 2009).* Moreover, the TRxR/TRx system contributes in regulating myocardial remodelling through the reversible

oxidation of signalling molecules. For example, adrenergic receptor activation-induced hypertrophy of adult rat cardiac myocytes is affected by the oxidation of cysteine thiols of Ras that can be reduced by TRxR1 *(Bellinger et al. 2009).* Levels of ROS influence, also, inflammatory gene expression, thus selenoproteins affect inflammatory responses by regulating the oxidative state of immune cells. GPxs and TRxRs are necessary for optimal function of immune cells by controlling oxidative stress and redox regulation but also other selenoproteins also have ROS-independent roles in modulating inflammatory responses *(Bellinger et al. 2009).* SelS, for example, is located in the ER membrane and its expression and secretion from liver cells is regulated by inflammatory cytokines as well as extracellular glucose concentrations. SelS has an antiapoptotic role and reduces ER stress in peripheral macrophages and brain astrocytes *(Bellinger et al. 2009).* Oxidative stress and generation of *reactive oxygen species* (ROS) are strongly implicated also in a number of neuronal and neuromuscular disorders, including stroke and cerebrovascular disease, Alzheimer's disease, Parkinson's disease, familial amyotrophic lateral sclerosis, and Duchenne muscular dystrophy *(Chen and Berry, 2003)* and selenoproteins, as GPx1 and SelP, are envolved in protection mechanisms against cellular demage - ROS induced. While GPx1 prevents lipid peroxidation on neuronal cell membranes, SelP has a function to chelate heavy metals (mercury, zinc, cadmium and silver), reducing their toxicity *(Chen and Berry, 2003)* and can also stimulate the survival of cultured central neurons *(Almondes et al. 2010).* Since the genotoxic damage caused by the accumulation of oxidative modifications in DNA bases and DNA single- or double-strand breaks are outstanding features in the development of many forms of cancer, selenium has been suggested to exert its anticarcinogenic function through ROS detoxification selenoenzymes *(Almondes et al. 2010).* There is evident correlation between cancer risk and a pattern of altered expression of selenoproteins: a single nucleotide polymorphism at codon 198 of GPx1 gene is correlated with increased risk of lung cancer *(Almondes et al. 2010),* while allelic loss of one of two GPx-1 alleles on chromosome 3p is a common event in the development of several types of cancer, including that of the lung, breast and cancers of the head and neck *(Zhuo and Diamond, 2010).* GPx1, GPx3 and SelP decrease expressions and GPx2 increase are observed in colorectal cancer progression *(Almondes et al. 2010).* Furthermore, methylation of CpG islands in the GPx3 promoter regions typically results in transcriptional silencing, gene down-regulation and to contribute to prostate cancer development *(Zhuo and Diamond, 2010).* GPx4 is another member of the glutathione peroxidase family of selenoproteins that has received considerable attention. Lower GPx4 protein levels have been observed in cell lines derived from pancreatic cancers compared to normal pancreatic tissue *(Zhuo and Diamond, 2010).* An examination of the dbEST database identified two polymorphic positions in Sep15 gene (C/T variation at position 811 and a G/A polymorphism at

position 1125), both included in the predicted SECIS element and correlated to *Malignant mesothelioma* (MM) *(Zhuo and Diamond, 2010)*. Selenoproteins role in cancer is much more complex; they appear to have roles in both preventing and promoting cancer. TRx1, for example, activates the p53 tumor suppressor, manifests other tumor suppressor activities, and is specifically targeted by carcinogenic electrophilic compounds, suggesting its major role in cancer prevention. On the other hand, TRx1 overexpression in many cancer cell lines and cancers, targeting by a number of anti-cancer drugs and potent inhibitors that alter cancer-related properties of malignant cells and its deficiency that reverses cell morphology point to a role of TR1 in cancer promotion *(Hatfield et al. 2009)*. The elucidation TRx roles, as well as, unknown function of other selenoproteins in cancer biology may yield some promising anti-cancer paradigms for new cancer therapeutic agent development.

2.3 OXIDATIVE STRESS AND CANCER - AN INFLAMMATORY NETWORK

2.3.1 *ROS definition and sources*

All animals need O_2 for efficient production of energy in mitochondria. This need for O_2 obscures the fact that it is a toxic mutagenic gas; aerobes survive only because they have evolved antioxidant defenses *(Barry Halliwell, 2006)*. The O_2 molecule is itself a free radical because has two unpaired electrons. Although this is the most stable, O_2 is, thermodynamically, a potent oxidizing agent, ready to accept a pair of electrons from non-radical molecules, like proteins, DNA and lipids *(Barry Halliwell, 2006)*. The resulting peroxidation and modification of these molecules can increase the risk of mutagenesis *(Reuter et al. 2010)*. So, r*eactive oxygen species* (ROS) is a collective term that includes both oxygen radicals and certain non radical derivatives of O2 that are oxidizing agents and/or are easily converted into radicals (HOCl, O_3, $^1O_2$, and H_2O_2). It should be noted that all oxygen radicals are ROS but not all ROS are oxygen radicals *(Barry Halliwell, 2006)*. The acronym ROS may include also several nitrogen-containing compounds or *RNS* (Reactive Nitrogen Species), such as nitric oxide (NO), nitroxyl anion (NO^-), and peroxynitrite ($ONOO^-$) *(Tafani aet al. 2016).* The cell is exposed to a large variety of ROS and RNS from both exogenous and endogenous sources. Major exogenous source of ROS is represented by ionizing and nonionizing irradiation and air pollutants such as car exhaust, cigarette smoke, and industrial contaminants that attack and damage the organism either by direct interaction with skin or following inhalation into the lung. Drugs are also a major source of ROS. There are drugs, such as belomycinem and adreamicine, whose mechanism of activity is mediated via production of ROS. A large variety of xenobiotics (eg, toxins, pesticides, and herbicides) and chemicals (eg, mustard gas, alcohol) produce ROS as a by-product of their metabolism in vivo. The invasion of pathogens, bacteria, and viruses might result in

the production of many ROS species by direct release from the invaders or an endogenous response induced by phagocytes and neutrophils. One of the major sources of oxidants is food for a large portion of the food we consume is oxidized to a large degree and contains different kinds of oxidants such as peroxides, aldehydes, oxidized fatty acids, and transition metals (*Kohen and Nyska, 2002) (Fig.7).*

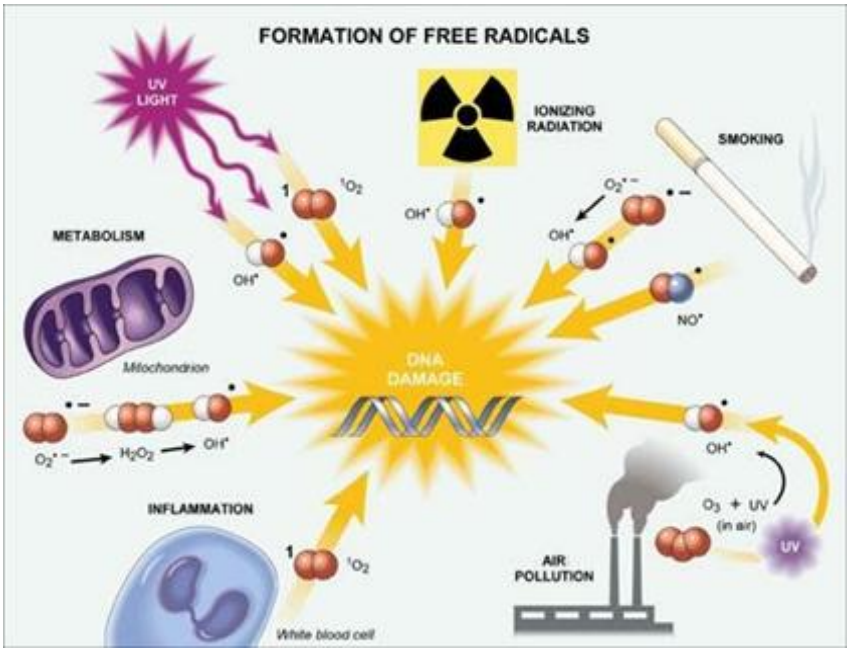

Figure 7. *Major exogenous sources of ROS*

Although the exposure of the organism to ROS is extremely high from exogenous sources, the exposure to endogenous sources is much more important and extensive, because it is a continuous process during the life span of every cell in the organism (*Kohen and Nyska, 2002)*. ROS are, also, products of a normal cellular metabolism and play vital roles in stimulation of signaling pathways in plant and animal cells in response to changes of intra- and extracellular environmental conditions *(Reuter et al. 2010)*. Five main cellular compartments contain ROS: ***mitochondria*** that produce in large quantities through oxidative phosphorylation for ATP synthesis, ***cytosol*** that produce ROS from many endogenous (growth factors, cytokines, and metabolisms) or exogenous sources (nutrients, radiation, microbiome, and xenobiotics), ***single membrane-bound organelles*** (peroxisomes, endosomes, and phagosomes) that can produce substantial amount of ROS as typically occurs in the respiratory burst of activated leukocytes (macrophages and eosinophils), ***exosomes***, released by plasma membranes by shedding and ***extracellular fluids*** (such as blood plasma and spermatic, peritoneal, and pleural fluid) in which ROS are released through aquaporins (hydroperoxides) and some anion channels (superoxides) and by secretion (external opening of

phagosomes and granules) as typically occurs in activated degranulating leukocytes *(Tafani et al. 2016) (Fig.8).*

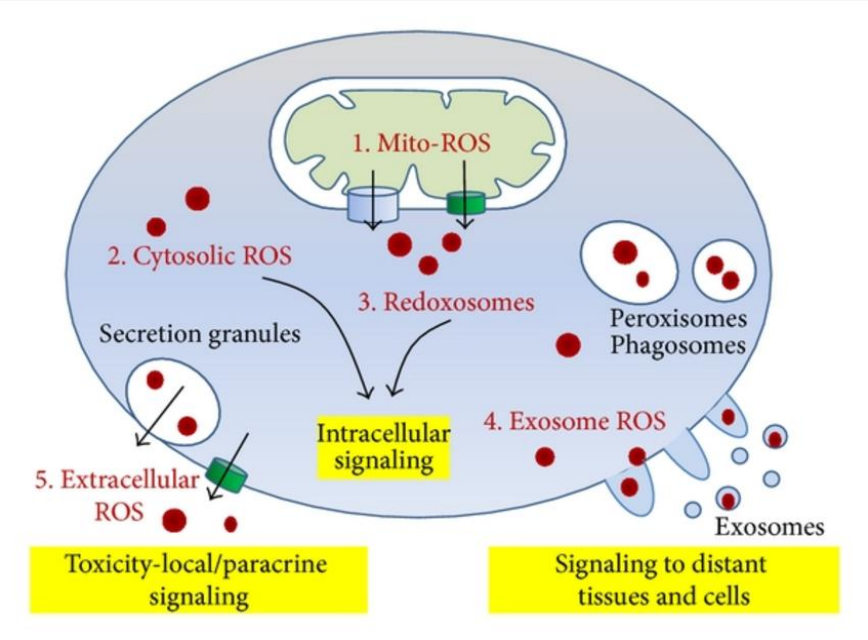

Figure 8. Subcellular compartmentation of ROS. ***1.*** *Mitochondrial ROS;* ***2.*** *Cytosolic ROS;* ***3.*** *Redoxosomes (Peroxisomes, Phaosomes);* ***4.*** *Exosome ROS that include vesicles shedding from damaged plasma membranes;* ***5.*** *Extracellular ROS in extracellular fluids and plasma (Tafani et al. 2016).*

2.3.2 *Physiological functions of ROS*

ROS has dual functionality in biological systems, with both beneficial and detrimental effects in cells *(Ramoutar et al. 2010)*. Beneficial effects of ROS occur at low or moderate concentrations and their '*stady state*' are determined by the balance between their rates of production and their rates of removal by various antioxidants *(Valko et al. 2007)*. A great number of physiological functions are controlled by redox-responsive signalling pathways. These, for example involve: (***i***) redox regulated production of NO; (***ii***) ROS production (*oxidative burst*) by NAD(P)H oxidase in activated neutrophils and macrophages in an inflammatory environment; (***iii***) ROS production by NAD(P)H oxidases in non-phagocytic cells, such as, fibroblasts, vascular smooth muscle cells, cardiac myocytes and endothelial cells, in order to regulate intracellular signalling cascades: regulation of cardiac and vascular cell functioning; (***iv***) modulation of the protein kinases functions and ion channels, as well as, regulation of vascular tone and the inhibition of platelet adhesion mediated by NO•; (***v***) ROS production as a sensor for changes of oxygen concentration and regulation of certain hormones such as erythropoietin, *VEGF* (vascular endothelial growth factor) and *IGF-II* (insulin-like growth factor), controlled by *HIF-1* (the transcription hypoxia inducible factor-1); (***vi***) redox regulation of cell adhesion that plays an important role in embryogenesis, cell growth,

differentiation and wound repair; (***vii***) activation of T lymphocytes and induction of their function, such as the IL-2 production; (***viii***) ROS-induced apoptosis, triggered internal signals: the intrinsic or mitochondrial pathway *(Valko et al. 2007).*

2.4 HARMFUL EFFECTS OF ROS

2.4.1 *ROS: common denominator of inflammation and cancer*

An imbalance between the continuous efflux of ROS from endogenous and exogenous sources and their elimination by protective mechanisms, referred to as *antioxidants (Fig.9)*, results in continuous and accumulative oxidative damage to cellular components, impairing many cellular functions and inducing somatic mutations *(Reuter et al. 2010; Kohen and Nyska, 2002)*. ROS may be important initiators and mediators in many clinical disorders: heart diseases, endothelial dysfunction, atherosclerosis and other cardiovascular disorders, burns, intestinal tract diseases, brain degenerative impairments, diabetes, eye diseases and ischemic and post-ischemic (eg, damage to skin, heart, brain, kidney, liver, and intestinal tract) pathologies (*Kohen and Nyska, 2002)*. ROS, also, are involved in chronic inflammation and in a wide variety of different cancers, indeed, epidemiological and experimental data have demonstrated how inflammation is strongly associated with an increased risk of several human cancers and how anti-inflammatory therapies show efficacy in cancer prevention and treatment *(Reuter et al. 2010)*. How oxidative stress placed in this context? And how it modulates these different stages of inflammation-induced carcinogenesis? Microbial and viral infections, exposure to allergens, radiation and toxic chemicals, autoimmune and chronic diseases, obesity, consumption of alcohol, tobacco use, and a high-calorie diet are sources both inflammation and ROS. During inflammation, mast cells and leukocytes are recruited to the site of damage, which leads to a '*respiratory burst*' due to an increased uptake of oxygen, and thus, an increased release and accumulation of ROS at the site of damage. On the other hand, inflammatory cells also produce soluble mediators, such as metabolites of arachidonic acid, cytokines and chemokines, which act by further recruiting inflammatory cells to the site of damage

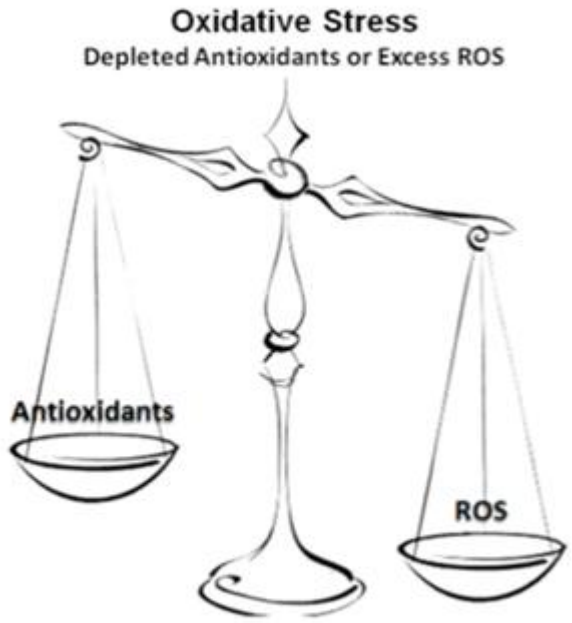

Figure 9. *Oxidative stress can occur when free radical production overwhelm the antioxidant system*

and producing more reactive species. These key mediators can activate signal transduction cascades, as well as, induce changes in transcription factors, such as *nuclear factor kappa B* (NF-κB) and *activator protein-1* (AP-1) that control transcription of DNA, cytokines production and cell survival. Also aberrant expression of inflammatory cytokines [*tumor necrosis factor* (TNF), *interleukin-1* (IL-1), IL-6] and chemokines, such as IL-8, *CXC chemokine receptor 4* (CXCR4) sustain oxidative stress-induced inflammation. This inflammatory/oxidative environment leads to a vicious circle, which can damage healthy neighboring epithelial and stromal cells and over a long period of time may lead to carcinogenesis *(Reuter et al. 2010)*.

2.4.2 *Hepatocellular Carcinoma (HCC) and Breast Cancer (BC): two examples of inflammation-related cancer*

Hepatitis B and C viruses (HBV and HCV) are the two global causes of hepatitis with 5 to 10 % of HBV infected and more than 70% of HCV infected patients developing chronic infection which alters the state of liver homeostasis inducing inflammatory responses. Chronic inflammatory state, during HBV and HCV infection, is associated with activated inflammatory cells and is mediated by various cytokines *(Mohammad Khalid Zakaria et al. 2014)*. Two are key mediators of HBV associated liver inflammation: IL-8 which maintains an inflammatory environment by activation of cyclooxygenase (COX)-2 gene and IL-29 which inhibits virus replication by PKr production, activated by NK cells subset and enriched in the HBV infected Liver *(Mohammad Khalid Zakaria et al. 2014)*. A transactivant and multifunctional viral protein, HBx, is responsible to viral genes expression, the activation of NFκB, AP1 and NF-AT transcription factors *(Mohammad Khalid Zakaria et al. 2014)* and upregulates some oncogenes (such as, Rab18 or Yes-associated protein). Moreover, induces apoptosis by upregulating FasL protein through activating MLK3/MKK7/JNKs signaling and integration of the *HBV* gene into the house genome (cancer-related genes such as *TERT*, *MLL4*, and *CCNE1* are integrated by HBV), an important mechanism that is responsible for HCC development *(Akinobu Takaki et al. 2015)*. HCV produces, by STAT3 signaling cascade, a several cytokines: RANTES, IL-8, MIP1α, MIP1β, converging on common inhibitor kappa beta (IκB)-kinase dependent kinases, thereby responsible to *interferon regulatory factor 3/7* (IRF3/7) and *Nuclear Factor Kappa Beta* (NFκB) activations. The letter driving the INFα/β and pro-inflammation cytokines synthesis which exacerbate inflammation. The continuous production of ROS, lipid peroxidation and the activation of *hepatic stellate cells* (HSC) are responsible to progression From Liver chronic desease to steatosis. The organ becomes more sensitive to apoptosis, injury and inflammation mediated by TNF-α, under the influence of pro-inflammatory and pro-fibrotic cytokine. The result of imbalance between

extracellular matrix production and degradation, as well as, of complex mechanisms of fibrinogenesis, cell proliferation, contractility, chemotaxis, degradation of matrix, and cytokine release, develop in fibrosis *(Mohammad Khalid Zakaria et al. 2014)*. The release of free radicals, chemokines and cytokines as a result of HBV and HCV infection and inflammation, result in DNA damage, cell proliferation, fibrosis and angiogenesis. Indeed, free radicals, for example, reactive nitrogen or oxygen species, can directly damage DNA and proteins, and indirectly damage these macromolecules via lipid peroxidation. The abrogation of p53-mediated apoptosis, both inhibition by HBx and for its mutation, as in HepG2 and HuH7 cells, infected with HCV, provide a selective clonal advantage for preneoplastic or neoplastic hepatocytes and contribute to hepatocellular carcinogenesis *(SP Hussain et al. 2007) (Fig.10)*.

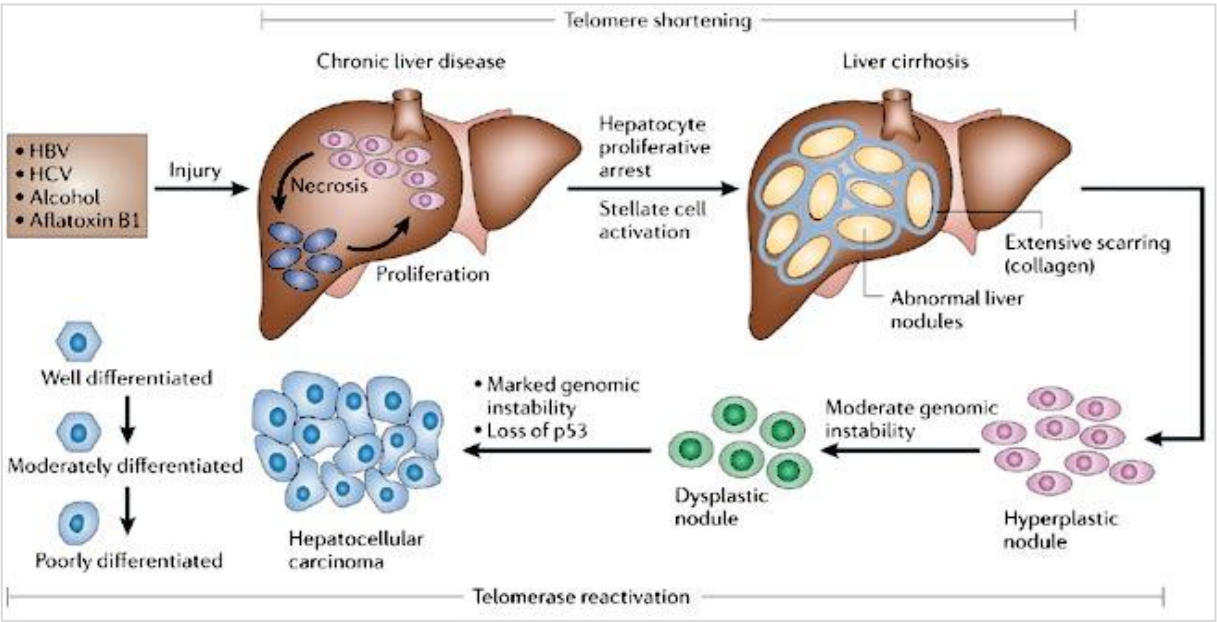

Figure 10. *Stages of Hepatocellular Carcinoma (HCC)*

The liver is also a hormone-sensitive organ and a several lines of evidence suggest that sex hormones, like estrogens, and their receptors play a role in liver carcinogenesis *(Wang et al. 2006)* by the formation of free radical-mediated DNA and RNA adducts. In humans, indeed, the chronic use of estrogens is associated with increased risk of developing liver neoplasms such as benign nodular hyperplasia and hepatic adenoma. Therefore, HCC could therefore be an estrogen-dependent cancer like breast cancer and the use of anti-estrogen drugs should control the growth of this tumor *(De Maria et al. 2002)*. Host microenvironment plays an important role also in breast cancer tumorigenesis. Mutations, like BRCA1/2 are responsible to extent of genomic instability in the malignant breast epithelium while, the letter, activate the tumor-associated stroma to foster

tumor growth by secreting growth factors, increasing angiogenesis or facilitating cell migration. The inflammatory component tends to exacerbate this context. Infiltration of lymphocytes, *tumor-associated macrophages* (TAM), mast cells and neutrophils have been shown to exert both direct and indirect tumoricidal action by their ability to express numerous tumor-promoting characteristics, such as growth factors for breast tumor cells, angiogenic mediators, ECM-degrading enzymes (MMPs), and inflammatory cytokines. For instance, monocyte-derived cytokines, such as TNF-a, may intensify chemokine expression by tumor or stroma cells besides possessing other promalignant properties. As for hepatocarcinogenesis, inflammatory cells can secrete cytokines, growth factors and chemokines, release reactive oxygen intermediates to stimulate proliferation, prevent apoptosis, direct morphogenesis and induce mutagenic changes that may increase tumor cell DNA damage. The overall effect of these activities may be a key factor in breast cancer metastasis and disease progression *(Artacho-Cordón et al.2012)*. Secretion of tumour necrosis factor-α by TAM is known to induce cellular oxidative stress. Oxidative stress trigger mitosis, in vitro, either by the activation of mitogen-activated protein kinases (MAPKs) or via MAPK independent mechanisms. Furthermore, persistent oxidative stress at sublethal levels may cause resistance to apoptosis. The induction of programmed cell death by ROS is dependent on p53 in both mouse and human cell lines. Constitutive oxidative stress within breast carcinoma cells may therefore accelerate the selection of p53 knockout tumour cell clones, which have an apoptosis resistant phenotype (Brown and Bicknell, 2001), fostering to progression from normal mammary gland to *ductal carcinoma in situ* (DCIS) and finally to *invasive ductal carcinoma* (IDC) *(Artacho-Cordón et al.2012) (fig.11)*.

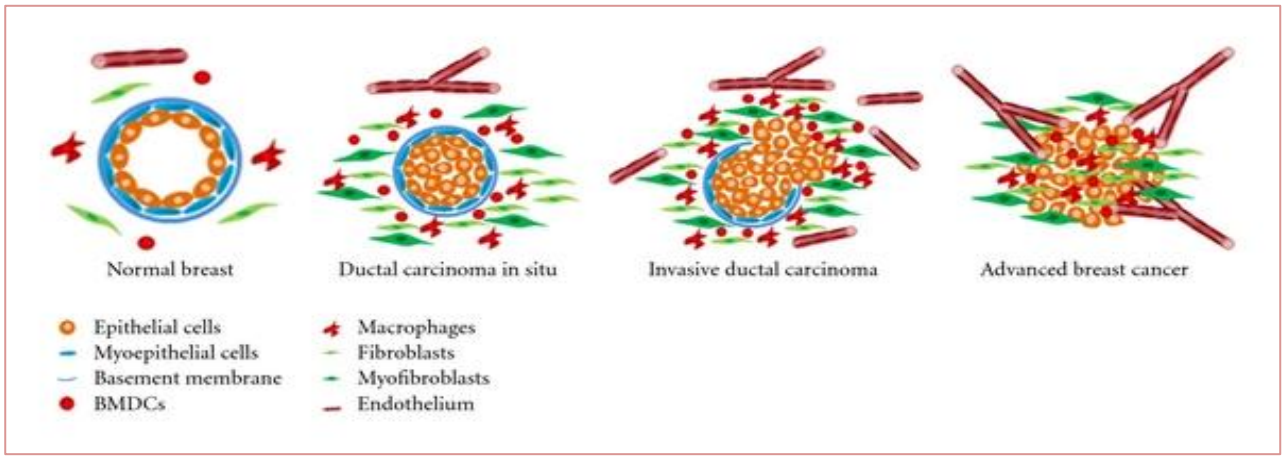

Figure 11. *Breast cancer progression*

3

MATERIALS AND METHODS

3.1 *HepG2 and Huh7 Cell Culture*

Human hepatoma cell lines (HepG2 and Huh7) were kept in culture and expanded at 37°C in a humidified atmosphere of 5% CO2 in culture medium DMEM (Dulbecco's Modified Eagle's Medium, Lonza, Verviers, Belgium), supplemented with FBS (Invitrogen, Camarillo, CA, USA) at 10%, Penicillin/Streptomycin 100x (Euroclone, Devon, UK), Glutamax 100x (Invitrogen) and non-essential amino acids 100x (Invitrogen) at 1%. Phosphate buffer (PBS phosphate buffered saline $Ca2^{+}$ and Mg^{2+} free) and trypsin (Ca^{2+} and Mg^{2+} free) were supplied by Euroclone. The cells (2×10^4) were seeded in 100 mm plates in 8 mL of culture medium and left to grow for 24 h at 37 °C to allow adhesion. Then, the cells were treated with *sodium selenite (*Na_2SeO_3*)*, dissolved in H2O, at the following concentrations 0.25 μM, 0.5 μM and 1 μM and incubated for 24 h. The experiments

were performed in duplicate and repeated for three times. However, the concentrations of sodium selenite used in this study were chosen concerning that human physiological concentration of selenium is less than 3 μM.

3.2 *Protein Extraction and Western Blot Analyses*

HepG2 and Huh7 cells were washed once in cold phosphate buffered saline (*PBS*) and lysed in a lysis buffer containing 20 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA and NP40 after 24 h of treatment with 0.25 μM, 0.5 μM and 1 μM of sodium selenite. The lysis buffer was complemented with protease inhibitor cocktail tablets (Roche Applied Science, Penzberg, Germany), diluted in H2O to obtain a stock solution 7X concentrated, and with phosphatase inhibitor cocktail tablets (Roche Applied Science), diluted in H2O to obtain a 100X concentrated stock solution. The lysates were clarified by centrifugation at 13,000 rpm for 15 min. Protein concentrations were estimated by a BioRad assay (Bio-Rad Laboratories, Hercules, CA, USA), based on the Method of Bradford, that is a simple and accurate procedure for determining concentration of solubilized proteins. Then the proteins were boiled for 5 min before electrophoresis in Laemmli Sample buffer (Bio-Rad) containing 62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulphate (*SDS*), 25% glycerol, 0.01% bromophenol blue complemented with 10% β-mercaptoethanol. 60 μg of proteins were subjected to SDS–polyacrylamide gel electrophoresis (SDS-*PAGE*) using 12% acrylamide concentrated gels under reducing condition. After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham Hyperfilm MP, High performance autoradiography, GE Healthcare, Hertfordshire, UK); complete transfers were assessed using prestained protein standards (Fermentas, Milano, Italy). After blocking with Tris-buffered saline 5% non fat dry milk (Bio-Rad), membranes were incubated ON at 4°C in shaking with the Goat anti GPX1 antibody (R&D Systems, Minneapolis, MN, USA), with the rabbit anti SELK antibody (ABCAM, Cambridge), and with the rabbit anti SELENBP1 antibody (ABCAM, Cambridge, UK) diluted 1:500, 1:500 and 1:1,000 overnight at 4°C, respectively, and then incubated with the horseradish peroxidase conjugated secondary antibody (1:3,000) for 60 min at room temperature; the reaction was detected with a Western blotting detection system (ECL; Amersham Biosciences, Little Chalfont, UK). To ascertain that equal amounts of protein were loaded, membranes were incubated with antibodies against the γ-tubulin protein (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

3.3 *Bio-Plex Assay for HepG2 and Huh7*

In our approach, the levels of a panel of numerous cytokines, chemokines and growth factors were evaluated at the same time by BioPlex assay. The simultaneous quantitative determination of a large panel of cytokines, able to report the correct ratios and dynamics between highly and poorly represented molecules, has emerged as an accurate, simple, specific, noninvasive, reproducible and less expensive method *(Costantini et al. 2010, Capone et al. 2010)*. The multiplex biometric ELISA-based immunoassay, containing dyed microspheres conjugated with a monoclonal antibody specific for a target protein was used, according to the manufacturer's instructions (Bio-Plex Bio-Rad), to evaluate the concentrations of different cytokines by Human Cytokine 27-Plex Panel after 24 h of incubation with sodium selenite in HepG2 and Huh7 supernatants. In particular, the following cytokines were evaluated: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin (CCL11), basic FGF, G-CSF, GM-CSF, IFN-γ, CXCL10, MCP-1, MIP-1α, MIP-1β, PDGF-ββ, RANTES, TNF-α and VEGF. Each experiment was performed in duplicate as previously described *(Costantini et al. 2010, Capone et al. 2010)*. Protein concentrations were determined using a Bio-Plex array reader (Luminex, Austin, TX, USA) that quantitates multiplex immunoassays in a 96-well format with very small fluid volumes. The analyte concentration was calculated using a standard curve, with software provided by the manufacturer (Bio-Plex Manager Software).

3.4 *Statistical Analysis*

The cytokines concentrations evaluated in HepG2 and Huh7 supernatants after 24 of incubation with sodium selenite were compared by T-test. Values of $p < 0.05$ were considered to be statistically significant. The statistical program Prism 4 (GraphPad Software, San Diego, CA, USA) was used.

3.5 *RNA preparation and Reverse Transcription-qPCR (RT-qPCR) analysis for seleno-transcriptoma in HepG2 and Huh7 cells*

Total RNA from hNHEPS® Human Hepatocytes (Lonza, Basel, Switzerland), HepG2 and Huh7 (Lonza, Basel, Switzerland) was obtained using the TRizol Reagent (Invitrogen, Milan, Italy) following the manufacturer's instructions. Each total RNA sample was treated with the DNa-free kit according to the manufacturer's instructions (Ambion). RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The mRNA levels of the analysed genes were measured by a RT-qPCR amplification procedure that was previously reported. Sequences for mRNAs from the nucleotide data bank (National Center for Biotechnology

Information, USA) were used to design primer pairs for RT-qPCR (Primer Express, Applied Biosystems, CA, USA). Amplicon sequences were aligned against the human genome by BLAST to check for specificity. Oligonucleotides were obtained from Sigma Aldrich. The primer sequences of 25 selenoprotein mRNAs are provided in *Table 1*. An appropriate region of 18S rRNA was used as control. Reverse transcriptase reactions (500 ng) were performed according to the manufacturer's instructions using iScriptTM Reverse Transcriptase Supermix for RT-qPCR (Biorad, Italia) and subsequently diluted with nuclease-free water (Ambion). RT-qPCR assays were run on an Opticon-4 machine (MJ, Research, Waltham, MT, USA). 2 μl of cDNA were amplified in a total volume of 20 μl containing 1X iQ SYBR Green Supermix (Bio-Rad) and 300 nM of forward and reverse primers. The thermal cycling conditions were as follows: 5 min of denaturation at 95°C followed by 40 cycles of a two-step program (denaturation at 95°C for 15 sec and annealing/extension for 1 min). For each target the primer sequences and the annealing temperature are reported in *Table 1*. Fluorescence threshold and baseline setting was adjusted with the aid of the Opticon Monitor software (Biorad). The efficiency of each assay was tested with serial dilutions of cDNA to obtain a standard curve. The PCR efficiency was calculated according to the following formula: $10^{(-1/slope)}$-1. All PCR efficiencies were above 95%. Dilutions of standards and test samples were run in triplicate. Each reaction was repeated at least three times. Standard deviations and coefficients of variation were calculated for the Cq values of replicated measurements using Microsoft Excel software. Relative quantities were calculated by the ΔΔCq method using the 18S rRNA as housekeeping gene for normalization. Statistical analyses (paired Student's t) were performed using Prism software (Graphpad Software, La Jolla, CA, USA). Significant differences in relative gene expression between hepatocytes and HepG2 or Huh7 are marked by* (p-value<0.05),** (p-value<0.01).

Gene	Tm [°C]	Ta [°C]	Sequence (5'→ 3')
DIO1	59.8	61	AGCTTACTCTGGCTTTGCCGA (21) TATTACCCGTCTTCTCGCCCA (21)
DIO2	59.8	60	CTTACTCTGGCTTTGCCGAGA (21) CAGGATGTTCCGCTTGACTCT (21)
DIO3	59.8	60	GGTAGTTTCCCCCGCTTGTTT (21) TTTAGGTGCTGCTTTGAGGCC (21)
GPX1	59.8	60	TTATGACCGACCCCAAGCTCA (21) ATGTCAATGGTCTGGAAGCGG (21)
GPX2	57.3	58	GGAGAATGAACCCAAGCGAA (20) CAGGTTTGTCACAGCCAGTGAT (22)
GPX3	59.8	60	TCTCATCCCATGTCCACCATG (21) TGCATCCATTTGTGCCAGG (19)
GPX4	59.8	60	AGAGATCAAAGAGTTCGCCGC (21) TCTTCATCCACTTCCACAGCG (21)
GPX5	57.9	58	TCCTTCCACGACAATGGTTCA (21) TGTGACTGTGACCCCATTGCT (21)
GPX6	59.8	61	CAGAAACCCCACCTCACATGA (21) TGCCATGACCTGAATGCACT (20)
GPX7	57.9	56	TTGGTCCCATCATTCTTGTGG (21) GGCTGGTGATTCACTGGTCAA (21)
SELI	56.7	59	AAAGGCCAGGTTCCCAGAA (19) CAATCCTGCTGCAGTCCAAGT (21)
SELK	57.3	59	AATCAATCATCTGCGTGGCC (20) TGGTCAGCCTTCCACTTCTTG (21)
SELM	57.9	61	TCACGCAGGACATTCCATTCT (21) CCTGCACTAGCGCATTGATCT (21)
SELO	59.8	60	CGGTTGTGTTGCGTGTAGCTT (21) TGCACTCGAATGTCGTTCCTC (21)
SELS	59.8	56	CAGCTGCTCGACTGAAAATGC (21) GCATGCTGTCCCACATTTCAA (21)
SELT	57.9	58	TCAATCCCACACCATCGATCA (21) ACAACGAGCCTGCCAAGAAAG (21)
SELV	57.9	59	GTGGATTCGTCATTTCCCATG (21) TTTGAGTCTGACTGCCATCCC (21)
SEP15	59.8	59	ATCGGAGGCATGCAGAGAGTT(21) TCTGCAATCAGGATCCAGCTG (21)
SEPHS2	57.3	60	CGGCTCGCTTTTGTTCTGAA (20) TCGCGGCTTGTCAATGATC (19)
SELN	59.8	59	AGGCAGATGCTCATTGTTCCC (21) CCCCAAATCCAGATGCAGACT (21)
SEPX1	59.8	61	AGCGGCTGTTGCTCCATAACT (21) ATTTCAGCATCACCCACCCTC (21)
TrxR1	57.9	60	CACAATTGGAATCCACCCTGT (21) GGTTTGCAGTCTTGGCAACA (20)
TrxR2	57.9	62	AGGACATTTGCTGGTCGAAGC (21) GGAATCCCCTGGAAAAACGTT (21)
SEPP1	59.8	57	TAGGAGCTGATGCTGCCATTG (21) ATGTTCTCCTCTGCCCGAAGT (21)
SEPW1	59.8	60	GTTTATTGTGGCGCTTGAGGC (21) CCATCACTTCAAAGAACCCGG (21)

Table 1. *Parameters for RT-qPCR Analysis*

3.6 *Bioinformatics analysis for HepG2 and Huh7 Selenotrascrittoma*

Biological functional analyses were performed using David and Panther tools whereas human pathway lists and networks were determined by Ingenuity Pathway Analysis (IPA) program setting at 0.001 the significance threshold of t-test. IPA is a system that transforms large data sets into a group of relevant networks containing direct or indirect relationships between genes based on known interactions in the literature and in the experimental studies. In our studies only the "direct relationships" option was selected.

3.7 *Tissue samples HepG2 and Huh7 cells*

Paraffin-embedded HCC tissues obtained by biopsy of 20 patients were subjected to immunohistochemical staining. All patients in this study provided informed consent, and the study was approved by the Second University of Naples Ethics Committee. All patients had HCV-related cirrhosis with poorly differentiated tumor. No information related to follow-up data of these patients is known.

3.8 *Tissue immunohistochemistry*

Briefly, xylene dewaxed and alcohol rehydrated paraffin sections were placed in Coplin jars filled with a 0.01M tri-sodium citrate solution and microwaved. After heating, slides were thoroughly rinsed in cool running water for 5 min. Sections were immersed in 3% H_2O_2 at room temperature for 30 min to block any endogenous peroxidase activity. They were then washed in Tris-buffered saline (TBS) pH 7.4 before incubating at 4°C O.N with monoclonal rabbit anti-human SELM (LifeSpan BioSciences), polyclonal rabbit anti-human GPX4 (LifeSpan BioSciences) and monoclonal mouse anti-human GPX7 (LifeSpan BioSciences), diluted 1:50, 10µg/mL and 10µg/mL respectively. After incubation with the primary antibody, tissue sections were stained with specie-specific biotinylated secondary antibodies, followed by peroxidase labeled streptavidine (Dako, Denmark); the signal was developed by using diaminobenzidine (DAB) chromogen (Dako) as substrate. Incubations omitting the specific antibody were used as negative controls.

3.9 *Cell culture for two human breast cancer cell lines and human non-cancerous mammary epithelial cell line*

Two human breast cancer cell lines, estrogen-receptor (ER)-positive MCF-7 (HTB-22, adenocarcinoma) and ER-negative MDA-MB231 (HTB-26, adenocarcinoma) and the human non-cancerous mammary epithelial cell line, MCF-10A (CRL-10317, fibrocystic disease) were kept in culture. In details, MCF-7 and MCF-10A were expanded at 37°C in a humidified atmosphere of 5%

CO_2 in culture medium DMEM (Dulbecco's Modified Eagle's Medium High Glucose with sodium pyruvate, without L-Glutamine), whereas MDA-MB231 in RPMI 1640 (RPMI 1640 Medium without L-Gluamine with Phenol Red), supplemented with FBS at 10% (Foetal Bovine Serum, Standard Quality, Australian origin, PAA Cell Culture Company), with 1% of Penicillin/Streptomycin 100× (Euroclone, Devon, UK) and 1% of Glutamine 200mM (100×) (PAA Cell Culture Company). Moreover, in the case of MCF-10A, the DMEM was supplemented also with human insulin 10 µg/mL (Life Technologies Corporation, Carlsbad CA, 92008 USA), human epidermal growth factor 20 ng/mL (Life Technologies), and hydrocortisone 0.5 μg/mL (Sigma-Aldrich 3050 Spruce St. St. Louis, MO 63103) according to the procedure reported in Rothwell et al (*Rothwell et al. 2014*).

3.10 *RNA preparation and quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) analysis in MCF-7, MDA-MB231 and MCF-10A*

Total RNA was extracted from MCF-7, MDA-MB231 and MCF-10A cells using the RNAeasy mini kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The extracted RNA was dissolved in diethylpyrocarbonate treated water, and its concentration and purity were assessed by measurement of optical density at 260⁄280 nm, using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). 2µg of total RNA of each sample was reverse-transcribed with SuperScript VILO cDNA Synthesis kit (Life Technologies-Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and subsequently diluted 1:20 with nuclease-free water (Life Technologies-Ambion). Reverse-trascriptions were performed in a Veriti® Thermal Cycler (Thermo Fisher Scientific) by incubating the samples at 25°C for 10 minutes (min), followed by a step at 42°C for 60 min, 85°C for 5 min and the samples were then chilled to 4°C. The reverse-transcribed products were used to perform a qPCR in order to evaluate the expression level of transcripts of seleno proteins. Sequences for mRNAs from the nucleotide data bank (National Center for Biotechnology Information, USA) were used to design primer pairs for RT-qPCR (Primer Express, Applied Biosystems, CA, USA) (Table 1). Oligonucleotides were obtained from Sigma Aldrich. The efficiency of each primer pair was calculated according to standard method curves using the equation $E=10^{-1/slope}$. Five serial dilutions were set up to determine Ct values and reaction efficiencies for all primer pairs. Standard curves were generated for each oligonucleotide pair using Ct values *versus* the logarithm of each dilution factor. RT-qPCR assays were run on an 7900HT Fast Real-Time PCR System (Applied Biosystems). Starting with 2 µg of total RNA, we have preparated a 20-fold dilution of the resulting cDNA to achieve the concentration equivalent of starting with 100 ng of RNA (Life Technologies-Invitrogen), according

to the manufacturer's instructions. 10 ng of cDNA were amplified in a total volume of 25 µL containing 1X SYBR Green PCR Master Mix (Applied Biosystems) and 300 nM of forward and reverse primers. The thermal profile conditions were as follows: 5 min of denaturation at 95°C followed by 44 cycles at 95°C for 30 sec and 60°C for 1 min. We have addicted one cycle for melting curve analysis at 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability, all RT-qPCR reactions were carried out in triplicate. For all RT-qPCR experiments, the data from each cDNA samples were normalized using β-actin mRNA as endogenous level (*Peirce et al. 2001*). Sample ΔCt values were calculated as the difference between the means of selenoprotein markers Ct and housekeeping assay Ct from the same sample. We determinated $2^{\Delta\Delta Ct}$ values in order to define the fold change of selenoprotein expression levels in tumor cells compared to the non-cancerous cells, MCF-10A. These data were also confirmed by REST tool (Relative expression software tool, Weihenstephan, Germany) based on the Pfaffi method *(Pfaffi, 2001; Pfaffi et al. 2002).*

3.11 *Bioinformatics analysis*

Network analysis was performed by Ingenuity Pathway Analysis (IPA) program and using the same procedure reported in our recent paper *(Costantini et al. 2013).* In details, IPA builds and explores transcriptional networks to identify regulatory events that lead from signaling events to transcriptional effects.

3.12 *Interactomic Studies*

Specific undirect or direct physical interactions among the molecules evidenced in this work and, biochemically different in their structure, have been assessed also by an interactomic analysis. In general, interacting molecules form molecular interaction networks that are classified by the nature of the compounds involved.

4

RESULTS

4.1 EVALUATIONS OF THE SELENOTRANSCRIPTOME ON HEPG2, HUH7 AND NORMAL HEPATOCYTE CELLS

The gene expression profiles of HepG2, Huh7 and normal hepatocyte cells by means of RT-qPCR have shown that in the two HCC cell lines there were three downregulated genes (DIO1, DIO2, and SELO) (*fig.12 Panel A*) and ten upregulated genes (GPX4, GPX7, SELK, SELM, SELN, SELT, SELV, SEP15, SEPW1, and TrxR1) (*Fig.12 Panel A*). In detail, two of the three downregulated genes showed a statistically significant difference between HepG2 and Huh7 versus hepatocytes. On the other hand, in the group of the of the ten upregulated genes five of them (GPX4, GPX7, SELK, SELM, and SEP15) have shown a statistically significant upregulation in HepG2. All the other twelve selenotranscripts have appeared unchanged *(Fig.12 Panel A).*

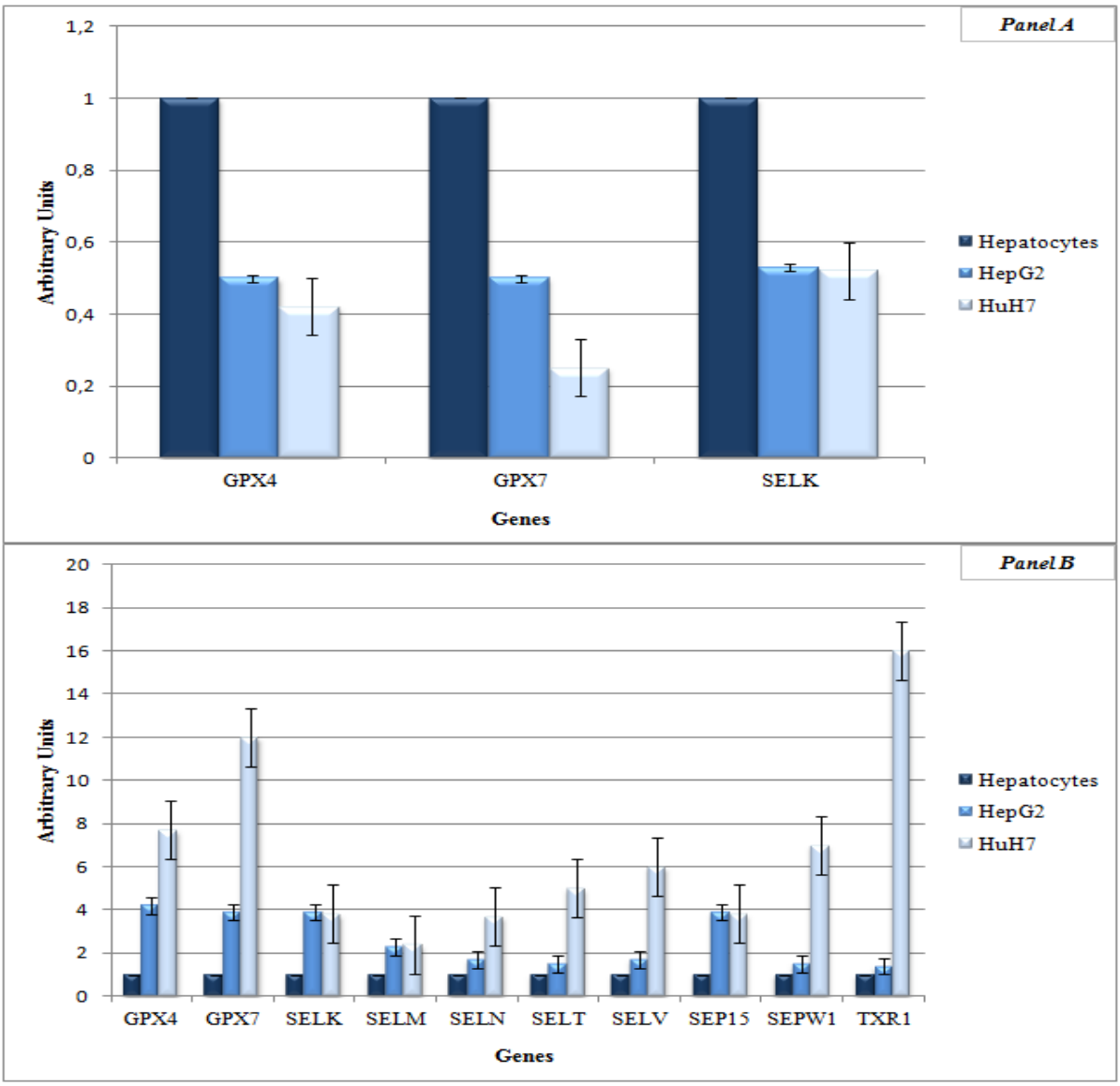

Figure 12. *Expression of Selenoprotein genes analyzed by means of RT-qPCR. The average value for the expression of these genes was obtained from three independent experiments.* ***Panel A*** *shows the down-regulated genes in HepG2 and Huh7 vs normal Hepatocytes (DIO1, DIO2, SELO) while* ***Panel B*** *the ten up-regulated genes (GPX4, GPX7, SELK, SELM, SELN, SELT, SELV, SEP15, SEPW1, TrxR1). In each reaction, the expression levels were normalized to the average of the control gene (18S rRNA) and expressed as arbitrary units. The mRNA levels in hepatocytes and HepG2 were evaluated by using the ΔΔCt method. Significant differences in relative gene expression between hepatocytes and HepG2 or Huh7 are marked by *(p-value<0.05) and ** (p-value<0.01).*

Biological functional analysis by *David*, *Panther* and *Ingenuity Pathway* (IPA) algorithms evidences that two of down-regulated genes, DIO1 and DIO2, are implicated in metabolic processes, and, in particular, DIO1 in drug metabolism, and DIO2 in small molecule biochemistry and amino acid metabolism. The analysis of top-canonical pathways shows that DIO1 and DIO2 are involved in thyronamine and iodothyronamine metabolism, thyroid hormone metabolism I (via

deiodination), thyroid hormone metabolism II (via conjugation and/or degradation) and TR/RXR activation (*Table 2*).

METABOLIC PROCESSES	PANTHER	INGENUITY PATHWAY
Metabolic process	DIO1, DIO2	
Thyronamina and Iodothyronamine metabolism	DIO1, DIO2	
Thyroid Hormone Metabolism I (Via Deiodination I)	DIO1, DIO2	
Thyroid Hormone Metabolism II (Via Deiodination II)	DIO1, DIO2	
TR/RxR Activation	DIO1, DIO2	

Table 2. *Biological functional analysis by David, Panther and IPA algorithms and metabolic processes in which down seleno-genes are involved.*

Functional analysis using the algorithms of David and Panther, shows four of the up-regulated genes (GPX4, GPX7, SELT, and TrxR1) involved into metabolic process as well as DIO1 and DIO2. In particular, GPX4 and GPX7 are involved in glutathione and arachidonate metabolisms, and in the response to stimulus of the immune system process. Concerning the analysis of the molecular and cellular functions by IPA, it has been highlighted that: ***a***) GPX4 and TrxR1 are involved in small compound biochemistry, ***b***) TrxR1 in amino acid metabolism, ***c***) GPX4 in lipid and drug metabolisms as well as DIO1. Moreover IPA analysis evidences that two of the up-regulated genes are associated to the following canonical pathways (*Table 3*): GPX4 in glutathione redox reactions I and mitochondrial dysfunction, while TrxR1 in thioredoxin pathway, vitamin C transport, antioxidant action of vitamin C and NRF2-mediated oxidative stress response.

METABOLIC PROCESSES	DAVID	PANTHER	INGENUITY PATHWAY
Response to stimolous	GPX4, GPX7		
Generation of precurson metabolities and energy	TrxR1		
Immune system process	GPX4, GPX7, TrxR1		
Metabolic process	GPX4, GPX7, TrxR1, SelT		
Glutathione metabolism		GPX4, GPX7	
Arachidonic acid metabolism		GPX4, GPX7	
Glutathione redox reaction I			GPX4, GPX7
Thireodoxine pathway			TrxR1
Mitochondrial dysfunction			GPX4, GPX7
Vitamin-C transport			TrxR1
Antioxidant action of Vitamin-C			TrxR1
NRF2-mediated oxidative stress response			TrxR1

Table 3. *Biological functional analysis by David, Panther and IPA algorithms and metabolic processes in which up-regulated seleno-genes are involved.*

4.1.1 *Network analysis for selenotranscriptome on HCC cells*

It has been used the IPA algorithm to study the correlation between down- and up-regulated genes. *Figure 13* shows that three down-regulated (DIO1, DIO2, and SELO) and eight up-regulated (GPX4, SELK, SELT, SELV, SEP15,–SEPN1, SEPW1, TrxR1) genes are connected in the same network named *"Amino acid metabolism, protein synthesis and small molecule biochemistry"* that presents some nodes that bind to our selenoprotein mRNAs: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (SMARCA4), specificity protein 1), SECISBP2 (sec insertion sequence binding protein (SP1), nuclear receptor co-repressor 2 (NCOR2) and (Transducin Beta-Like Protein 1X TBL1X). In details, ***a***) SECISBP2 interacts with the up-regulated SELO, SELK, SELV, SEP15, GPX4, SELN (SEPN1), SELT, SEPW1, while TrxR1 interacts with SMARCA4 and SP1, ***b***) SP1 with diverse genes among which it is important to highlight TrxR1 and SMARCA4, ***c***) SMARCA4 with SP1 and TrxR1 and finally ***d***) NCOR2 and TBL1X with DIO1.

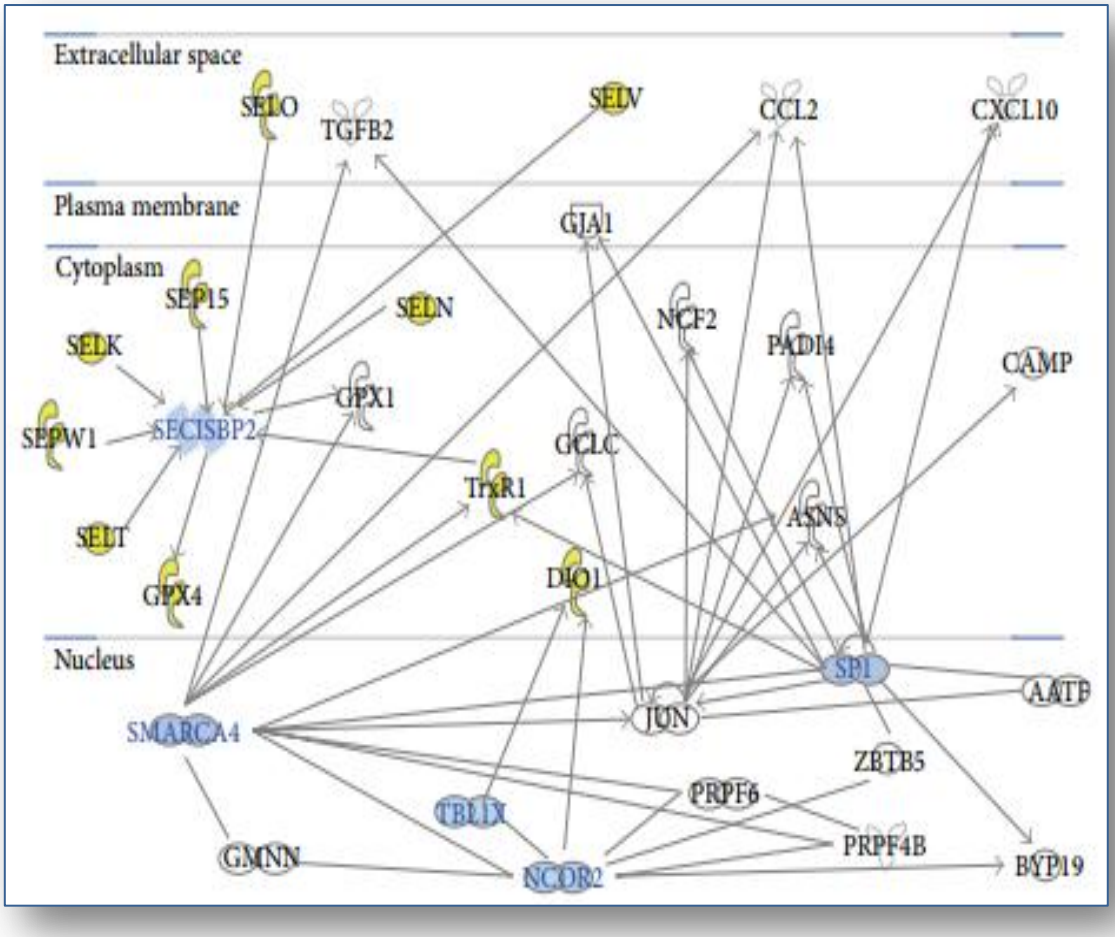

Figure 13. *Network analysis: down- and upregulated genes are evidenced by yellow symbols, HUB nodes by cyan symbols, whereas all other genes by white symbols.*

4.1.2 *Validation of the up-expression of SELM, GPX4 and GPX7 by immunohistochemistry*

Twenty biopsy tissues from HCC patients were subjected to SELM, GPX4 and GPX7 staining and the expression of these genes in liver cancer cells were compared to their related cirrhotic counterparts used as control. In details, the HCC tissues have shown to be positively stained for SELM (*fig.14*) while in the cirrhotic area the staining was negative. On the other hand, in the case of GPX4 and GPX7 (*fig.15*), the staining of hepatocytes shows light positivity in cirrhotic tissues and strong positivity in the cancerous area. Therefore, these evaluations have highlighted that the increase of SELM, GPX4 and GPX7 in HCC tissues is in excellent agreement with the increased levels of mRNA expression in HepG2 and Huh7 cells compared to normal hepatocytes.

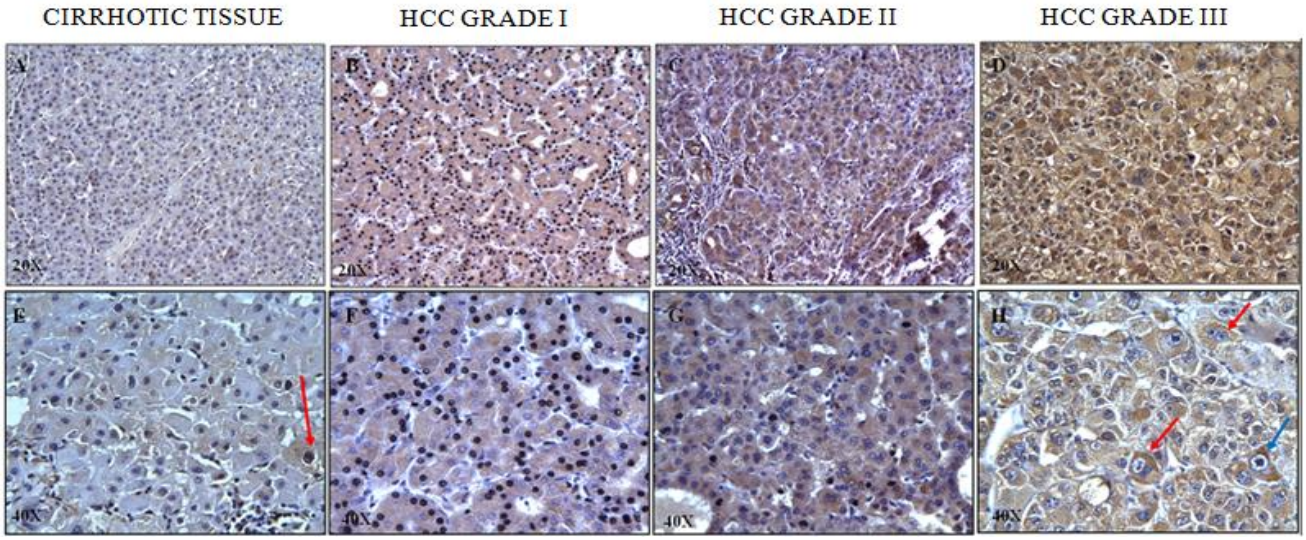

Figure 14. *Immunohistochemical observations of SELM expression at 20X (the first line) and 40X (the second line) in HCC tissues with different malignity grades (I, II and III grade) in comparison with cirrhotic tissue (used as control)*

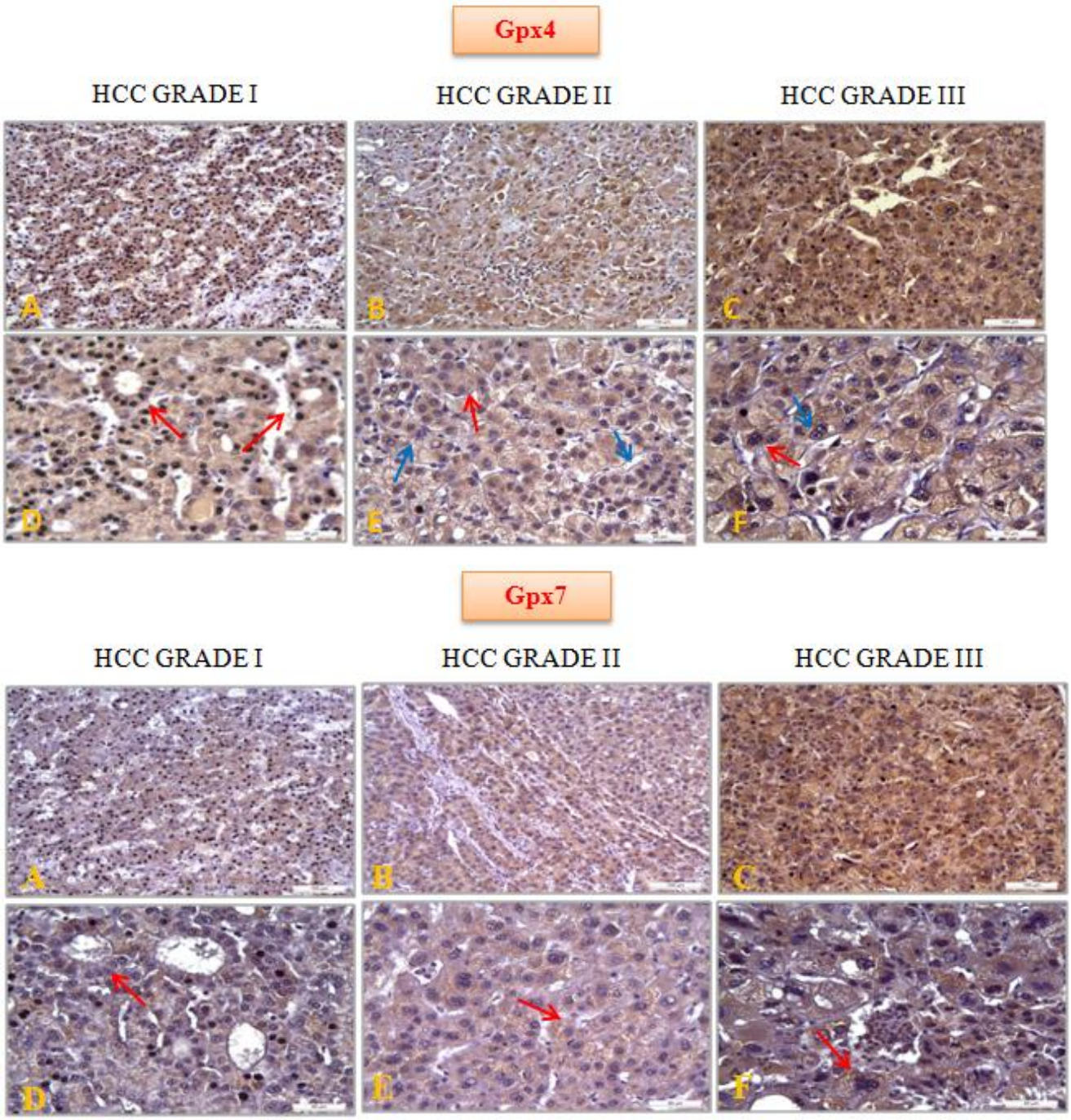

Figure 15. *Immunohistochemical observations of Gpx4 and Gpx7 expressions at 20X (the first line) and 40X (the second line) in HCC tissues with different malignity grades (I, II and III grade)*

4.2 EVALUATION OF THE SODIUM SELENITE EFFECT ON HCC CELLS

4.2.1 *SELENBP1, SELK and GPX1 Expression in HepG2 and Huh7 Cells after stimulation with sodium selenite*

It has been evaluated the SELENBP1, SELK and GPX1 protein expression in HepG2 and Huh7 after 24 h stimulation with different concentrations of sodium selenite (0.25 μM, 0.5 μM and 1 μM) compared to untreated cells. In untreated HepG2 and Huh7 cells SELENBP1 is not expressed (f*ig.16*).

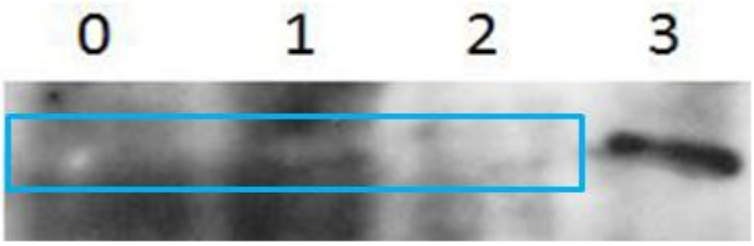

Figure 16. *SELENBP1 protein expression analysis by western blot in the untreated HepG2 cells (0) [light blue box], in HepG2 cells after 24hrs of treatment with sodium selenite at the indicated doses, 0.25 mM (1) and 1 mM (2) [light blue box] and in untreated HeLa cells (3)*

It has been used, as positive control for anti SELENBP1, antibody HeLa cells, known to express high levels of this protein. Instead, SELK and GPX1 are expressed in the untreated HepG2 and Huh7 cells but the expression of SELK is less than that of GPX1 *(Fig.17)*. This is in agreement with the gene expression analysis which evidenced that SELK was found down-regulated in HepG2 when compared to normal hepatocytes, whereas the expression of GPX1 was similar in normal and cancerous cells (*Fig.18*). Moreover the treatment with sodium selenite in both cell lines produces an increase in the 11 KDa SELK as well as 23 KDa GPX1 protein expression already at low doses of sodium selenite and levels of both proteins were found constant, independently from used concentrations (*Fig.17*). In the meantime, the 40 KDa SELENBP1 protein was not appreciable.

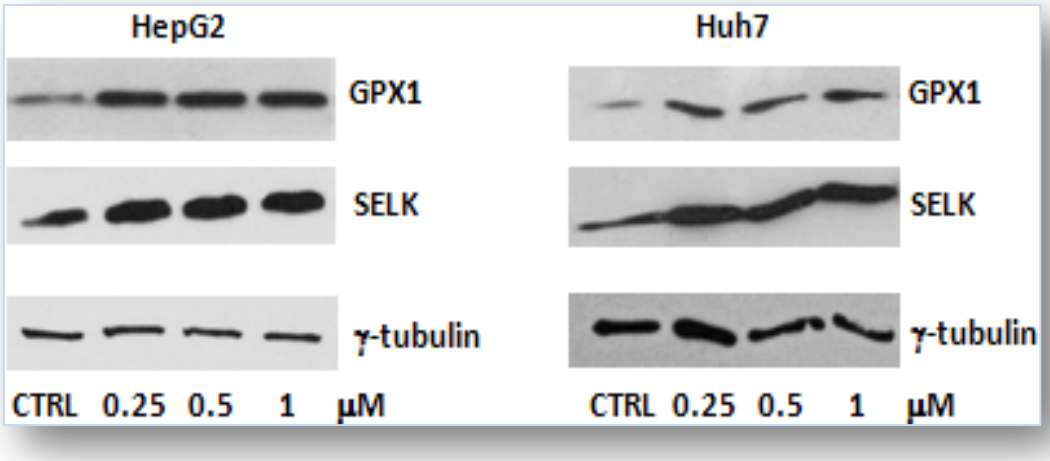

Figure 17. *SELK and GPX1 protein expression analysis by Western blot in the HepG2 and Huh7 cell lines after 24 h of treatment with sodium selenite at the indicated doses. γ-tubulin was used as loading control*

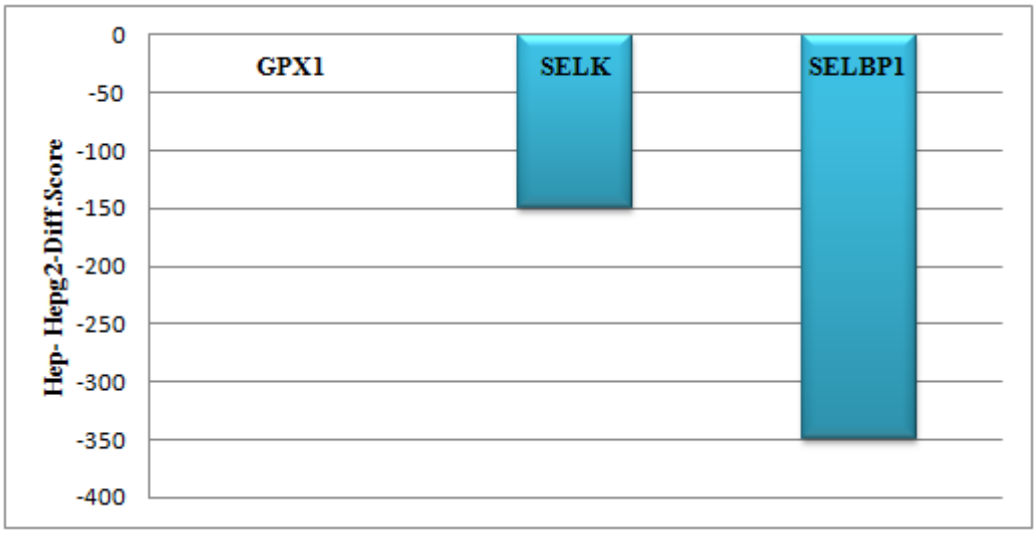

Figure 18. *Difference score (Diff. score) between hepatocytes and HepG2 cells*

4.2.2 *Evaluation of Selenium Concentrations*

Since SELK and GPX1 protein expression increase already at low doses of selenite and, then, remained constant, it has been evaluated, by atomic absorption spectrometry, the concentration of selenium in the same protein extracts from HepG2 and Huh7 after stimulation with different concentrations of selenite compared to untreated cells in order to understand if the cells absorbed only the selenium present in SELK and GPX1 or also other amounts. As shown in *figure 19*, the selenium concentration increases with increasing sodium selenite concentrations (with $p < 0.05$) in both cell lines even if in different amount and through different trend.

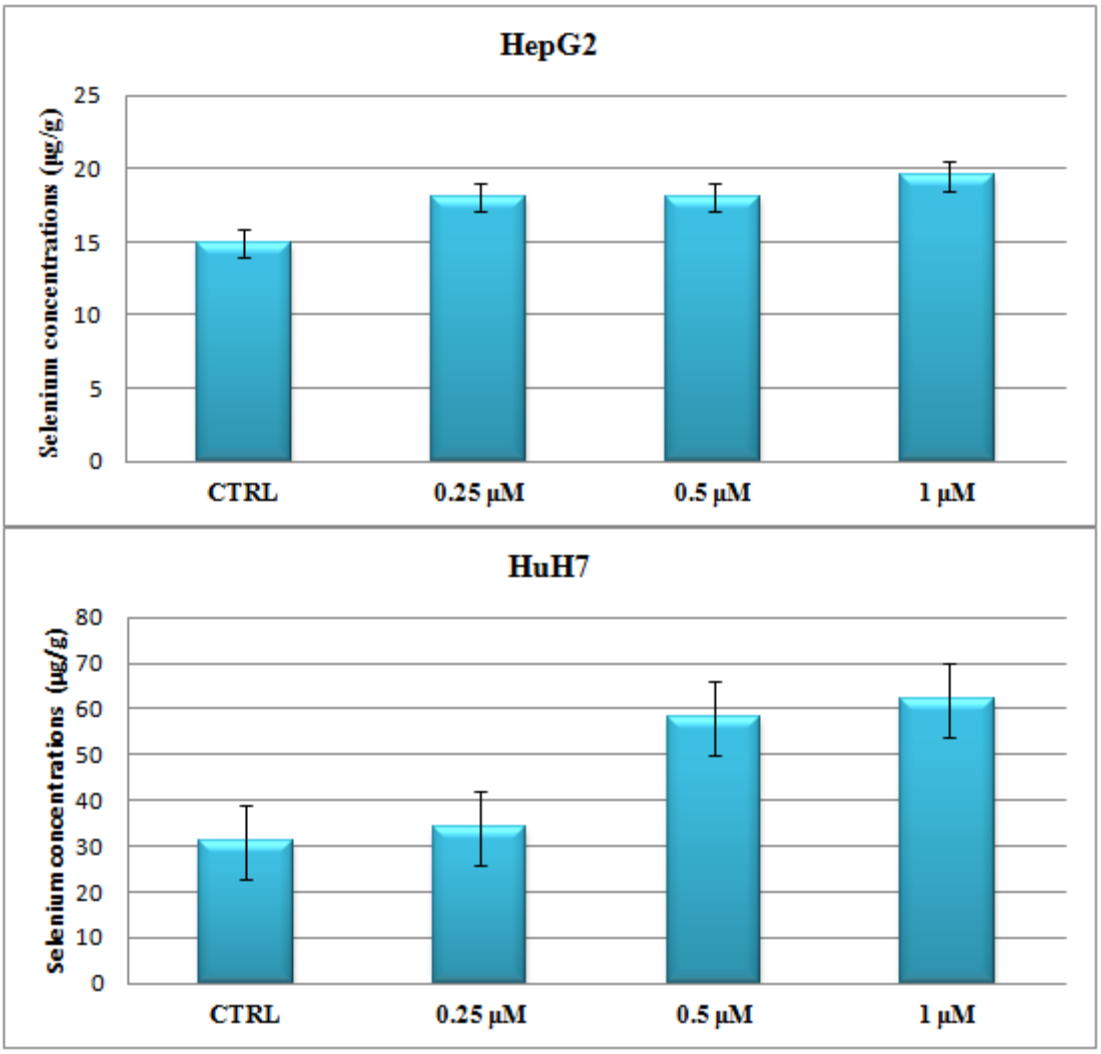

Figure 19. *Selenium concentrations (µg/g) in protein extracts of HepG2 and Huh7 after 24 h of treatment with sodium selenite at the indicated doses*

4.2.3 *Bio-Plex assay on HepG2 and Huh7 cells*

Since in HCC the constant inflammation resulted to play an important role in the transition from chronic liver disease to neoplastic process, we also studied the immuno-modulatory role of the selenite on the cytokines production in cellular hepatocarcinoma supernatants by a multiplex biometric ELISA-based immunoassay. In details, it has been evaluated the cytokine levels in HepG2 and Huh7 supernatants after incubation with sodium selenite at 24 h. The obtained results were compared with untreated cells used as control. These experiments showed that the levels of VEGF and three pro-inflammatory interleukins, like IL-6, IL-8, and IL-17, decreased in statistically significant way at increasing concentrations of sodium selenite *(Fig.20)*. Studies on HCC patients, conducted in our laboratory, showed that high levels of IL-8 and IL-6 correlated with tumor size suggesting that these two proteins could have a role during the HCC progression and can be considered as markers of tumor invasiveness.

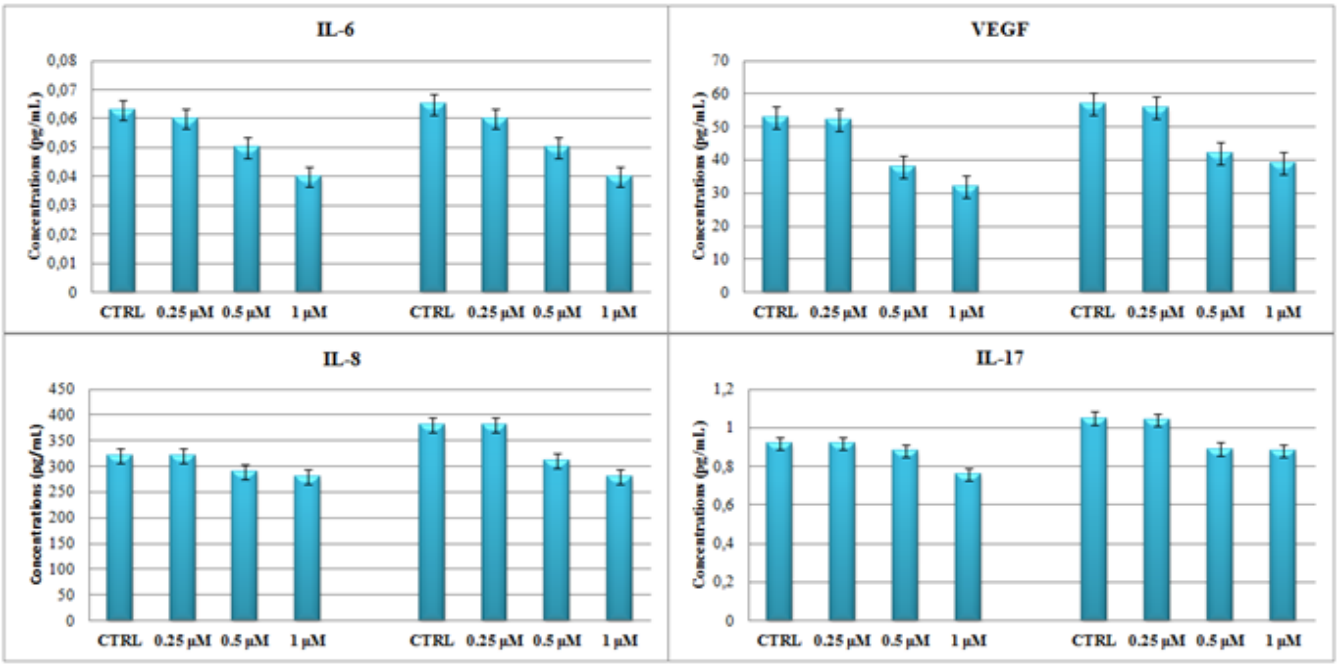

Figure 20. *Significant cytokine levels (with p < 0.05) in HepG2 and Huh7 cells line after 24h of treatment with sodium selenite*

4.2.4 *Interactomic Studies*

Specific undirect or direct physical interactions among the molecules, biochemically different in their structure, have been assessed also by an interactomic analysis. In general, interacting molecules form molecular interaction networks that are classified by the nature of the compounds involved. The four significant cytokines and the two seleno-proteins, SELK and GPX1, were analysed by *Ingenuity Pathway Analysis* 7.1 (Ingenuity Systems, Inc., Redwood City, CA, USA). The network is generated by associated functions and data mining from experimental data reported in literature and these molecules have been found involved into a network named ―*Tissue Development, Gene expression, Cell Death and Survival*‖ where IL-6, IL8, IL-17, VEGF and GPX1 are connected with two hub genes, correlated between them such as SMARCA4 and encoding chromatin remodeling complex components and *tumor protein 53* (TP53) that responds to different types of cellular stresses to regulate target genes by inducing cell cycle arrest, apoptosis, senescence, DNA repair, or metabolic changes (*fig.21*).

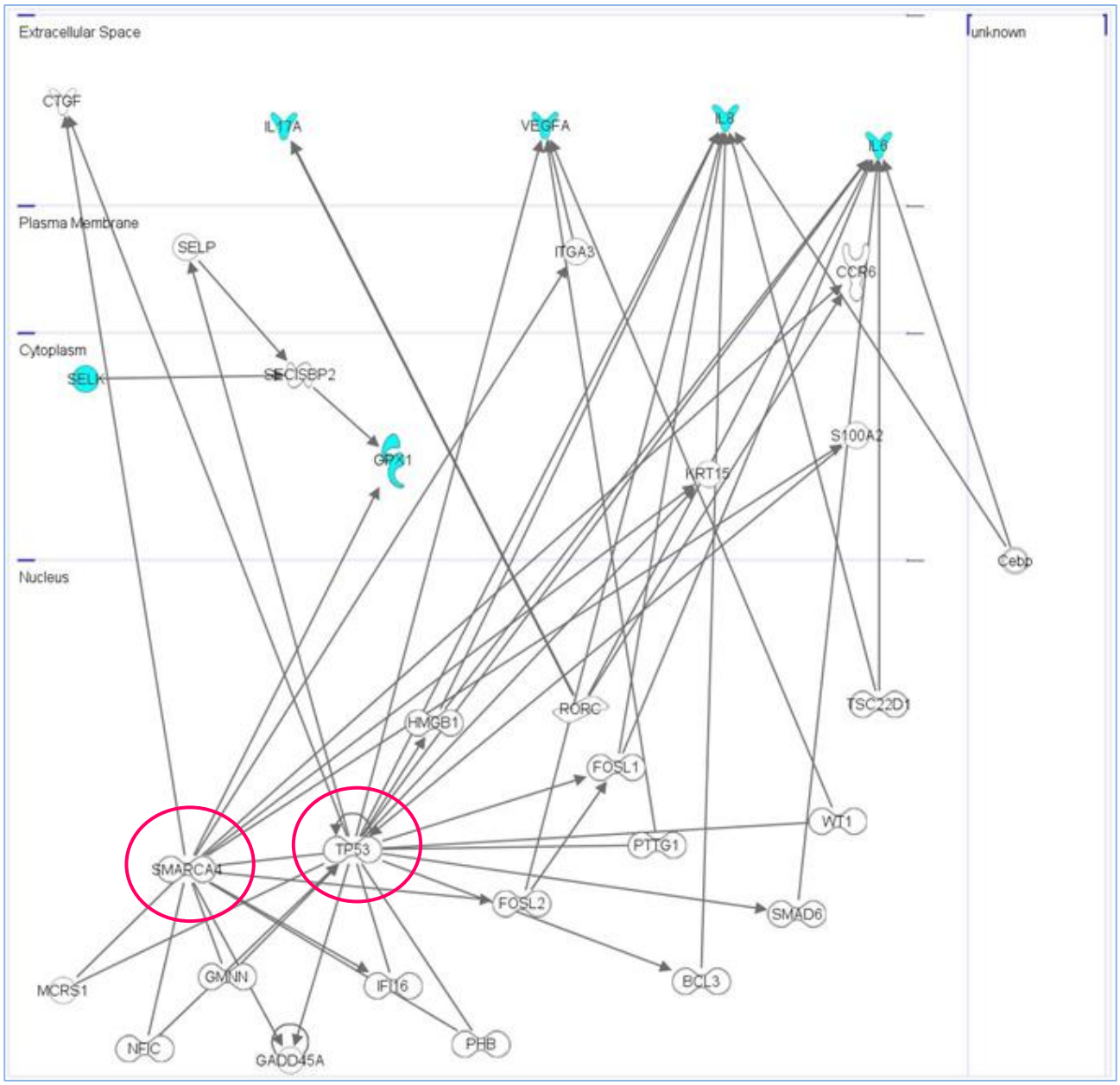

Figure 21. *Interactomic analysis by Ingenuity Pathway Analysis (IPA) of significant molecules. The interactome shows the close functional association between SELK, GPX1 and significant cytokines, (evidenced with cyan symbols) as well as the paths in which other functionally relevant molecules are also involved (evidenced with white symbols).*

4.3 EVALUATION OF THE SELENOTRANSCRIPTOME ON MCF-7, MDA-MB231 AND HUMAN NON-CANCEROUS BREAST CELL LINE (MCF-10A)

The gene expression profiles of MCF-7, MDA-MB231 in comparison with non-cancerous breast cells MCF-10A by means of RT-qPCR (*Fig.20*) have shown: ***a***) four down-regulated (GPX1, GPX4, GPX5 and GPX7) and eight up-regulated (DIO1, DIO2, DIO3, SELT, SEPW1, SEPX1, GPX2 and GPX3) genes in MCF-7 cells and ***b***) five down-regulated (GPX1, GPX4, GPX5, GPX6 and GPX7) and four up-regulated (DIO2, SEP15, GPX2 and GPX3) genes in MDA-MB231cells. A 1 x-fold expression level was chosen as the threshold for significance of selenoprotein expression levels. Moreover, the comparison between the two breast cancer cell lines has evidenced that four down-regulated (GPX1, GPX4, GPX5 and GPX7) and three up-regulated (DIO2, GPX2 and GPX3) genes are in common (*Fig.21*).

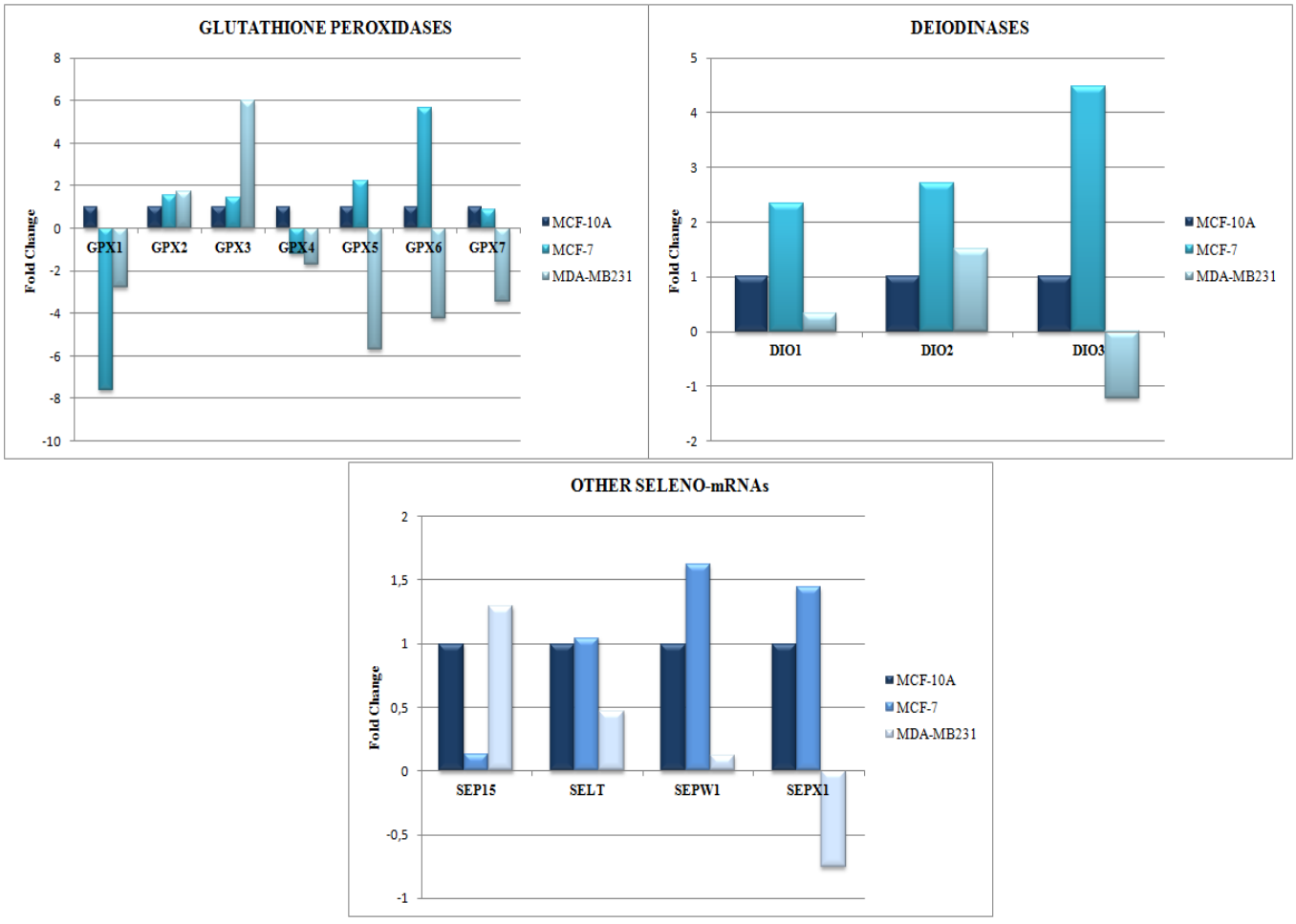

Figure 21. *The histograms show the differences in the expression levels followed by RT-qPCR of (**A**) diodinase, (**B**) other seleno-mRNAs and (**C**) glutathione peroxidases in MCF-7 (in light grey) and MDA-MB231 (in dark grey) compared to MCF-10A cells. Fold changes greater than ±1 are considered significant*

4.3.1 *Network Analysis for MCF-7, MDA-MB231 and MCF-10A selenotranscriptome*

To evaluate the ability of these down- and up-regulated genes to interact among them as well as to identify putative HUB nodes able to exercise a direct control over them, it has been used the IPA algorithm to study these correlations. *Figure 22* shows that all the differentially regulated genes, with the unique exception of SELT, are implicated in the same network that presents three HUB nodes interconnected to our selenoprotein mRNAs: i.e., TP53, *ESR1* (estrogen receptor 1) and *CTNNB1* (Catenin beta 1).

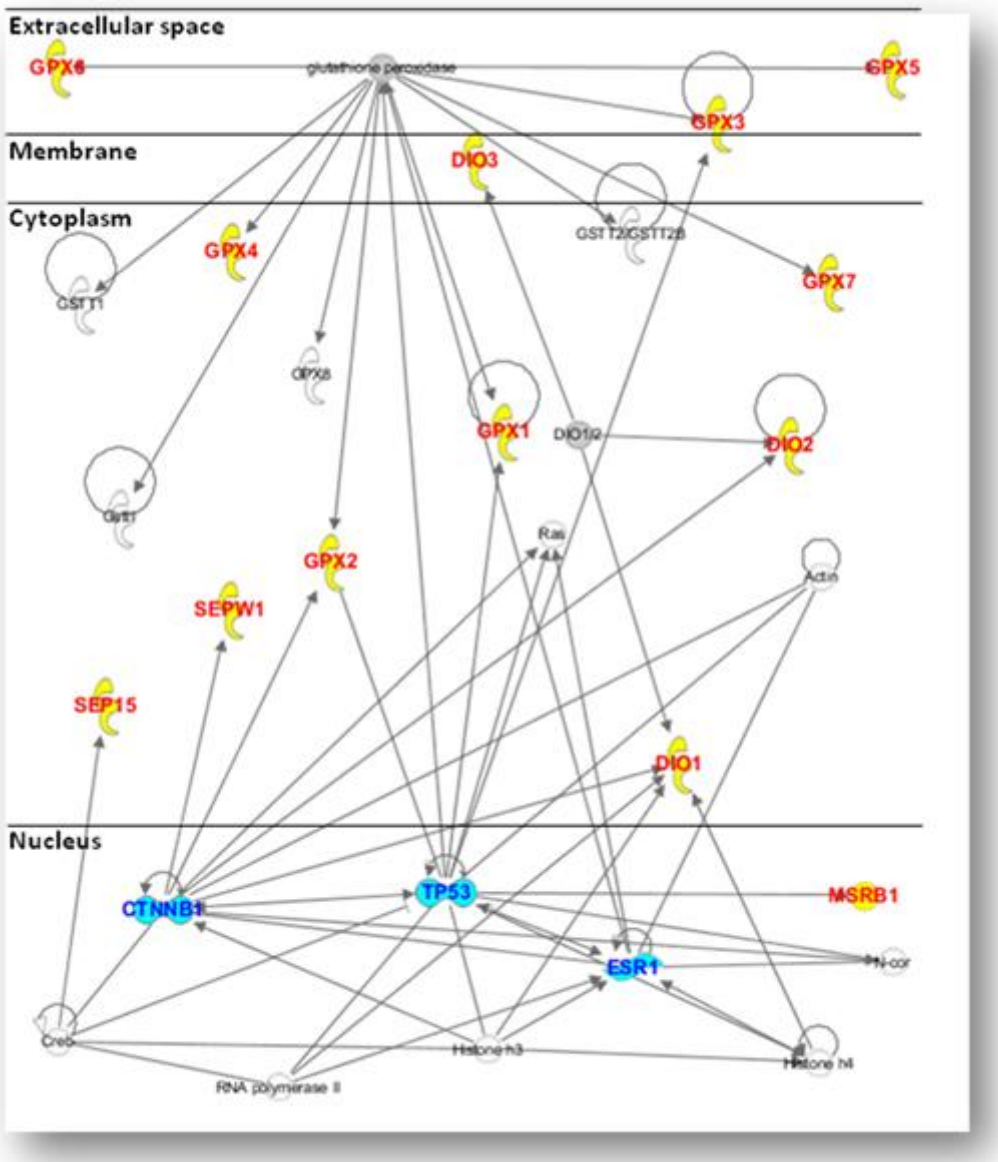

Figure 22. *Network analysis: down- and up-regulated genes are evidenced by yellow symbols, HUB genes by cyan symbols whereas all other genes by white symbols*

5

DISCUSSIONS

The strong association between etiologic agents, chronic liver disease (hepatitis and cirrhosis), progression to *hepatocellular carcinoma* (HCC) and individuals with known risk factors allowed the identification of a possible strategy in a several of chemopreventive agents in lowering the current morbidity and mortality associated with HCC *(Castello et al. 2010)*. In particular, from some studies, conducted both in vivo and in vitro, selenium emerged as a promising molecule that inhibits carcinogenesis with a pleiotropic mode of action affecting cellular proliferation and growth, apoptosis, inflammation, invasion, angiogenesis and metastasis by a complex system that involves the expression of a wide range of selenium-containing proteins *(Huang et al. 2012)*. The gene expression profiles of Hetocellular carcinoma cell lines, HepG2, Huh7, and normal hepatocyte cells by means of RT-qPCR have shown that in the two HCC cell lines there were three down regulated genes (DIO1, DIO2 and SELO) and ten up regulated genes (GPX4, GPX7, SELK, SELM, SELN, SELT, SELV, SEP15, SEPW1, and TrxR1). Two of the three down regulated genes showed a statistically significant difference between HepG2 and Huh7 versus hepatocytes, while GPX4, GPX7, SELK, SELM, and SEP15 have shown a statistically significant up regulation in HepG2 compared to HuH7 *(Guariniello et al. 2015)*. It is important to underline that differences in the gene expression found for HepG2 and Huh7 could be due to differences between these two liver cancer cell lines. An important distinction element between two hepatoma lines is in p53 gene. HepG2 cells carry wild type p53, whereas Huh7 cells show a high level of p53 with a point mutation at the third position of codon 249^{ser} resulting in a G:C to T:A transversion and are characterized by a more malignant phenotype. Consequently, this means that Huh7 cells are more aggressive than HepG2 and present a more enhanced inflammatory status *(Hussain et al. 2007)*. Selenoprotein W (SEPW1) is a highly conserved small thioredoxin-like protein required for cell cycle progression. Hawkes et al. had demonstrated that in breast cancer cells (MCF-7) SEPW1 controlled p21 by modulating levels of the p53 transcription factor *(Hawkes et al. 2011)*. Furthermore, p53 was increased in cells growth-arrested by siRNA-mediated SEPW1 silencing resulting that p53 was inversely related to SEPW1 mRNA in cell lines stably over-expressing or under-expressing SEPW1 (Hawkes et al. 2012). Moreover, SEPW1 presents a thioredoxin like domain as well as SELN, SELT, SELV, and TrxR1 and all these five selenoproteins play an important role in the regulation of the redox signal. Therefore, this can explain why the Huh7 cells showed a higher level of these five genes compared

to HepG2 cells *(Guariniello et al. 2015)*. Correlation study between down- and up regulated genes by IPA 7.1 had associated three down regulated (DIO1, DIO2 and SELO) and eight up regulated (GPX4, SELK, SELT, SELV, SEP15, SELN, SEPW1, and TrxR1) genes in the same network named "amino acid metabolism, protein synthesis, and small molecule biochemistry" that presents some nodes (HUB nodes) that bind to selenoprotein mRNAs: *SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4* (SMARCA4), *specificity protein 1* (SP1), *sec insertion sequence binding protein* (SECISBP2), *nuclear receptor corepressor 2* (NCOR2) and *transducin beta-like protein 1X* (TBL1X) *(Guariniello et al. 2015)*. SWI/SNF complexes are evolutionarily conserved multi-subunit complexes that utilize the energy of ATP hydrolysis to mobilize nucleosomes and remodel chromatin. These approximately 2 MDa complexes are made up of 12–15 subunits; they contain one of the two catalytic ATPase subunits, SMARCA4/BRG1 or SMARCA2/BRM, several core subunits including SMARCB1/SNF5/INI1/BAF47 and SMARCC1/BAF155 that are present in all SWI/SNF complexes *(Helming et al. 2014)*. SMARCA4 interacts with a diverse group of nuclear proteins involved in various cellular processes including transcriptional regulation, cell cycle control, proliferation, DNA repair and recombination *(Shilpa et al. 2000)*. At least eight genes encoding subunits of SWI/SNF complexes have been identified as recurrently mutated in 20% of all human cancers *(Helming et al. 2014)*. In particular, SMARCA4 subunit proposed to be a tumor suppressor via diverse biological mechanisms, often, presents inactivating mutations in a variety of tumor cell lines (Wong *et al. 2000)*. Reintroduction of SMARCA4 into SMARCA4-deficient tumor cells resulted in Rb-dependent cell cycle arrest and a flattened morphology, suggesting SMARCA4 may function as a tumor suppressor. *SMARCA4* heterozygotes are predisposed to differentiated epithelial tumors, which supports that SMARCA4 plays an important role in the regulation of cellular proliferation. In addition, SMARCA4 has shown to involve in tumor suppression by physical interaction with other tumor suppressors including pRb, p53, and c-Myc. Furthermore, *SMARCA4* has also been found under expressed in human hepatocellular carcinoma while it has been seen that *SMARCA4* rs11879293 A allele presented a significantly decreased risk of HCC compared with rs11879293 G allele in both the detection and validation stages *(Zhong et al. 2014)*. Thus, the pivotal role of SMARCA4 in the selenonetwork as well as its propensity to mutate suggest that the progression of HCC might be much more complex and elusive than believed, and, hence, future researches need to unravel this important point *(Guariniello et al. 2015)*. SP1 recruits SMARCA4 subunit on the c-FOS promoter and acts in synergy with other transcription factors such as NF-kB and RELA subunit, probably, regulating the inflammation. Moreover, in the literature it is reported that SP1 up expression leads to p53-dependent apoptosis in

cancer cells evidencing the correlation between SP1 and p53. This finding evidences the linking between the seleno-mRNAs, the HUB genes, and p53, and, hence, this findings can explain because it has been a different expression of some seleno-mRNAs between HepG2 and Huh7 cells that have wild-type and mutated p53, respectively *(Guariniello et al. 2015).* The nuclear receptor corepressor (N-CoR) and the silencing mediator for retinoic and thyroid receptors (SMRT), referred to also NcoR2, were identified initially as corepressors for nuclear receptors such as thyroid hormone receptors (TRs) and retinoic acid receptors. Have been identified two additional N-CoR/ SMRT-associated proteins, GPS2, a protein involved in intracellular signaling, and transducin-like 1 receptor (TBLR1). TBL1/TBLR1, complexed with SMRT and N-CoR, stabilizing the quaternary structure of the corepressor assemblage through additional contacts with HDAC3 and bind to histones H2B and H4 to assist in chromatin substrate recognition *(Choi et al. 2008).* Therefore, these data suggest that it is the binding of DIO1 to NCOR2 and TBL1X to raise its down expression. DIO2, together with DIO1, catalyzes the removal of an iodine residue from the prohormone thyroxine (T4), producing either the active form triiodothyronine (T3; activation) or the inactive metabolites (reverse T3; inactivation). It is also reported that DIO2, as well as DIO1, is downexpressed in nearly all papillary thyroid carcinomas and in the liver carcinoma when compared with normal tissue. The involvement of this pathway in HepG2 and Huh7 cell lines is in accord with a recent paper where it has been reported that the thyroid hormone receptors promote metastasis of the human hepatoma cells and that the disruption of the cellular thyroid hormone signaling triggers chronic liver diseases, including alcoholic or nonalcoholic fatty liver disease and HCC *(Arnaldi et al. 2005).* So, the attention has been focused on selenoproteins whose levels were found reduced in according to the grade of the HCC *(Raucci et al. 20111 and Di Stasio et al. 2011).* In details, *selenium binding protein-1* (SELENBP1), expressed in various cell types, such as liver, heart and kidney, was found down regulated in a vast number of human cancers, such as colorectal cancer, lung adenocarcinomas, ovarian cancer, gastric cancer and HCC, underlying a solid connection between SBP1 and cancer *(Huang et al. 2012).* In tissue samples of HCC patients the studies provided evidence that this protein, as well as selenium, is down-regulated in the liver tissue of HCC patients and that its gradual loss is associated with an increased malignant grade *(Castello et al. 2010).* Glutathione peroxidase 1 (*GPX1*) is also an important selenium-containing protein *(Huang et al. 2012)* present as selenocysteine. The literature observations related to the levels of GPX1 are contradictory because some papers emphasized that GPX1 levels are increased in HCC patients, but others stated that they were reduced according to the grade of the HCC *(Castello et al. 2010).* Indeed, allelic loss at the GPX1 locus is a common event in cancer development but in vitro data indicate that reduced GPX1 expression can sensitize mammalian cells to DNA damage and that

over expression can have the opposite effect, supporting a role for GPX1 levels in determining cancer risk *(Fang et al. 2010)*. Coimmunoprecipitation exeperiments showed that GPX1 and SBP1 form a physical association that facilitates their interactions and although it is known that SBP1 binds *hypoxia response element in its promoter region* (HIF1α), their possible roles in cancer development are still unknown. It is probably that decreased SBP1 could promote HCC invasion/metastasis and lead to poor prognosis through the enhancement of GPX1 activity and the downregulation of HIF-1a *(Huang et al. 2012).* Moreover, *selenoprotein K* (SelK), biological function of which has been little characterized, is a novel endoplasmic reticulum (ER) resident protein, involved in the cellular redox state control and signaling as well in the control of oxidative stress, ER calcium flux through endoplasmic reticulum, and *ER-associated protein degradation* (ERAD) pathway together p97(VCP) and *selenoprotein S* (SelS), moving out the misfolded proteins from the ER to the cytosol *(Potenza et al. 2016).* Although no detailed information is present in the literature about its involvement in cancer, a recent study showed that in HepG2 cells SelK was regulated by the two ER stress agents, tunicamycin and β-mercaptoethanol and its gene silencing could significantly aggravate HepG2 cell death and apoptosis induced by the ER stress agent. This suggests that SelK plays an important role in protecting HepG2 cells from ER stress agent-induced apoptosis *(Du et al.2010).* Furthermore, in *Rusolo et al. 2013*, it has been underlying SELK expression was correlated with the increase of the sodium selenite concentration in two HCC cell lines, HepG2 and HuH-7 *(Rusolo et al. 2013)*. Furthermore, several studies have demonstrated that miRNAs are involved in human cancer, such as lung, breast, brain, liver, colorectal cancer, and leukemia. By targeting different genes in tumor development, miRNAs can function as either oncogenes or tumor suppressor *(Di Leva et al. 2014)*. Since it has been seen that SELK was induced by sodium selenite *(Rusolo et al. 2013)*, Potenza et al. have also investigated whether some miRNAs was responsive to selenium, finding that both miR-544a and SELK expression were conversely regulated by sodium selenite. In particular, after the treatment of HCC cell lines with sodium selenite, the gene expression trend of SELK was towards increasing, while those of miR-544a towards a decrease, thus suggesting that the selenium is able to block the ability of miR-544a to negatively regulate the SELK expression and have a role in metabolic regulation exerted by micronutrients *(Potenza et al. 2016)*. GPX1, SELK and SELENBP1 up expression, observed after sodium selenite treatment, triggered effects on HepG2 and HuH7 cell cytokinome (*Rusolo et al. 2013*). These experiments, performed by a multiplex biometric ELISA-based immunoassay, showed that the levels of VEGF and three pro-inflammatory interleukins, like IL-6, IL-8, and IL-17, decreased in statistically significant way at increasing concentrations of sodium selenite. Studies on HCC patients, conducted in C.R.O.M's *Molecular Immunology and*

Immunomonitoring Laboratory, showed that high levels of IL-8 and IL-6 correlated with tumor size suggesting that these two proteins could have a role during the HCC progression and can be considered as markers of tumor invasiveness (*Rusolo et al. 2013*). In particular, in HCC, IL-17+ T cells have been found in increased numbers within tumors and correlate with poor survival and increased postoperative recurrence, indicating that Th17 cells and IL-17 may promote tumor progression in HCC. It achieves its effects through IL-6 induction and subsequent signal transducer and activator of transcription 3 (STAT3) activation. IL-6 and other members of the IL-6 family of cytokines, including IL-11, in activating the JAK-STAT3 pathway leading to cancer-promoting inflammation. Furthermore, IL-17 up-regulate the production of proinvasive factors in HCC cells, including IL-8, MMP2, and VEGF, master regulators of cell dissemination, proliferation and angiogenesis and promoting through STAT3 phosphorylation, neutrophil infiltration and increased tumor vascularity *(Gu FM et al. 2011).* IL-6, IL-8, IL-17 and VEGF, down regulated after sodium selenite treatment, not only evidences that four interleukins are certainly correlated between them, but they have been found involved in the same network named ―*Tissue Development, Gene expression, Cell Death and Survival*― afte following the analysis by Ingenuity Pathway Analysis 7.1 *(Rusolo et al. 2013).* IL-6, IL8, IL-17, VEGF and GPX1 are connected with two hub genes, correlated between them such as SMARCA4 and encoding chromatin remodeling complex components and *tumor p53* (TP53) that responds to different types of cellular stresses to regulate target genes by inducing cell cycle arrest, apoptosis, senescence, DNA repair, or metabolic changes. Their related proteins are known to create a protein complex. In details, SMARCA4 is connected with GPX1 and VEGF via *integrin alpha3* (ITGA3) whereas TP53 with VEGF, IL-6, IL-8 and SELP that, together with SELK, interacts with SECISBP2 that functions as a *sec insertion sequence* (SECIS) binding protein useful for the incorporation of selenocysteine also into GPX1. On the other hand, IL-17 is correlated with IL-6 through RORC that decrease the expression of IL-17 and IL-6 in differentiating human naïve T lymphocytes. Because TP53 is modulated by NF-κB, network analysis confirms that the four cytokines are strictly correlated between them and the decrease of their levels can induce NF-κB inactivation and, hence, underlying a possible anti-inflammatory role for the selenite (*Rusolo et al. 2013*). In an attempt to identificate new markers, useful and necessary to improve HCC diagnosis and prognosis, it has started to point the attention on selenoproteins which, from RT-qPCR analysis were found to change, not only between human hepatocellular carcinoma cells compared to healthy hepatocytes, but also accurate signature, intended to characterization of differences between human tumoral cell lines, HepG2 and Huh7, both derived from human hepatoma without HBV or HCV infections. This allowed to extend the speech from cellular to much more complex and complete tissue feature**.** It has started evaluating expression of

two glutathione peroxidases, GPX4 and GPX7, through IHC, in cirrhotic tissue samples – HCV related, used like control and compared with HCC tissue sections. Results evidenced that GPX4 and GPX7 had a statistically significant overexpression in HCC tissues compared to cirrhotic counterparts used as non tumor tissues and that their expression was higher in grade III HCC tissues with respect to grade I-II samples *(Guerriero et al. 2015)*. GPx4 is supposed to attenuate cancer development, reducing lipid peroxides formed through ROS-mediated oxidation of unsaturated lipids. In colorectal cancer cells, lipid peroxides increase AP-1 activity and VEGF formation, as well as, in cultured HCC cells *(Rohr-Udilova et al. 2012)*. Elevation of AP-1 components c-jun and c-fos and activation of expression of its target genes VEGF and IL-8 in HCC seem to be control by antagonist effects of GPX4. Increased GPx4 levels were associated with reduced VEGF and AP-1/c-fos expression and with a decline in tumor growth. Importantly, selenium levels inversely correlated with VEGF and IL-8 serum levels and tumor size in HCC patients *(Rohr-Udilova et al. 2012)*. As GPX4, also GPX7 can reduce oxidative stress generated from polyunsaturated fatty acid metabolism and recently it has been demonstrated that GPX7 possesses tumor suppressor functions in esophageal adenocarcinoma (EAC), supporting the idea that GPX7 deficiency leads to systemic oxidative stress, increased tumor incidence and shortened life span *(Peng et al. 2014)*. Loss of expression and dysfunction of GPX7 are frequent in EAC and its precancerous lesions. GPX7 can suppress activation of NF-κB in esophageal epithelial cells upon exposure to TNF-α, responsible to upregulation of NF-κB target genes, including proinflammatory cytokines and chemokines, like IL-8 that promote cancer cell proliferation, survival and migration which are associated with tumor progression, metastasis and size *(Peng et al. 2014)*. GPX4 in colon cancer tissues in comparison with healthy tissues, as well as in HCC tissues, and GPX7 could ensure defense against oxidative stress and may support survival mechanisms suppressing apoptosis in the tumor cells, especially within more advanced tumors. So, selenoproteins may limit damage in DNA, which has been directly linked to apoptosis resistance *(Yagublu et al. 2011)*. The tissue, also, were subjected to SELM staining. IHC was resulted positive and highly specific for hepatocytes cytoplasm. SELM expression resulted positive in all the stained HCC tissues whereas a light and discontinuous positivity was observed in the corresponding cirrhotic tissues *(Guerriero et al. 2014)*. SelM levels increases protect against oxidative stress and, probably, its decrease would render cells more vulnerable to oxidative challenge. Indeed, knock-down of SelM in HT22 cells and primary cortical cells increased oxidative damage. Furthermore, in the absence of oxidative challenge, SelM knock-down resulted in decreased cell viability and increased ROS, further demonstrating the functional importance of SelM in preventing oxidative stress *(Reeves et al. 2010)*. SelM overexpression significantly induce the ERK MAPK pathway and promote *(Yim et al. 2009)* cell proliferation,

migration and survival. Its association with HCC progression can play important roles during HCC development. So, all these data suggested a possible correlation among SELM, selenoproteins and HCC *(Guerriero et al. 2014)*. Also the etiology of breast cancer is multifactorial and multiple intrinsic and extrinsic risk factors and their interactions are involved in its development and progression. Among the many factors influencing development, progression and metastasis, oxidative stress has an important role in the initiation and preservation of breast cancer progression *(Jezierska-Drutel et al. 2013)*. I radicali liberi, accumulandosi, danneggiano lipidi, protein e dna e anche in assenza di BRCA1/2 mutations, genomic instability in the cancer stroma might facilitate a microenvironment that promotes genomic instability in the epithelium, inducendo la trascrizione di oncogeni come RAS e MYC and, subsequently, neoplastic transformation *(Artacho-Cordón et al. 2012)*. Ne segue A strong inflammatory response mediata da *tumor - associated macrophages* (TAM), involved in hypoxia-triggered formation of new blood vessels and in chemokines production, neutrophil-derived substances that promote tumor growth and degradation of the *extracellular matrix* (ECM) components mediated by matrix metalloproteases (MMPs) family, which allows to tumor cells to invade local tissue and to develop distant metastases promoting angiogenesis, through control bioavailability of VEGF *(Artacho-Cordón et al. 2012)*. From the above, considerable interest is arise to research in selenoproteins, not only, a possible rule in inflammation control, in an attempt to slow down the progression from normal mammary gland to ductal carcinoma in situ (DCIS) to invasive breast cancer, but also chemopreventive properties *(Zhuo et al. 2009)*. It has been performed the analysis of the global expression of the seleno-transcriptome family in two human breast cancer cells (MCF-7 and MDA-MB231) compared to healthy breast cell (MCF-10A) by RT-PCR analysis that has shown a different expression pattern between two tumor cell lines and in comparison to no tumor cell line. Had been found four down-regulated (GPX1, GPX4, GPX5 and GPX7) and eight up-regulated (DIO1, DIO2, DIO3, SELT, SEPW1, SEPX1, GPX2 and GPX3) genes in MCF-7 cells and five down-regulated (GPX1, GPX4, GPX5, GPX6 and GPX7) and four up-regulated (DIO2, SEP15, GPX2 and GPX3) genes in MDA-MB231cells. Since recent advances in cancer research are showing that this pathological process cannot be understood simply through genetic mutations of cancerous cells but that gene/protein inter-relationships which correlate and regulate metabolic processes should be also considered. It is well known that the thyroid hormone, as well as the estrogens, acts via nuclear receptors and its mRNA level is altered in breast cancer tissues *(Silva et al. 2002)*. However, it is also known that the thyroid hormone receptors can bind to an estrogen response element of target gene promoters, so affecting the estrogen-dependent gene transcription *(Hall et al. 2008)*. Studies on breast cancer cell lines have shown that thyroid hormone stimulates cell growth and division in the ER positive MCF-

7 cell line, but not in the ER negative MDA-MB231 cells *(Debski et al. 2007)*. This highlights how strictly correlated to the thyroid hormone is the expression of the three diodinases, so explaining their only over-expression for the MCF7 cells. Concerning the GPX family, the best characterized member of is GPX1 that uses reducing equivalents from glutathione to detoxify peroxides *(Bera et al. 2014)* and is resulted lower in tumor cells compared to their normal counterparts *(Wang et al. 2003)*. These results for GPX1 in the two cell lines relative to breast cancer are in agreement with data reported for other cancers while about GPX5, which is androgen-regulated *(Brigelius-Flohé and Maiorino, 2013)*, was found to be associated with overall survival among patients with non-small cell lung cancer *(Li et al. 2011)*. Since no data of its possible involvement in breast cancer are reported, it was suggested suggest, for the first time, its role in breast cancer. Moreover, for GPX4, GPX6 and GPX7, the results confirm the general observations that they are strongly inter-correlated from a functional point of view and thus this can explain why all three have been found down-regulated in these experiments. In fact, they share some structurally related features *(Scheerer et al. 2007* and *Brigelius-Flohé et al. 2013)* and all the three enzymes are functionally involved in countering the effects of oxidative damage particularly to membrane lipids and often found down-expressed in cancers *(Brigelius-Flohé et al. 2013)*. In details, GPX4 was found with a reduced expression in a number of cell lines such as MCF-7 breast cancer cell line, colon cancer HT29 and ovarian cancer cell line A2780 *(Ding et al. 2007)*, while the absence of NPGPX (non-selenocysteine phospholipid Hydro-peroxide glutathione peroxidase complex with GPX6) expression was found in the majority of breast cancer cell lines *(Rodrigues et al. 2014)*, and the potential role in alleviating oxidative stress induced by dietary consumption of fatty acids was suggested for GPX7 in breast cancer cells *(Utomo et al. 2004)*. Conversely, it possible to explain why in these experiments, GPX2 and GPX3 have been found up-regulated in both ER-positive MCF-7 and ER-negative MDA-MB231 cells on the basis of their known effects in cancers. GPX2 is required in healthy tissues to maintain the normal self-renewing of the gastrointestinal epithelium also depressing inflammatory processes, therefore, GPX2 should inhibit the carcinogenesis initiation but once a cell has been programmed to proliferate in an uncontrolled way, it supports its growth by inhibiting apoptosis *(Brigelius-Flohé et al. 2012)*. This view appears to be consistent also with results of experiments. GPX3, we have to consider that it is a major scavenger of ROS produced during normal metabolism or after oxidative insult, was found up-regulated in these experiments. It also found down-regulated by promoter hyper-methylation in several human cancers, and thus suggesting a function as a tumor suppressor *(Liu et al. 2015)*, but at the same time, the same gene was found also highly up-regulated in the epithelial ovarian carcinoma, where was proposed as molecular marker, highly specific for clear cell carcinomas *(Hough et al. 2000)*. In these case, knowing that GPX3 targets directly the

gene of the estrogen receptor alpha in the white adipose tissue, it has been indicated as an important mediator of the estrogen effects in relation to fat mass *(Lundholm et al. 2008),* and considering the link between visceral fat and breast cancer initiation and progression *(Rohan et al. 2013),* it is possible to find reasonable its up-expression. About Sep15, SEPW1 and SELT, the three proteins share a similar structural feature because they present a thioredoxin-like domain in common. However, SEPW1 has been found involved in the cell cycle progression *(Hawkes and Alkan, 2011)* where it facilitates the G1 to S-phase transition by down-regulating the expression of the cyclin-dependent kinase inhibitor p21 through the control of the levels of the TP53 transcription factor *(Hawkes and Alkan, 2011).* In the literature it is also reported that SELT is involved in the cell adhesion, in the expression of several oxidoreductase genes, in the cell structure organization, in the redox regulation and cell anchorage but the same authors found also that SEPW1 may functionally compensate for SELT expression *(Sengupta et al. 2009).* Therefore these considerations demonstrate that SEPW1 and SELT are inter-correlated and can explain because we have found both up-expressed. Moreover, Sep15 has been found involved in redox function, as well as, in the carcinogenesis process *(Kasaikina et al., 2011),* where it has been demonstrated the role of Sep15 allelic loss in the development of the breast cancer and hence its involvement in breast cancer *(Nasr et al. 2003)* as conformed also by these data. On the other hand, SEPX1 belongs to the methionine sulfoxide reductase B (MSRB) family, and for this reason it is named also as MSRB1. A pivotal role for Sp1 in the constitutive expression of SEPX1 was demonstrated, and SEPX1 promoter activity appeared to be controlled also by epigenetic modifications, such as methylation *(De Luca et al. 2007).* However, high levels of its transcript were detected in MCF7 human breast cancer cells whereas more low levels were detected in the highly aggressive MDA-MB231 cells *(De Luca et al. 2007),* and once again these data are in excellent agreement with previous studies. To evaluate the ability of these down- and up-regulated genes to interact among them, as well as, to identify putative HUB nodes able to exercise a direct control over them, it has been used the IPA algorithm to study these correlations. It shows that all the differentially regulated genes, with the unique exception of SELT, are implicated in the same network that presents three HUB nodes interconnected to our selenoprotein mRNAs: i.e., *tumor protein 53* (TP53), estrogen receptor 1 (ESR1) and *Catenin beta 1* (CTNNB1). In details, analyzing the correlations between down- and up-regulated genes by means of the IPA algorithm it has been found that ESR1 binds TP53 and CTNNB1; TP53 binds the most part of GPXs and SEPX1 (MSRB1) and, finally, CTNNB1 binds DIO1, DIO2, GPX2, SEPW1 and SEP15 through *cAMP response element-binding protein* (CREB). In general, CTNNB1 regulates the coordination of cell-cell adhesion and gene transcription and acts in the Wnt signaling pathway as an intracellular signal. However, CTNNB1 is known to bind ESR1

by an involvement in estrogen signaling *(Kouzmenko et al. 2004)*, and to increase the transcriptional activity of the human TP53 *(Damalas et al. 1999)*, which is the most frequently mutated gene in human cancer and often referred to as "the guardian of the genome" *(Hawkes and Alkan, 2011)*. In response to genomic stresses, TP53 causes cell cycle arrest to allow time for genomic damage to be repaired before cell division or induces apoptosis to eliminate irreparably damaged cells *(Hawkes and Alkan, 2011)*. Moreover, TP53 activation is influenced from the cellular transcription factor, named CREB, that binds SEP15 *(Ariumi et al. 2000; Wilkinson et al. 2009)* whereas its inactivation from expression of SEPX1-mRNA involves also the oncogenic mutant HRAS protein *(Boiko et al. 2006)*. In the literature it is also reported that CTNNB1 is involved in the expression of human SEPW1-mRNA *(Lin et al. 2001)*, in that of DIO1 *(Tan et al. 2006)*, and in that of GPX2 and DIO2-mRNA by the binding with human T-cell signaling factor *(Schwartz et al. 2003)*. In conclusion, the network analysis suggests a reasonable role for CTNNB1 as upstream regulator because it is an important link connecting both the diodinases, the gluthathione peroxidases, the seleno-mRNAs belonging to the thioredoxin-like family, the tumor suppressor TP53 and the estrogen receptor that plays an important role in the estrogen-receptor-positive MCF-7. The presence of CTNNB1 and TP53, with extensive interrelationships, suggests an effective control of the biological activities of the selenoprotein family by means of these two proteins, but opens also to consideration that the great potential molecular adaptability exerted by these two proteins to always new metabolic environments, at the same time, suggests a close dependence of selenoproteins from metabolic changes, also coming from far away.

6

CONCLUSIONS AND FUTURE PROSPECTIVES

It has become clear that the physiological effects of selenium occur mainly through the function of selenoproteins. Recent advances in understanding the complex regulation of synthesis and function of several members of the selenoproteins family have contributed to an improved comprehension of the role of selenium in human health. At the physiological level, these enzymes are involved in diverse functions: from antioxidant defense to fertility, from muscle development and function to thyroid hormone metabolism and from immune function to cancer chemopreventive agent *(Moghadaszadeh and Beggs, 2012)*. This latter aspect has aroused the interest of many researchers. In low doses, selenium functions as an essential component of SeCys in various specific selenoproteins and promotes cell proliferation, important for immune response, meanwhile, in high but nontoxic doses, selenium may reduce the risk of cancer by blocking tumor cell cycle, stimulating apoptosis and inhibiting tumor cell migration and invasion *(Almondes et al. 2010)*. The studies conducted until now have also highlighted how the defects of selenium intake, aberrant mutations and polymorphisms (SNPs) in the selenoproteins genes, as well as, their impaired expression can be associate to wide range of pathologies, as cancer. An example is Hepatocellular carcinoma (HCC) that develops in the liver with severe impairment of cellular antioxidant systems *(Rusolo et al. 2013)*. these, in turn, are responsible of an imbalance between production of free radicals and reactive Metabolites (ROS) and their elimination by protective mechanisms, referred to as antioxidants. Ros, producted long, not only, significantly, damage cell structures and functions but may induce somatic mutations and neoplastic transformation. The scenery complicates because in damage site are are recruited, for the first, cells of innate immune response and, later, those of acquired response (mast cells and leukocytes) that trigger chronic inflammation, characterized, on one side, the increased release and accumulation of ROS, on the other side, the production of soluble mediators, such as, cytokines and chemokines, which recruit other inflammatory cells to the site of damage, producing more reactive species e damaging healthy neighboring epithelial and stromal cells and contributing to carcinogenesis *(Reuter et al. 2010)*. Effective HCC chemoprevention or treatment strategies are still lacking, often due to of their side effects, therefore *(Shaaban et al. 2014)* it became necessary to identify new possible chemo-preventive alternatives and/or therapeutic strategies. The use of dietary antioxidants and micronutrients has been recently proposed for successful HCC management *(Halliwell, B. 2011)*, for which reason, this thesis started

to focus attention on some of the most important cellular antioxidant systems, that of selenoproteins. It has started from global analysis of the selenotranscriptome expression in HepG2 and Huh7 cells compared to the normal human hepatocytes by reverse transcription-qPCR (RT-qPCR) which traced a clear and different signature on two cancer cell lines cellular compared to healthy hepatocytes *(Guariniello et al. 2015).* This allowed to think whether the treatment with sodium selenite, with concentrations comparable to physiological ones, can modulate both seleno-transcripts and corresponding selenoproteins. Emphasized those selenoproteins whose levels were found reduced in according to the grade of the HCC, it has been analyzed GPX1, SELK and SELENBP1 in HepG2 and Huh7 cells, compared to non treated cell, used as control and it has been evaluated the cytokine concentrations in order to evaluate the pro- or anti-inflammatory effects of sodium selenite. The data obtained evidenced that sodium selenite induced the increase of GPX1 and SELK protein expression in both HepG2 and Huh7 cells treated with increasing selenite concentrations and the related decrease of VEGF and of three pro-inflammatory cytokines, *i.e.*, IL-6, IL-8, and IL-17, confirming, by an interactomic analysis, the involvement of these molecules in functional pathway alone *(Rusolo et al. 2013).* The investigation is continued by *immunohistochemistry* (IHC) for some selenoproteins, whose transcripts were found over-expressed, through RT-qPCR analysis, on two cell lines of cellular hepatoma compared to the normal human hepatocytes. In detalis, it has been evalueted expression livels of GPX4, GPX7 and SELM in human HCC tissues compared to the cirrhotic liver tissue, used as control. The experiments have demonstrated that these selenoproteins was associated with an increased malignancy grade, highlighting the possible use of GPX4, GPX7 and SELM as markers for improving HCC diagnosis/ prognosis *(Guerriero et al. 2015).* As HCC, also *Breast Cancer* (BC) is not only an isolated group of mutated somatic cells but it is a microenvironment system comprising breast cancer cells, fibroblasts, adipocytes, immune and endothelial cells. Among the many factors influencing development, progression and metastasis, also in this case, oxidative stress and the impairment of antioxidant systems have an important role in the initiation and preservation of breast cancer progression *(Jezierska-Drutel et al. 2013).* So, before every other thing, this thesis has to prefix to perform analysis of the global expression of the seleno-transcriptome family in two human breast cancer cells (MCF-7 and MDA-MB231) compared to healthy breast cells (MCF-10A) by RT-PCR analysis. This suggested a signature of seleno-mRNAs specific for human breast cells showing the genes that change their expression on the basis also of their ER positivity or negativity, and the HUB nodes, that represent the correlation centers and control over the coordinated genes, suggesting mainly the possible important role of *catenin beta 1* (CTNNB1). However, it is planning new studies, which will regard the evaluation of the seleno-transcriptome in bioptic tissues of breast

cancer patients to confirm the results obtained and to suggest new markers to improve the diagnosis and prognosis of this cancer. But the liver is, also, a hormone-sensitive organ indeed, sex hormones and their receptors play a role in liver carcinogenesis (*Wang et al. 2006)*. Furthermore, it seems that the hepatic parenchyma presents a similar architecture, in its organization and cellular complexity, to the mammary gland. This can allow to think that the responsiveness of hepatocytes and hepatic parenchymal cells to impaired secretion of sex hormones from the mammary gland could influence the hepatocarcinogenesis evolution which, in turn, would influence the progression of breast cancer and its metastasis. Moreover, Breast Cancer cells, preferentially, home to the liver (with additional organotropism) *(Taylor et al. 2014)*. So, the continuous oxidative stress and un-regulation of redox enzymes in HCC, as well as in BC, impaired synthesis and secretion of sex hormones laid the foundations to research in selenoproteins a great help not only in mitigating these mechanisms but also in modulating the hormone signaling exacerbating hepatocarcinogenesis. Furthermore, the possibility to control the inflammation and stress oxidative mechanisms responsible of these diseases could ensure, on one side, a slowdown of unregulated synthesis of sex hormones from breast cancer and, on the other side, to restrain further stressor events of hepatocarcinogenesis. To do this, it might think to work of co-cultures that will allow the growth of more than one distinct cell type in a combined cultures, useful for modeling and studying the interaction and signaling between different cell types e to monitor intercellular communication, cell migration dynamics, and stimulation and maintenance of cell function and differentiation. Among future prospective that will contribute to evolution and improvement of the work conducted until now, three dimensional cell culture models overlook this scenery. 3D models of Hepatorcinoma and Breast Cancer cell lines, not only, it will be a system able to model more accurately the cells in vivo, but these will allow to follow, in Live Imaging by *Light sheet Fluorescence Microscopy (LSFM)*, how the treatment or depletion of selenium, or micronutriente combination with specific chemotherapeutic molecules, can influence the localization and expression of selenoproteins and the pathways responsible of cell lines tumorigenicity. In such system, the effect of a drug will can be investigate in an environment similar to the target tissue and the combination to the LSFM technology will allow dynamic long-term observations of living three-dimensional cultures, due to its ability to ensure, over several days, cell viability through a special perfusion chamber, where are tightly controlled, temperature, medium flow, pH and gas exchange. *LSFM*, such as, realization and the study of 3D cell culture models, are techniques that i had the opportunity to learn during my permanence at European Molecular Biology Laboratory (*EMBL*) in Heidelberg, woking wih Francesca Peri and Martin Jachlinger's Groups. Such, these in vitro models allowed to move from a vision of mono dimensional, far from reality and static cell biology to a new, live and prospect vision that will can

provide a more physiologically relevant way of demonstrating in vivo-like tissue morphology and function.

7

BIBLIOGRAPHY

- Almondes, KG.; Leal, GV.; Cozzolino, SM.; Philippi, ST.; Rondó, PH**.** The role of selenoproteins in cancer. *Rev Assoc Med Bras*. 2010, 4, 484-8;
- Ariumi, Y.; Kaida, A.; Lin, JY.; Hirota, M.; Masui, O.; Yamaoka, S.; Taya, Y.; Shimotohno, K. HTLV-1 tax oncoprotein represses the p53-mediated trans-activation function through coactivator CBP sequestration. *Oncogene*. 2000, 19,1491-9;
- Arnaldi, LA.; Borra, RC.; Maciel, RM.; Cerutti, JM. Gene expression profiles reveal that DCN, DIO1, and DIO2 are underexpressed in benign and malignant thyroid tumors. *Thyroid*. 2005, 15, 210-21;
- Artacho-Cordón, A.; Artacho-Cordón, F.; Ríos-Arrabal, S.; Calvente, I.; Núñez, MI. Tumor microenvironment and breast cancer progression: a complex scenario. *Cancer Biol Ther*. 2012, 13, 14-24;
- Bellinger, FP.; Raman, AV.; Reeves, MA.; Berry, MJ. Regulation and function of selenoproteins in human disease. *Biochem J.* 2009, 422, 11-22;
- Benstoem, C.; Goetzenich, A.; Kraemer, S.; Borosch, S.; Manzanares, W.; Hardy, G.; Stoppe, C. Selenium and its supplementation in cardiovascular disease--what do we know? *Nutrients.* 2015, 7, 3094-118;
- Bera, S.; Weinberg, F.; Ekoue, DN.; Ansenberger-Fricano, K.; Mao, M.; Bonini, MG.; Diamond, AM. Natural allelic variations in glutathione peroxidase-1 affect its subcellular localization and function. *Cancer Res*. 2014, 74, 5118-26;
- Boiko, AD.; Porteous, S.; Razorenova, OV.; Krivokrysenko, VI.; Williams, BR.; Gudkov, AV. A systematic search for downstream mediators of tumor suppressor function of p53 reveals a major role of BTG2 in suppression of Ras-induced transformation. *Genes Dev*. 2006, 20, 236-52;
- Brenneisen, P.; Steinbrenner**,** H.; Sies, H. Selenium, oxidative stress, and health aspects. *Mol Aspects Med.* 2005, 26, 256-67;
- Brigelius-Flohé, R.; Maiorino, M. Glutathione peroxidases. *Biochimica et Biophysica Acta*. 2013, 1830, 3289-3303;

- Brigelius-Flohé, R.; Müller, M.; Lippmann, D.; Kipp, AP. The yin and yang of nrf2-regulated selenoproteins in carcinogenesis. *Int J Cell Biol*. 2012, 486147;
- Brigelius-Flohe, R.; Sies, H. Diversity of Selenium Functions in Health and Disease. *CRC Press*. 2015;
- Brown, KM.; Arthur, JR. Selenium, selenoproteins and human health: a review. *Public Health Nutr.* 2001, 4, 593-9;
- Brown, NS.; Bicknell, R. Hypoxia and oxidative stress in breast cancer. Oxidative stress: its effects on the growth, metastatic potential and response to therapy of breast cancer. *Breast Cancer Res*. 2001, 3, 323-7;
- Burk, R.F. Selenium in man. Prasad, A.S. (ed.), *Trace Elements in Human Health and Disease*, Academic Press, New York. 1976, 105–133;
- Capone, F.; Costantini, S.; Guerriero, E.; Calemma, R.; Napolitano, M.; Scala, S.; Izzo, F.; Castello, G. Serum cytokine levels in patients with hepatocellular carcinoma. *Eur. Cytokine Netw*. 2010, 21, 99-104;
- Castello, G.; Costantini, S.; Scala, S. Targeting the inflammation in HCV-associated hepatocellular carcinoma: a role in the prevention and treatment. *J Transl Med*. 2010, 3, 8, 109;
- Chen, J.; Berry, MJ. Selenium and selenoproteins in the brain and brain diseases. *J Neurochem.* 2003, 86, 1-12;
- Choi, HK.; Choi, KC.; Kang, HB.; Kim, HC.; Lee, YH.; Haam, S.; Park, HG.; Yoon, HG. Function of multiple Lis-Homology domain/WD-40 repeat-containing proteins in feed-forward transcriptional repression by silencing mediator for retinoic and thyroid receptor/nuclear receptor corepressor complexes. *Mol Endocrinol*. 2008, 22, 1093-104;
- Costantini, S.; Capone, F.; Guerriero, E.; Maio, P.; Colonna, G.; Castello, G. Serum cytokine levels as putative prognostic markers in the progression of chronic HCV hepatitis to cirrhosis. *Eur. Cytokine Netw*. 2010, 21, 251-256;
- Costantini, S.; Capone, F.; Maio, P.; Guerriero, E.; Colonna, G.; Izzo, F.; Castello, G. Cancer biomarker profiling in patients with chronic hepatitis C virus, liver cirrhosis and hepatocellular carcinoma. *Oncol Rep*. 2013, 29, 2163-8;
- Damalas, A.; Ben-Ze'ev, A.; Simcha, I.; Shtutman, M.; Leal, JF.; Zhurinsky, J.; Geiger, B.; Oren, M. Excess beta-catenin promotes accumulation of transcriptionally active p53. *EMBO J*. 1999, 18, 3054-63;

- De Luca, A.; Sacchetta, P.; Nieddu, M.; Di Ilio, C.; Favaloro, B. Important roles of multiple Sp1 binding sites and epigenetic modifications in the regulation of the methionine sulfoxide reductase B1 (MsrB1) promoter. BMC Mol Biol. 2007, 8, 39;
- Debski, M.G.; Pachucki, J.; Ambroziak, M.; Olszewski, W.; Bar-Andziak, E. Human breast cancer tissue expresses high level of type 1 5'-deiodinase. *Thyroid*. 2007, 17, 3-10;
- Di Leva, G.; Garofalo, M.; Croce, CM. MicroRNAs in cancer. *Annu Rev Pathol*. 2014, 9, 287-314;
- Di Stasio, M.; Volpe, M.G.; Colonna, G.; Nazzaro, M.; Polimeno, M.; Scala, S.; Castello, G.; Costantini, S. A possible predictive marker of progression for hepatocellular carcinoma. *Oncol. Lett.* 2011, *2*, 1247–1251;
- Ding, W.Q.; Lind, S.E. Phospholipid hydroperoxide glutathione peroxidase plays a role in protecting cancer cells from docosahexaenoic acid-induced cytotoxicity. *Mol Cancer Ther*. 2007, 6, 1467-74;
- Dreosti, I. Selenium. *J. Food Nutr.* 1986, 43, 60-78;
- Du, S.; Zhou, J.; Jia, Y.; Huang, K. SelK is a novel ER stress-regulated protein and protects HepG2 cells from ER stress agent-induced apoptosis. *Arch. Biochem. Biophys.* 2010, *502*, 137–143;
- Fang, W.; Goldberg, ML.; Pohl, NM.; Bi, X.; Tong, C.; Xiong, B.; Koh, TJ.; Diamond, AM.; Yang, W. Functional and physical interaction between the selenium-binding protein 1 (SBP1) and the glutathione peroxidase 1 selenoprotein. *Carcinogenesis.* 2010, *31*, 1360–1366;
- Farazi, PA.; DePinho, RA. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nature Reviews Cancer*. 2006, 6, 674-87;
- Gu, FM.; Li, QL.; Gao, Q.; Jiang, JH.; Zhu, K.; Huang, XY.; Pan, JF.; Yan, J.; Hu, JH.; Wang, Z.; Dai, Z.; Fan, J.; Zhou, J. IL-17 induces AKT-dependent IL-6/JAK2/STAT3 activation and tumor progression in hepatocellular carcinoma. *Mol Cancer*. 2011, 15, 10-150;
- Guariniello, S.; Di Bernardo, G.; Colonna, G.; Cammarota, M.; Castello, G.; Costantini, S. Evaluation of the selenotranscriptome expression in two hepatocellular carcinoma cell lines. *Anal Cell Pathol (Amst)*. 2015, 2015-419561;
- Guerriero, E.; Accardo, M.; Capone, F.; Colonna, G.; Castello, G.; Costantini, S. Assessment of the Selenoprotein M (SELM) over-expression on human hepatocellular carcinoma tissues byimmunohistochemistry. *Eur J Histochem*. 2014, 1, 58-2433;

- Hall, LC.; Salazar, E.P.; Kane, SR.; Liu, N. Effects of thyroid hormones on human breast cancer cell proliferation. *J Steroid Biochem Mol Biol*. 2008, 109, 57-66;
- Halliwell, B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol*. 2006, 141, 312-22;
- Hatfield, DL.; Yoo, MH.; Carlson, BA.; Gladyshev, VN. Selenoproteins that function in cancer prevention and promotion. *Biochim Biophys Acta*. 2009, 1790, 1541-5;
- Hawkes, WC.; Alkan, Z. Delayed cell cycle progression from SEPW1 depletion is p53- and p21-dependent in MCF-7 breast cancer cells. *Biochem Biophys Res Commun*. 2011, 413, 36-40.
- Hawkes, WC.; Printsev, I.; Alkan, Z. Selenoprotein W depletion induces a p53- and p21-dependent delay in cell cycle progression in RWPE-1 prostate epithelial cells. *J Cell Biochem*. 2012; 113; 61-9;
- Heinrich, PC.; Behrmann, I.; Haan, S.; Hermanns, HM.; Muller-Newen, G.; Schaper, F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J*. 2003, 374, 1-20;
- Helming, KC.; Wang, X.; Roberts, CW. Vulnerabilities of mutant SWI/SNF complexes in cancer. *Cancer Cell*. 2014, 26, 309-17;
- Hough, CD.; Cho, KR.; Zonderman, AB., Schwartz, DR.; Morin, PJ. Coordinately up-regulated genes in ovarian cancer. *Cancer Res*. 2001, 61, 3869-76
- Huang, C.; Ding, G.; Gu, C.; Zhou, J.; Kuang, M.; Ji, Y.; He, Y.; Kondo, T.; Fan, J. Decreased Selenium-Binding Protein 1 Enhances Glutathione Peroxidase 1 Activity and Downregulates HIF-1α to Promote Hepatocellular Carcinoma Invasiveness. *Clin. Cancer Res.* 2012, *18*, 3042–3053;
- Hussain, SP.; Schwank, J.; Staib, F.; Wang, XW.; Harris, CC. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene*. 2007, 26, 2166-76;
- Hussain. SP.; Harris, CC. Inflammation and cancer: an ancient link with novel potentials. *Int J Cancer*. 2007, 121, 2373-2380;
- *Jan Trofast* Berzelius' Discovery of Selenium "Undersökning af en ny Mineral-kropp, funnen i de orenare sorterna af det vid Fahlun tillverkade svaflet." *Chemistry international.* 2011 Vol. 33 No. 5

- Jezierska-Drutel, A.; Rosenzweig, SA.; Neumann, CA. Role of Oxidative Stress and the Microenvironment in Breast Cancer Development and Progression. *Adv Cancer Res.* 2013, 119, 107-25;
- Jezierska-Drutel, A.; Rosenzweig, SA.; Neumann, CA. Role of oxidative stress and the microenvironment in breast cancer development and progression. *Adv Cancer Res*. 2013, 119, 107-25;
- Kadam, S.; McAlpine, GS.; Phelan, ML.; Kingston, RE.; Jones, KA.; Emerson, BM. Functional selectivity of recombinant mammalian SWI/SNF subunits. *Genes Dev*. 2000, 14, 2441-51;
- Kasaikina, MV.; Fomenko, DE.; Labunskyy, VM.; Lachke, SA.; Qiu, W.; Moncaster, JA.; Zhang, J.; Wojnarowicz, MW Jr.; Natarajan, SK.; Malinouski, M.; Schweizer, U.; Tsuji, PA.; Carlson, BA.; Maas, RL.; Lou, MF.; Goldstein, LE.; Hatfield, DL.; Gladyshev, VN. Roles of the 15-kDa selenoprotein (Sep15) in redox homeostasis and cataract development revealed by the analysis of Sep 15 knockout mice. *Journal of Biological Chemistry*. 2011, 286, 33203-12;
- Kohen, R.; Nyska, A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol*. 2002, 30, 620-50;
- Kouzmenko, AP.; Takeyama, K.; Ito, S.; Furutani, T.; Sawatsubashi, S.; Maki, A.; Suzuki, E.; Kawasaki, Y.; Akiyama, T.; Tabata, T.; Kato, S. Wnt/beta-catenin and estrogen signaling converge in vivo. *J Biol Chem*. 2004, 279,40255-8;
- Labunskyy, VM.; Hatfield, DL.; Gladyshev, VN. Selenoproteins: molecular pathways and physiological roles. *Physiol Rev*. 2014, 94, 739-77;
- Leonardi, GC.; Candido, S.; Cervello, M.; Nicolosi, D.; Raiti, F.; Travali, S. Spandidos, DA; Libra, M. The tumor microenvironment in hepatocellular carcinoma (review). *Int J Oncol*. 2012, 40, 1733-47;
- Levander, O. A.; Baumann, C. A. Selenium metabolism VI. Effect of arsenic on excretion of selenium in the bile. *Toxicol. Appl. Pharmacol.* 1966, 9, 106-15;
- Li, Y.; Sun, Z.; Cunningham, JM.; Aubry, MC.; Wampfler, JA.; Croghan, GA.; Johnson, C.; Wu, D.; Aakre, JA.; Molina, J.; Wang, L.; Pankratz, VS.; Yang, P. Genetic variations in multiple drug action pathways and survival in advanced stage non-small cell lung cancer treated with chemotherapy. *Clin Cancer Res*. 2011, 17, 3830-40;
- Lin, YM.; Ono, K.; Satoh, S.; Ishiguro, H.; Fujita, M.; Miwa, N.; Tanaka, T.; Tsunoda, T.; Yang, KC.; Nakamura, Y.; Furukawa, Y. Identification of AF17 as a downstream gene of

the beta-catenin/T-cell factor pathway and its involvement in colorectal carcinogenesis. *Cancer Res*. 2001, 61, 6345-9;

- Liu, Q.; Jin, J.; Ying, J.; Sun, M.; Cui, Y.; Zhang, L.; Xu, B.; Fan, Y.; Zhang, Q. Frequent epigenetic suppression of tumor suppressor gene glutathione peroxidase 3 by prom oterhypermethylation and its clinical implication in clear cell renal cell carcinoma. *Int J Mol Sci*. 2015, 16, 10636 - 49;
- Lu, J.; Holmegren, A. Selenoproteins. *J Biol Chem*. 2009, 284-723-7;
- Lundholm, L.; Putnik, M.; Otsuki, M.; Andersson, S.; Ohlsson, C.; Gustafsson, JA.; Dahlman-Wright, K. Effects of estrogen on gene expression profiles in mouse hypothalamus and white adipose tissue: target genes include glutathione peroxidase 3 and cell death-inducing DNA fragmentation factor, alpha-subunit-like effector A. *J Endocrinol*. 2008, 196, 547-57;
- Mahalingaiah, P.K and Singh, K.P. Chronic Oxidative Stress Increases Growth and Tumorigenic Potential of MCF-7 Breast Cancer Cells**.** *PLoS One*. 2014, 9, e87371;
- Margis, R.; Dunand, C.; Teixeira, F.K.; Margis-Pinheiro, M.; Glutathione peroxidase family - an evolutionary overview. Review. *FEBS J*. 2008, 275, 3959-70;
- Mariotti, M.; Ridge, PG.; Zhang, Y.; Lobanov, AV.; Pringle, TH.; Guigo, R.; Hatfield, DL.; Gladyshev, VN. Composition and evolution of the vertebrate and mammalian selenoproteomes. *PLoS One.* 2012, 7:e33066;
- Martin, M.; Herceg, Z. From hepatitis to hepatocellular carcinoma: a proposed model for cross-talk between inflammation and epigenetic mechanisms. *Genome Med*. 2012, 4-8;
- Moghadaszadeh, B.; Beggs, AH. Selenoproteins and their impact on human health through diverse physiological pathways. *Physiology (Bethesda)*. 2006, 21, 307-15;
- Mohammad, KZ.; Anurag, S.; Ashraf A.; Kaneez, F.; Esam, A.; Ishtiaq, Q. HBV/HCV Infection and Inflammation. *J Genet Syndr Gene Ther*. 2014, 5, 241;
- Nasr, MA.; Hu, YJ.; Diamond, AM.;. Allelic loss at the SEP15 locus in breast cancer. *Cancer Therapy*. Vol 1. 2003, 293-298;
- Papp, LV.; Lu, J.; Holmgren, A.; Khanna, KK. From selenium to selenoproteins: synthesis, identity, and their role in human health. *Antioxid Redox Signal*. 2007, 9, 775-806;
- Peirce, S.K., Chen, W.Y., Chen, W.Y. Quantification of prolactin receptor Mrna in multiple human tissues and cancer cell lines by real time RT-PCR. *J Endocrinol*. 2001, 171, R1-4;

- Peng, DF.; Hu, TL.; Soutto, M.; Belkhiri, A.; El-Rifai, W. Loss of glutathione peroxidase 7 promotes TNF-α-induced NF-κB activation in Barrett's carcinogenesis. *Carcinogenesis*. 2014, 35, 1620-8;
- Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001, 29, e45;
- Pfaffl, M.W.; Horgan, G.W.; Dempfle, L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res*. 2002, 30, e36;
- Potenza, N.; Castiello, F.; Panella, M.; Colonna, G.; Ciliberto, G.; Russo. A.; Costantini, S. Human MiR-544a Modulates SELK Expression in Hepatocarcinoma Cell Lines. *PLoS One*. 2016, 8, 11-6;
- Ramoutar, RR.; Brumaghim JL. Antioxidant and anticancer properties and mechanisms of inorganic selenium, oxo-sulfur, and oxo-seleniumcompounds. *Cell Biochem Biophys*. 2010, 58, 1-23;
- Raucci, R.; Colonna, G.; Guerriero, E.; Capone, F.; Accardo, M.; Castello, G.; Costantini, S. Structural and functional studies of the human selenium binding protein-1 and its involvment in hepatocellular carcinoma. *Biochim. Biophys. Acta*. 2011, *1814*, 513–522;
- Reeves, MA.; Bellinger, FP.; Berry, MJ. The neuroprotective functions of selenoprotein M and its role in cytosolic calcium regulation. *Antioxid Redox Signal*. 2010, 1, 809-18;
- Reuter, S.; Gupta, S.C.; Chaturvedi, M.M.; Aggarwal. B.B. Oxidative stress, inflammation, and cancer: How are they linked? *Free Radic Biol Med*. 2010, 49, 1603-1616;
- Robinson, M.F.; McKenzie, J.M.; Thomson, C.D.; van Rij, A.L. Metabolic balance of zinc, copper, cadmium, iron, molybdenum and selenium in young New Zealand women. *Br. J. Nutr*. 1973, 30, 195-205;
- Rodrigues, P.; de Marco, G.; Furriol, J.; Mansego, M.L.; Pineda-Alonso, M.; Gonzalez-Neira, A.; Martin-Escudero, J.C.; Benitez, J.; Lluch, A.; Chaves, F.J.; Eroles, P. Oxidative stress in susceptibility to breast cancer study in Spanish population. *BMC Cancer*. 2014, 14, 861.
- Rohan, TE.; Heo, M.; Choi, L.; Datta, M.; Freudenheim, JL.; Kamensky, V.; Ochs-Balcom, HM.; Qi, L.; Thomson, CA.; Vitolins, MZ.; Wassertheil-Smoller, S.; Kabat, GC. Body fat and breast cancer risk in postmenopausal women: a longitudinal study. *J Cancer Epidemiol*. 2013, 754815.

- Rohr-Udilova, N.; Sieghart, W.; Eferl, R.; Stoiber, D.; Björkhem-Bergman, L.; Eriksson, LC.; Stolze, K.; Hayden, H.; Keppler, B.; Sagmeister, S.; Grasl-Kraupp, B.; Schulte-Hermann, R.; Peck-Radosavljevic, M. Antagonistic effects of selenium and lipid peroxides on growth control in early hepatocellula r carcinoma. *Hepatology*. 2012, 55, 1112-21;
- Roman, M.; Jitarub, P.; Barbante, C. Selenium biochemistry and its role for human health. *Metallomics*. 2014, 6, 25-54;
- Rothwell, D.G.; Li, Y.; Ayub, M.; Tate, C.; Newton, G.; Hey, Y.; Carter, L.; Faulkner, S.; Moro, M.: Pepper, S.; Miller, C.; Blackhall, F.; Bertolini, G.; Roz, L.; Dive, C.; Brady, G. Evaluation and validation of a robust single cell RNA-amplification protocol through transcriptional profiling of enriched lung cancer initiating cells. *BMC Genomics*. 2014, 15, 1129;
- Rusolo, F.; Capone, F.; Pasquale, R.; Angiolillo, A.; Colonna, G.; Castello, G.; Costantini, M.; Costantini, S. Comparison of the seleno-transcriptome expression between human non-cancerous mammary epithelial cell line and two human breast cancer cell lines. *Oncology letters*. 2016;
- Rusolo, F.; Pucci, B.; Colonna, G.; Capone, F.; Guerriero, E.; Milone, MR.; Nazzaro, M.; Volpe, MG.; Di Bernardo, G.; Castello, G.; Costantini, S. Evaluation of selenite effects on selenoproteins and cytokinome in human hepatoma cell lines. *Molecules*. 2013, 18, 2549-62;
- Scheerer, P.; Borchert, A.; Krauss, N.; Wessner, H.; Gerth, C.; Höhne, W.; Kuhn, H.; Structural basis for catalytic activity and enzyme polymerization of phospholipid hydroperoxide glutathione peroxidase-4 (GPx4). *Biochemistry*. 2007, 46, 9041-9;
- Schwartz, DR.; Wu, R.; Kardia, SL.; Levin, AM.; Huang, CC.; Shedden, KA.; Kuick, R.; Misek, DE.; Hanash, SM.; Taylor, JM.; Reed, H.; Hendrix, N.; Zhai, Y.; Fearon, ER.; Cho, KR.. Novel candidate targets of beta-catenin/T-cell factor signaling identified by gene expression profiling of ovarian endometrioid adenocarcinomas. *Cancer Res*. 2003, 63, 2913-22;
- Sengupta, A.; Carlson, B.A.; Labunskyy, V.M.; Gladyshev, V.N.; Hatfield, D.L. Selenoprotein T deficiency alters cell adhesion and elevates selenoprotein W expression in murine fibroblast cells. *Biochem Cell Biol*. 2009, 87, 953-61;
- Shaaban, S.; Negm, A.; Ibrahim, EE.; Elrazak, AA. Chemotherapeutic agents for the treatment of hepatocellular carcinoma: efficacy and mode of action. *Oncol Rev*. 2014, 28, 8, 246;

- Silva, JM.; Dominguez, G.; Gonzalez-Sancho, JM.; Garcia, JM.; Silva, J.; Garcia-Andrade, C.; Navarro, A.; Munoz, A.; Bonilla, F. Expression of thyroid hormone receptor/erbA genes is altered in human breast cancer. *Oncogene*. 2002, 21, 4307-4316;
- Tafani, M.; Sansone, L.; Limana, F.; Arcangeli, T.; De Santis, E.; Polese, M.; Fini, M.; Russo, MA. The Interplay of Reactive Oxygen Species, Hypoxia, Inflammation, and Sirtuins in Cancer Initiation andProgression. *Oxid Med Cell Longev*. 2016, 2016:3907147;
- Takaki, A.; Yamamoto, K. Control of oxidative stress in hepatocellular carcinoma: Helpful or harmful? *World J Hepatol*. 2015, 7, 968-79;
- Tan, X.; Behari, J.; Cieply, B.; Michalopoulos, GK., Monga, SP. Conditional deletion of beta-catenin reveals its role in liver growth and regeneration. *Gastroenterology*. 2006, 131, 1561-72;
- Taylor, DP.; Clark, A.; Wheeler, S.; Wells, A. Hepatic nonparenchymal cells drive metastatic breast cancer outgrowth and partial epithelial to mesenchymal transition. *Breast Cancer Res Treat*. 2014, 144, 551-60;
- Terry, EN., Diamond, AM. Selenium. eds. Present Knowledge in Nutrition. 10th ed. 2012, 568-87;
- Thomson, C.D. and Robinson, M.F. Urinary and faecal excretions and absorptions of a large supplement of selenium: superiority of selenate over selenite. *Am. J. Clin. Nutr*. 1986, 44, 659-663;
- Utomo, A.; Jiang, X.; Furuta, S.; Yun, J.; Levin, D.S.; Wang, Y.C.; Desai, K.V.; Green, J.E.; Chen, P.L.; Lee, W.H. Identification of a novel putative non-selenocysteine containing phospholipid hydroperoxide glutathione peroxidase (NPGPx) essential for alleviating oxidative stress generated from polyunsaturated fatty acids in breast cancer cells. *J Biol Chem*. 2004, 279, 43522-9;
- Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, MT.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol*. 2007, 39, 44-84;
- Villa, E.; Camellini, L.; Dugani A.; Zucchi, F.; Grottola, A.; Merighi, A.; Buttafoco, P.; Losi, L.; Manenti, F. Variant estrogen receptor messenger RNA species detected in human primary hepatocellular carcinoma. *Cancer Res*. 1995, 55, 498-500;
- Wang, AG.; Lee, KY.; K, SY.; Choi, JY.; Lee, KH.; Kim, WH.; Wang, HJ.; Kim, JM.; Park, MG.; Yeom, YI.; Kim, NS.; Yu, DY.; Lee, DS. The Expression of Estrogen Receptors in Hepatocellular Carcinoma in Korean Patients. *Yonsei Med J*. 2006, 6, 811-816;

- Wang, AG.; Lee, KY.; Kim, SY.; Choi, JY.; Lee, KH.; Kim, WH.; Wang, HJ.; Kim, JM.; Park, MG.; Yeom, YI.; Kim, NS., Yu, DY., Lee, DS. The expression of estrogen receptors in hepatocellular carcinoma in Korean patients. *Yonsei Med J*. 2006, 47, 811-6;
- Wang, H.P.; Schafer, F.Q.; Goswami, P.C.; Oberley, L.W.; Buettner, G.R.; Phospholipid hydroperoxide glutathione peroxidase induces a delay in G1 of the cell cycle. *Free Radic Res*. 2003, 37, 621-30;
- Whanger, P.; Xia, V.; Thomson, C. Metabolism of different forms of selenium in humans. *J. Trace Elem. Electrolytes Health Dis.* 1993, 7, 121-125;
- Wilkinson, M.B., Xiao, G., Kumar, A., LaPlant, Q., Renthal, W., Sikder, D., Kodadek, T.J., Nestler, EJ. Imipramine treatment and resiliency exhibit similar chromatin regulation in the mouse nucleus accumbens in depression models. *J Neurosci*. 2009, 29, 7820-32;
- Wong, AK.; Shanahan, F.; Chen, Y.; Lian, L.; Ha, P.; Hendricks, K.; Ghaffari, S.; Iliev, D.; Penn, B.; Woodland, AM.; Smith, R.; Salada, G.; Carillo, A.; Laity, K.; Gupte, J.; Swedlund, B.; Tavtigian, SV.; Teng, DH.; Lees, E. BRG1, a component of the SWI-SNF complex, is mutated in multiple human tumor cell lines. *Cancer Res*. 2000, 60, 6171-7;
- Yagublu, V.; Arthur, JR.; Babayeva, SN.; Nicol, F.; Post, S.; Keese, M. Expression of selenium-containing proteins in human colon carcinoma tissue. *Anticancer Res*. 2011, 31, 2693-8;
- Yang, G.; Zhou, R.; Yin, S.; Gu, L.; Yan, B.; Liu, Y.; Liu, Y.; Li, X. Studies of safe maximal daily dietary selenium intake in a seleniferous area of China. Selenium intake and tissue selenium levels of the inhabitants, *Trace Elem. Electrolytes Health Dis.* 1989, 37, 77-87;
- Yim, SY.; Chae, KR., Shim, SB.; Hong, JT.; Park, JY.; Lee, CY.; Son, HJ.; Sheen, YY.; Hwang, DY. ERK activation induced by selenium treatment significantly downregulates beta/gamma-secretase activityand Tau phosphorylation in the transgenic rat overexpressing human selenoprotein M. *Int J Mol Med*. 2009, 24, 91-6;
- Zhong, R.; Liu, L.; Tian, Y.; Wang, Y.; Tian, J.; Zhu, BB.; Chen, W.; Qian, JM.; Zou, L.; Xiao, M.; Shen, N.; Yang, H.; Lou, J.; Qiu, Q3.; Ke, JT.; Lu, XH.; Wang, ZL.; Song, W3.; Zhang, T.; Li, H.; Wang, L.; Miao, XP. Genetic variant in SWI/SNF complexes influences hepatocellular carcinoma risk: a new clue for the contribution of chromatin remodeling in carcinogenesis. *Sci Rep*. 2014, 21, 4-4147;

- Zhuo, P.; Diamond, AM. Molecular mechanisms by which selenoproteins affect cancer risk and progression. *Biochim Biophys Acta*. 2009, 1790, 1546-54.