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ROLE OF PARAOXONASE-2 IN BLADDER CANCER: EFFECT OF GENE SILENCING AND OVEREXPRESSION ON CELL PROLIFERATION, MIGRATION AND CHEMORESISTANCE.

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1.1.1 Epidemiology

Bladder cancer (BC) is the ninth most common cancer in the world. In 2012, a total of 430,000 cases were diagnosed representing 3.1% of all new cancer cases [Ferlay J. et al, 2012]. The frequency of this neoplastic pathology is gender-related, meaning that it is 3–4 times higher in men than in women. BC is the fourth most frequent tumor in men with percentages of 6%, 10% and 12% in the age groups 0-49 years, 50-69 years and 70+ years, respectively. In women, the tumor is less frequent and is responsible for 1% of all female cancers in the same age groups. Among male urological tumors, only prostate cancer has a frequency higher than that of the bladder (12.3%), while all the others are less frequent: 2.3% kidney, 2.26% penis, 1.1% testis, 0.01% paratholic tissue.

In Italy, in the last 2 years, 27,000 new cases of bladder cancer were recorded (21,700 among men and 5,300 among women), equal to 7% of all incidence. In Italy, the incidence in men shows the highest values in the southern regions (83.2 /100,000 inhabitants) compared to northern and central Italy (78.6 and 66.9 per 100,000, respectively); in women the incidence is much lower and shows more homogeneous values in the areas examined (14.9 in the north, 12.4 in the center and 12.7 in the south). In 2014, in our country, there were 5610 deaths from bladder cancer, equal to 3% of deaths from cancer: 4369 in men (4.4%) and 1241 in women (1.5%). The mortality trend is slightly down in men (-1% per year) and slightly higher in women (+1.4% per year) [AIOM, 2017].

The main risk factor for bladder cancer is advanced age: the most number of cases occur in people aged over 75 [International Agency for Research on Cancer, 2012]. At the time of diagnosis, approximately 70% of urothelial tumors are superficial while the remaining 30% already present with infiltration of the muscle layer.

Patients treated with radical cystectomy, approximately 57%, have a disease infiltrating the musculature already at the time of diagnosis, while the remaining 43% develop this condition at a later time, despite the treatments carried out [Antoni S. et al, 2017]. BC (superficial and infiltrating) shows a 5-year survival of 79%,

without significant gender differences (80% in men, 78% in women) [AIRTUM, 2017]. However, there is a gradient by age: the 5-year survival is 96% in young people (<45 years) and is reduced to 66% in old people (>75 years). Unlike many tumors, there is no geographical gradient for the bladder cancer patients: the survival is 80-81% in the north and 78-79% in the center-south in men and 77-79% in north and 75-79% center-south in women. In the last few years, in Italy, 296,000 people were estimated living with a previous diagnosis of bladder cancer (240,000 men and 56,000 women). The prevalence rates per 100,000 inhabitants are on average higher in the north than in the south in both men and women, and concerning male gender center regions display the lowest values (587 cases/100,000). Over 60% of the prevalent cases have been diagnosed for over 5 years. The largest proportion of cases is observed in the 75+ age group (1984 cases/100,000 inhabitants) [AIRTUM,2016].

1.1.2 Risk factors

The risk of developing bladder cancer is low in the younger age groups and increases progressively with increasing age in both genders. It is not considered a pathology with a family predisposition, however many data describe families with multiple cases with some genetic polymorphisms and rare genetic alterations [Aben KK et al, 2002]. Extrinsic or environmental risk factors include: cigarette smoke, prolonged exposure to various chemical substances, including some drugs, infections and chronic inflammation of the urinary tract and exposure to ionizing radiation [Cogliano VJ et al, 2011]. Several risk factors are involved in the aetiology of bladder cancer: among these the most important are cigarette smoking and exposure to aromatic amines [Negri E, La Vecchia C, 2001].

- **Tobacco smoke**

To tobacco smoke is attributed 2/3 of the overall risk in males and 1/3 in women [IARC, 2004]. The risk of smokers to contract this tumor is 4 to 5 times higher than that of non-smokers and increases both with the duration and with the number of cigarettes smoked, in both sexes [Puente D et al, 2006]. The risk is reduced with the cessation of smoking, returning after about 15 years to that of non-smokers. These data have been validated in a 2016 meta-analysis of 89 observational studies performed over the last 50 years [IARC, 2004]. It is estimated that cigarette smoking is responsible for about 50% of new bladder cancer cases and 40% of deaths from this cancer [Dietrich H et al, 2001]. Among the numerous mutagenic substances contained in cigarette smoke, it has not yet been identified which the most responsible is and what precise mechanism of damage leads to the urothelium. Smoker cancer patients, including those with urothelial bladder tumors, show higher mortality rates, are more refractory to treatments, display an increased risk of complications and ofter develop a second cancers than non-smokers. Therefore preventive and dissuasive interventions from smoking by urologists and oncologists, both with counseling and with pharmacological treatments should always be carried out [Karam-Hage M et al, 2014], better if during the perioperative period [Nayan S et al, 2013]. Regarding passive smoking, a recent review showed a weak association in both sexes [OR = 1.13 (CI 95% 0.98-1.30)] [Lee PN et al, 2016].

- **Workplace exposure**

Occupational exposure to chemicals constitute another significant risk factor. Already since the last century, the increased incidence of bladder cancer among the workers in the aniline-based dye industries was known [Dietrich H et al, 2001]. Two important meta-analyzes [Cumberbatch MGK et al, 2015; Reulen RC et al, 2008] reported a great impact of exposure to occupational factors in males, related to tobacco processing, rubber production, use of dyes, textiles, paints and chemicals. Thanks to the development of laws for the protection of workers, these substances today contribute minimally to the incidence of bladder cancer, as confirmed by a study conducted in various European countries between 1976 and 1996 [Kogevinas M et al, 2003].

- **Other causes**

In North Africa and East Asia, most bladder carcinomas are caused by schistosomiasis, responsible for about 10% of bladder carcinomas in developing countries and 3% of global cases [Parkin DM et al, 2006]. A recent review confirmed a positive association with chronic cystitis and *Schistosoma*-related infections which, in countries as Egypt, may be responsible for 70% of bladder tumors [Ferrís J et al, 2013]. A positive association between human papilloma virus (HPV) infection and bladder cancer is also reported in the literature [Shigehara K et al, 2011].

A large-scale study has identified some genes predisposing to the development of urothelial carcinoma in European populations [Rafnar T et al, 2011]: genetic factors, in general, are responsible for 7% of bladder tumors. An association between bladder tumors, obesity and sausage consumption has been described (RR = 1.22) [Al-Zalabani AH et al, 2016] while the intake of Vitamins A, D, E, folate and selenium appears to be associated with a reduced risk (RR = 0.61 for selenium, 0.84 for folates) [Zelefsky MJ et al, 2012]. Physical activity also represents a protective factor for bladder cancer (RR = 0.86) as well as fruit (RR = 0.77) and vegetables (RR = 0.83) consumption. Some studies have found a potentially carcinogenic role ofn the intake of water with a high content of chlorine and arsenic [Ros MM et al, 2011; Christoforidou EP et al, 2013].

Among drugs, the use of cyclophosphamide was related to the development of infiltrating bladder cancer with a latency period of 6-13 years. This increase is independent of the development of hemorrhagic cystitis linked to the treatment itself and is also confirmed by a more recent review [Ferrís J et al, 2013]. An association between pioglitazone use and bladder cancer occurrence has been found in numerous studies [Tuccori M et al, 2016]. The European Medicines Agency (EMA), having analyzed the available data, concluded that pioglitazone remains a "valid therapeutic option for some patients with type 2 diabetes, but that there is a slight increase in cases of bladder cancer". Therefore, "careful selection of patients with periodic review of the efficacy and safety of treatment in the individual patient" is required. In particular, this therapy should not be started in subjects with bladder cancer or with a history of bladder neoplasm or with unexplored macroscopic hematuria.

1.1.3 Anatomo-pathological features of bladder cancer

The urinary bladder is within the pelvic cavity. The bladder lies behind the symphysis pubis. It is below the parietal peritoneum. Urine produced in the kidneys flows into the bladder via the ureters. The urine is ultimately excreted from the bladder through the urethra. The inner most portion of the urinary bladder is the mucosa (**Figure 1**). The histology of the mucosa is composed of transitional epithelium and connective tissue. The epithelium lies upon connective tissue called *lamina propria*.

The lamina propria is composed of areolar connective tissue. It contains blood vessels, nerves, and, in some regions, glands. 90% percent of bladder cancers derived from transitional epithelium and they are called transitional cell carcinoma (TCC). The other 10% are squamous cell carcinoma, adenocarcinoma, sarcoma, small cell carcinoma, and secondary deposits from cancers elsewhere in the body.

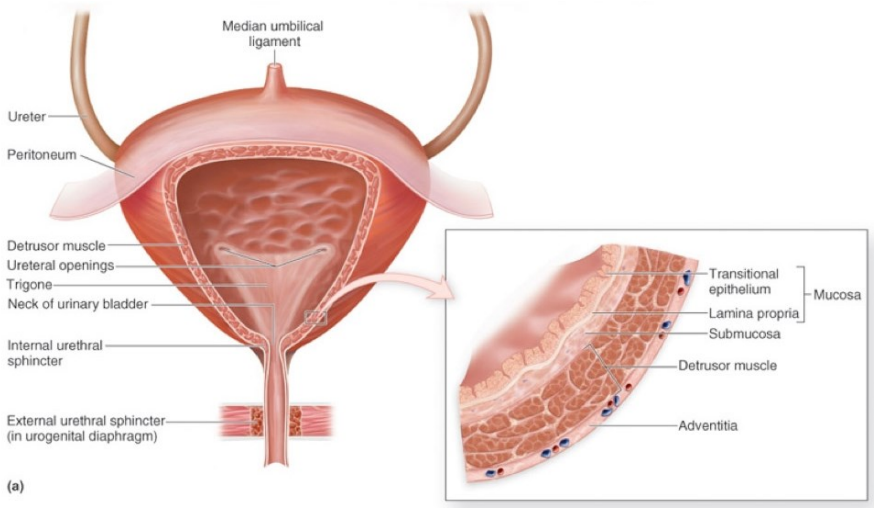


Figure 1. Structure and conformation of the urinary cavity

In the urinary tract, epithelia have continuously decreasing permeability from the extremely permeable kidney glomerular membrane down to the transitional epithelium of the ureter and bladder. The urinary epithelium is specialized, the cells form a continuous layer and are joined with tight junctions. They have specialized cell-surface proteins, and ion pumps plus proteoglycans and glycoproteins, all of which function together to maintain the impermeability of the membrane [Parsons, C.L et al, 1990]. These same mechanisms also present an active defence against bacterial colonisation.

The urothelium, once categorized as “transitional” type, is a stratified epithelium consisting of a minimum of two layers of cells; a superficial layer of capping or *umbrella cells*, and a *basal layer* separated from the submucosa/lamina propria by a continuous extracellular basal lamina (**Figure 2**). In histological preparations, these two layers usually appear to be separated by one or more layers of “intermediate” cells.

The superficial cells or *umbrella cells* have large nuclei with prominent nucleoli and dispersed chromatin, are often multinucleate and are linked to their neighbours at their luminal aspect by tight junctions and small desmosomes. They have an irregular and angular profile due to their content in a family of transmembrane particulate proteins, the uroplakins, which stiffen the membrane forming plaques that confer resistance to bladder wall distension and impermeability.

The deeper cells of the urothelium are closely related to the underlying *basal lamina*. Their cell bodies are generally columnar, with numerous folds and villi on the lateral and apical surface, and they adhere to each other only by means of small scattered desmosomes; they are therefore well adapted to adjust their shape to the degree of stretch applied to urothelium.

The *intermediate cells* have similar cytological characteristics to basal cells and can be identified only by their lack of contact with basal lamina.

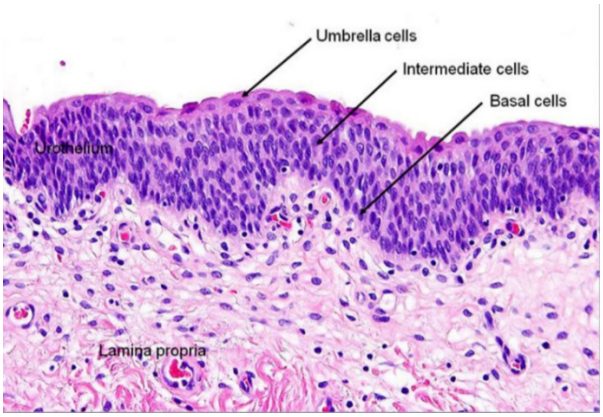


Figure 2. Representative image of normal urothelium

The below tissue layers of the bladder wall are represented by the muscolaris mucosae and by the own muscular layer (detrusor muscle) consisting of smooth muscle fibers and elastic fibers of loose connective tissue. About

95% of bladder neoplasms have epithelial origin. The remaining part is mainly represented by squamous and glandular neoplasms.

Clinical feature of bladder cancer

Urothelial cell carcinoma is subdivided into *non-infiltrating urothelial neoplasms* and *infiltrating urothelial carcinomas.* At the time of the first diagnosis, about 70% of bladder tumors are composed of non-infiltrating urothelial neoplasms [Kirkali Z et al, 2005]. The 2016 World Health Organization (WHO) classification of bladder tumors followed the 2004 edition, which had perfected the criteria already published in 1998 [Humphrey PA et al, 2016; Epstein JI et al, 1998]. This classification system officially recommended by the WHO is the International Cancer Control Partnership (ICCP) and the guidelines of the European Urology Association (UAE). However, the UAE guidelines also recommend the use of the 1973 WHO classification, which differently divides papillary-type tumors [Mostofi FK, Sobin LH & Torloni H, 1973).]. Furthermore, it is believed that maintaining the old criteria alongside the new ones facilitates the comparison of data. On the basis of the WHO 2016 classification it is possible to distinguish two forms of urothelial neoplasms: non-muscle-invasive bladder cancer (NMIBC), the tumor is confined to the transitional epithelium or to the submucosal and muscle-invasive bladder cancer (MIBC), invading and/or exceeding the lamina propria and and the muscularis mucosae [Epstein JI, Amin MB, Reuter VR, Mostofi FK, 1998].

1.1.3.1 Non-muscle-invasive bladder cancer

Non-invasive neoplasms can be distinguished into *papillary* and *flat lesions* (**Figure 3 and Tables 1 and 2**).

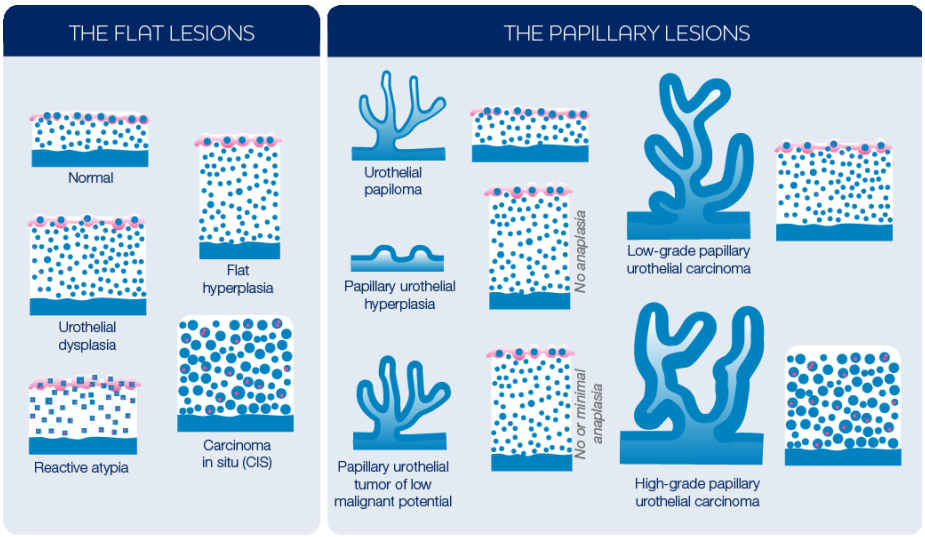


Figure 3. Classification of non-invasive neoplasms.

Table 1. Flat Tumors

Flat urothelial hyperplasia	Markedly thickened mucosa without cytological atypia
Reactive atypia	Nuclear abnormalities occurring in acutely or chronically inflamed urothelium.
Atypia of unknown significance	In some cases it is difficult to differentiate between reactive and neoplastic atypia. There may be a greater degree of pleomorphism and/or hyperchromatism out of proportion to the extent of the inflammation.
Dysplasia: low-grade intraurothelial neoplasia	Presents appreciable cytological and structual changes considered preneoplastic, but not reaching the diagnostic threshold for in situ transitional cell carcinoma (CIS).
Carcinoma in situ high-grade intraurothelial neoplasia	Consist in a flat lesion of the urothelium that is a documented precursor of invasive cancer in some cases.

Table 2. Papillary tumors.

Urothelial papilloma	Refers to the exophytic variant of papilloma, defined as a discrete papillary growth with a central fibrovascular core lined by urothelium of normal thickness and cytology.

Inverted urothelial papilloma	Inverted papilloma shares features with exophytic urothelial papilloma. Rarely, cases are hybrid in which significant portions of the lesion resemble exophytic urothelial papillomas and others inverted urothelial papillomas
Papillary urothelial neoplasm of low malignant potential (PUNLMP)	This is a papillary urothelial lesion with an orderly arrangement of cells within papillae with minimal structural abnormalities and minimal nuclear atypia irrespective of cell thickness. This lesion is not associated with invasion or metastases, except in rare cases.
Papillary urothelial carcinoma, low grade	Low grade papillary urothelial carcinomas are characterized by a variation in structural or cytological features. It is not unusual to see the fusion of adjacent papillae; the fused papillary nuclei can also be subjected to overgrade.
Papillary urothelial carcinoma, high grade	These tumors are characterized by a predominantly or totally disordered, with structural and cytological anomalies. The cells appear irregularly grouped and the epithelium is disorganized.

1.1.3.2 Muscle-invasive bladder cancer

Underlying the mucosa there is the connective tissue layer known as the lamina propria, which may contain an interrupted layer of smooth muscle known as the muscularis mucosae [Ro JY, Ayala AG, El-Naggar, 1987]. The muscle wall of the bladder contains larger muscle bundles and is referred to as either the muscularis propria or detrusor muscle. Invasive urothelial neoplasms differenciate according to the invasion site: *lamina propria* or *muscularis propria* (**Figure 4**) [Nat Rev Urol., 2019].

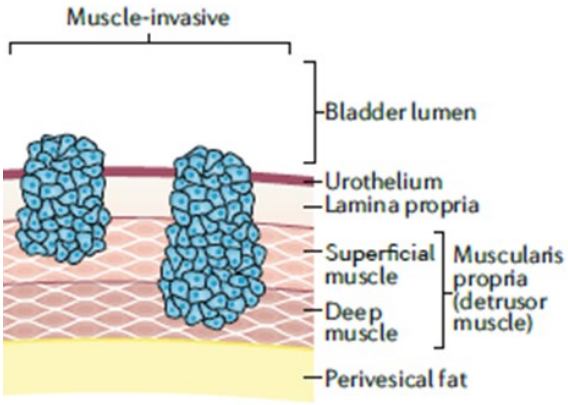


Figure 4. Clinical features of muscle-invasive bladder cancer

Lamina propria invasion: Lamina propria invasion is characterized by the presence of urothelial nests, clusters, or single cells within the lamina propria. Another feature of invasive tumor that is not always conspicuous is an associated desmoplastic or inflammatory stromal response. In low grade papillary carcinomas, large rounded nests of urothelium with peripheral pallisading within the lamina

propria, yet surrounded by stroma with a normal appearance, represent an inverted growth pattern of noninvasive carcinoma.

Muscularis Propria (Detrusor Muscle) Invasion: Muscularis propria invasion is diagnosed when tumor is seen infiltrating thick smooth muscle bundles. During transurethral resection, the distinction of muscularis mucosae from muscularis propria invasion may occasionally be difficult. These include cases with extensive infiltrating tumor where scattered wisps of muscle could either represent muscularis mucosae or disrupted and distorted muscularis propria. When there is uncertainty as to the presence of muscularis propria invasion the transurethral resection is recommended.

1.1.4 Staging and grading

The clinical staging system currently in use for bladder cancer is the tumor, node, metastasis classification (TNM) approved in 2002 by the Union for International Cancer Control (UICC) and updated in 2017 [Sobin LH, et al. 2009; Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, 2010]. The purpose of staging is to identify the extension, local invasion and the possible spread of the neoplasm at the time of diagnosis (**Table 3**). Staging is fundamental because it influences the patient's prognosis and determines the therapeutic choices to be offered. The TNM staging system makes it possible to classify bladder tumors according to local extension (parameter "T"), the involvement of regional lymph nodes (parameter "N") and the presence of metastases (parameter "M") and, therefore, to define the pathological stage (**Table 3 and 4**).

Table 3. Tumor, node, metastasis classification of bladder cancer

T- Tumor	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ: ‘flat tumour’
T1	Tumour invades subepithelial connective tissue

T2	Tumour invades muscularis propria **pT2a** Tumour invades superficial muscularis propria (inner half) **pT2b** Tumour invades deep muscularis propria (outer half)
T3	Tumour invades perivesical tissue **pT3a** Microscopically **pT3b** Macroscopically (extravesical mass)
T4	Tumour invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall **T4a** Tumour invades prostatic stroma, uterus, vagina **T4b** Tumour invades pelvic wall, abdominal wall

N - Regional lymph nodes (N)	
Nx	Lymph nodes cannot be assessed
N0	No lymph node metastasis
N1	Single regional lymph node metastasis in the true pelvis
N2	Multiple regional lymph node metastasis in the true pelvis
N3	Lymph node metastasis to the common iliac lymph Nodes

M- Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

Table 4. Pathological stage of bladder cancer.

Group	T	N	M
Stage 0a	Ta	N0	M0
Stage 0is	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2a T2b	N0 N0	M0 M0
Stage III	T3a T3b T4a	N0 N0 N0	M0 M0 M0

Stage IV	T4b All T All T	N0 N1-3 All N	M0 M0 M1

Grading is a parameter that defines the morphological aspects of bladder biopsy. The evaluation takes into account how much the tumor cells differ from those of the tissue of origin, the speed of growth and tumor progression. The tumors can thus be classified as low, high grade and papillary urothelial neoplasm of low malignant potential (PUNLMP).

Low-grade tumors have low growth rates, rarely progress and remain localized for a long time. Conversely, those with high grade, are characterized by a high mitotic index and high aggressiveness, exposing the patient to a high risk of progression and invasion of the surrounding organs and at a distance. The PUNLMP category, is represented by papillary-type lesions with increased cell proliferation but without relevant cellular alterations and low probability of progression and recurrence (**Figure 5**).

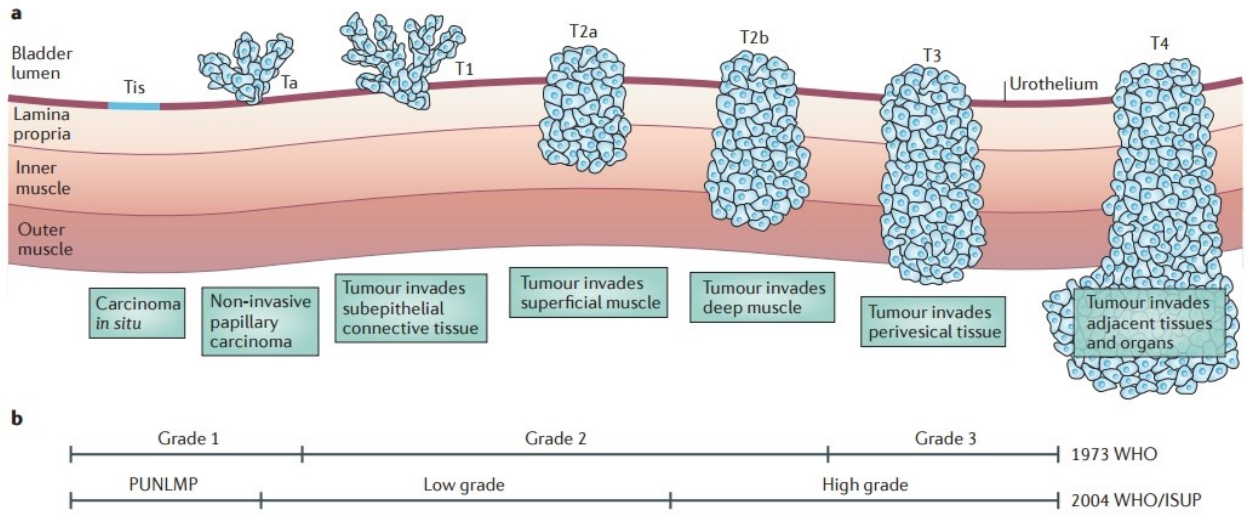


Figure 5. Bladder cancer grading system.

The WHO 2016 classification system [WHO, 2016], reproduced the WHO 2004 one [WHO, 2004], eliminating the intermediate degree (grade 2) introduced in the 1973 classification, and distinguishing neoplasms exclusively between low grade and high grade (**Table 5**).

Table 5. Comparison between old and new WHO classification.

WHO/AFIP 1973		WHO 2004 e WHO 2016
Urothelial papilloma	→	Urothelial papilloma
Transitional cell carcinoma (TCC) grade 1	↘	PUNLMP
		Urothelial carcinoma, low grade
TCC grade 2	→	Urothelial carcinoma, low or high grade
TCC grade 3	→	Urothelial carcinoma, high grade

Despite the best histological definition of the low grade and the introduction of the PUNLMP typology, the reproducibility of the new classification seems to be only moderately higher than the previous one, at least from the statistical point of view, due to a documented and non-negligible variability. From the clinical point of view it seems appropriate to maintain both classifications. In particular for pT1 carcinomas, they are classified by pathologists as high-grade urothelial carcinomas according to WHO 2016, while according to the 1973 classification, half of the cases are tracked to grade 2 and the rest to G3 with a more accurate prognostic definition [Otto W, Denzinger S, Fritsche HM et al. 2011; Pellucchi F, Freschi M, Colombo R. 2015; Van Rijn BW, Musquera M, Liu L, et al,2015].

1.1.5 Molecular genetics

The molecular mechanisms and development of bladder cancer include interactions between tumour suppressor genes, oncogenes, growth factors, adhesion molecules and angiogenic factors. The resultant of these interactions is the acquisition of the malignant phenotype by normal transitional cells.

The initiation and the development of the tumour is the result of an initial damage that involves a genetic disorder. Tumour progression follows the acquisition by the transformed normal cell further development capability (mediated by growth factors and angiogenicfactors), as well as the ability to invade and overcome the lamina propria (adhesion molecules and motility factors) thus spreading to distany sites. Repeated and sequential stages of initiation and promotion lead to the selection of cell populations increasingly affected by proliferating power. It is thought that the development of bladder cancer is a process resulting from the

evolutionary stages, passing through the stages of hyperplasia and dysplasia to malignant urothelial transformed cell [Boccardo F, Silvestrini R, 2005.]. Cytogenetic studies have revealed both chromosomal abnormalities and specific correlations between genetic alterations and biological behavior of the tumour. The most common alterations of low-grade, non-invasive tumours include the deletion of chromosome 9 and the point mutation within the gene encoding the fibroblast growth factor receptor 3 (FGFR3). The muscle-invasive bladder tumours show a broad spectrum of genetic alterations. Many studies have identified the RB1 and TP53 tumour suppressor genes, which are altered in the majority of these tumours. The involvement of TP53 is perhaps the main difference between this group of tumours and the low-grade Ta, being identified many different mutations of this gene [Bentley J, L'Hôte C, Platt F, Hurst CD, Lowery J, Taylor C, Sak SC, Harnden P, Knowles MA, Kiltie AE, 2009]. Finally, numerous deletions on chromosome arms (9q, 11p, 17p) characterize bladder carcinomas. There are also correlations between the degree of malignancy and specific deletions (3p, 17p). The stadium is associated with deletions of 3p, 17p, and with the altered expression of the RB (retinoblastoma) gene, and vascular invasion correlates with the 17p deletion [Imad Fadl-Elmula,2005].

1.1.6 Symptoms and clinical diagnosis

Macro-hematuria is the main, and often the only, initial sign of observable disease in patients with bladder urothelial neoplasia. The superficial forms (Ta-T1) rarely manifest themselves with only irritative-type disorders (urination urgency, pollakiuria) which are, on the contrary, frequently found in patients with carcinoma in situ. Tumors arising near the bladder neck or involving the urethra can also cause acute urine retention. It should be noted that the extent of hematuria do not correlate with size or clinical stage of the neoplasm. The presence of pelvic pain and hydroureteronephrosis may instead be indicative of a locally advanced disease [Shirodkar SP, Lokeshwar VB, 2008]. Hematuria is commonly associated with other disorders of the urinary system such as kidney stones and acute inflammation, so this should be supplemented with the patient's clinical history. Microhematuria is a condition that must be taken into consideration in all those cases in which the patient belongs to one of the categories defined as high risk (smoker, age greater than 40 years, etc.) [Campbell-Walsh Urology,2012]. To date, the cytological examination of the urinary sediment remains the only marker universally used in clinical practice for the diagnosis of urothelial neoplasms and the

follow-up of high-grade bladder neoplasms. This is a low-cost, non-invasive examination characterized by high sensitivity in high-grade tumors (84%), low sensitivity in low-grade (16%) and high-specificity (84-100%) tumors [Yafi FA, Brimo F, Steinberg J, Aprikian AG, Tanguay S, Kassouf W. 2015].

1.1.6.1 Imaging

The first investigation, in the field of diagnostic imaging, is the ultrasound that presents an overall accuracy of between 80 and 95% accompanied by very high specificity. The limits of this method reside in the difficulty of detecting flat lesions inside the bladder and in the impossibility of adequately studying the high excretory path, which nevertheless easily shows the possible expansion upstream. Ultrasound is normally associate with endoscopic examination. The persistence of the clinical suspicion (monosymptomatic hematuria) or the positive urinary cytology after negative endoscopic examination, must direct towards the execution of a computed tomography associated with the performance of urographic scans (Uro-CT), that has replaced traditional urography [Amis ES,1999]. Uro-CT can only document the macroscopic involvement of perivescical fat and neighboring organs, while it is not able to assess the extension of the initial stages.

The staging is defined, at bladder level, with transurethral resection (TUR) which obtains in a single time the immediate therapeutic result (resection), the histological grading and the staging data. When requested, the exam to evaluate the local extension is magnetic resonance (MR). As regards the lymph node involvement both methods (Uro-CT and MR) are able to evaluate the volume increase of lymph nodes and define a morphology (round or elongated) that helps to hypothesize a possible neoplastic involvement.

The PET-CT with Fluoro-Desoxy-Glucose (FDG) is the only imaging method able to determine with sufficient accuracy the neoplastic commitment of the lymph nodes through their uptake, if affected by disease, of the radiopharmaceutical. However, the evidence currently available is not

yet sufficient to consider PET-CT as a routine investigation in clinical practice (not clinical utility demonstration) [WooS, Shh CH, Kim SY et al 2017].

1.1.6.2 Endoscopy

Urethrocystoscopy

The urethrocystoscopy (diagnostic or follow-up) generally precedes the Uro-CT in the diagnostic procedure. In the report of the endoscopy, a careful description of the position, size, number, appearance (papillary or solid) of the bladder cancer (s) found, as well as any abnormalities of appearance of the bladder mucosa, must be reported.

Fluorescence urethrocystoscopy (PDD)

Photodynamic cystoscopy with hexaminolevulinic acid (HAL) can be used to perform guided biopsies in cases with positive urinary cytology and negative imaging and in the follow-up after TUR of non-invasive high-grade neoplasms.

Fluorescence cystoscopy showed, in various studies, greater sensitivity to identify exophytic lesions and especially flat lesions (compared to standard cystoscopy in white light) [Kołodziej A1, Krajewski W1, Matuszewski M1 et al, 2016]. The main limits of the method are represented by a substantial rate of false positives and elevated costs (drug and technical equipment).

Narrow-band imaging (NBI)

Some studies have documented an increased detection rate (compared to standard cystoscopy in white light), thanks to the new diagnosis of small invasive non-muscle lesions and in the follow-up after TUR [Denziger S, Burger M, Walter B, Knuechel R, Roessler W, Wieland WF, Filbeck T, 2007].

Endoscopic bladder resection

The histological diagnosis can be performed during the endoscopic resection of the lesion itself (TUR). The endoscopic resection intervention, in addition to a diagnostic intent, also has a staging and therapeutic intent.

1.1.6.3 Potential molecular markers

Considering the fluctuating sensitivity of urinary cytology and the relative invasiveness of cystoscopy, researchers have launched an extensive research aimed at identifying non-invasive tests that can be performed on urinary specimens, which have sensitivities and specificities that can be used both for the screening of subjects at risk and in the primary diagnosis of neoplasia or in the subsequent follow-up. Numerous potential molecular markers have therefore emerged. They are divided into soluble (**Table 6**) and cellular markers (**Table 7**).

Table 6. List of soluble markers for bladder cancer diagnosis.

Soluble markers	
BTA STAT and BTA TRAK	(bladder tumor antigen), a protein correlated to the H factor of the complement.
NMP22	(nuclear matrix protein 22), a nuclear matrix protein involved in chromatin distribution during DNA replication.
BLCA1	by using an antibody specific for the BLCA 1 protein, can determine urine protein levels.
Survivin	one of the most important anti-apoptotic proteins
Hyaluronic acid (HA) and hyaluronidase (HAase)	molecules involved in the processes of angiogenesis and tumor invasion, which therefore increase during bladder cancer

Table 7. List of cellular markers for bladder cancer diagnosis.

Cellular markers	
Telomerase	enzymes capable of preserving telomere length and determining immortality in neoplastic cell lines. PCR test.
Aneuploidies typical	affecting chromosomes 3, 7, 9 and 17 → UroVysion test using fluorescent in situ hybridization (FISH) (Têtu B. 2009).
DNA microsatellite analysis	used to detect alterations such as the loss of heterozygosity (LOH) of chromosome 9 and genomic instability → determination by PCR.
DD23	a tumor-associated antigen expressed by neoplastic bladder tissue but not by healthy urothelium, detected by a monoclonal antibody.
High molecular weight **carcinoembryonic antigen** and the **mucinous antigens**	expressed by most bladder neoplastic cells → ImmunoCyt / uCyt test.

Most of these tests have a higher sensitivity than urinary cytology in identifying low-intermediate grade forms but too low specificity to be considered a valid alternative to urinary cytology [Van Rhijn BW et al, 2005].

1.1.7 Prognosis

The 75-80% of urothelial carcinomas present at the first diagnosis the non-muscle-invasive form. In most cases these are papillary-type low-level lesions within the basement membrane (stage Ta), which tend to recur, but rarely progresses. Conversely, the tumors that invade the subepithelial connective tissue (T1) are more frequently high grade, with higher recurrence rates and higher mortality [Colombel M, Soloway M, Akaza H, Bohle A, Palou J, Buckley R, Lamm D, Brausi M, Witjes JA, Persad R., 2008]. Carcinoma in situ accounts for 10% of non-muscle invasive tumors but if untreated progresses to muscle-invasive carcinoma in 54% of cases [Lamm DL, 1992]. The 25% of patients with bladder cancer have a muscle-invasive form. Of these, 20% represents the progression of a non-muscle-invasive carcinoma treated with intravesical therapy and 80% are first diagnosed. In order to predict the short and long term risk of recurrence and progression of patients with NMIBC tumor (Ta and T1 stage), who underwent transurethral resection, the European Organisation for Research and Treatment of Cancer (EORTC) studied a risk stratification system, having the purpose of helping the urologist to choose the most suitable treatment [Sylvester RJ, van der Meijden AP, Oosterlinck W, Witjes JA, Bouffioux C, Denis L, Newling DW, Kurth K, 2006]. The system is based on six risk-predicting parameters that include: the number of tumors, the diameter (> / <3 cm), the previous recurrence rate, the stage, the concomitant presence of CIS and the grade (WHO 1973).

1.1.8 NMIBC therapy and follow-up

For patients with small superficial papillary tumors, endoscopic resection (TUR) is the best diagnostic and therapeutic approach. The resection operation is a surgical maneuver, conducted in loco-regional anesthesia, aimed at the complete removal of the exophytic portion of the tumor, its implant base and the surrounding margins. Despite the success of the TUR, the risk of recurrence and progression of the disease cannot be

completely averted [Brausi M, Collette L, Kurth K, van der Meijden AP, Oosterlinck W, Witjes JA, Newling D, Bouffioux C, Sylvester RJ, 2002]. 70% of patients experience a relapse and, in 25% of these, the tumor progresses to a muscle-invasive form within 5 years of TUR. When patients undergoing a first TUR experience incomplete resection, they must undergo a second transurethral resection (Re-TUR) within 2-6 weeks of the first surgery. In patients with NMIBC, follow-up is essentially based on performing regular cystoscopies with random biopsies. The most important prognostic factor is the outcome of cystoscopy performed 3 months after surgery. [Colombel M, et. al, 2008; Lamm DL, 1992; Sylvester RJ, et.al, 2002; Grimm MO et.al, 2003; Babjuk M, et al, 2016]. Cytological examination of the urinary sediment can be performed every 3-6 months in the first 2 years, after surgery, and subsequently at increasing time intervals. The high rates of recurrence and progression following TUR have necessitated the use of adjuvant therapies, chemotherapy and intravesical immunotherapy, to reduce this risk. The intravesical therapy needs to maximize the exposure of tumors located in the bladder cavity to therapeutic agents, limiting systemic exposure and, therefore, reducing toxicity. For most of the drugs in use, with the lowest molecular weights, it is believed that less than 5% of the therapeutic dose is found in the circulation [De Bruijn E A, Sleeboom H P, van Helsdingen P J, van Oosterom A T, Tjaden U R, and. Maes R A,1992; Chai M, Wientjes M G, Badalament R A, Burgers J K, and Au J L. 1994].

1.1.8.1 Chemotherapy

Intravesical chemotherapy consists of intravesical instillation, within 6 hours of resection, of one of the chemotherapy drugs approved in Italy: mitomycin C, epirubicin or doxorubicin [Sylvester RJ, Oosterlinck W, Witjes JA,2008; Böhle A,2004; Solsona E, Iborra I, Ricós JV, Monrós JL, Casanova J, Dumont R,1999; Rajala P, Kaasinen E, Raitanen M, Liukkonen T, Rintala E,2002]. In some cases, especially for patients defined as intermediate or high risk, intravesical chemotherapy continues to be administered even after surgery following precise therapeutic schemes. This treatment has been shown to reduce the risk of post-TUR recurrence [Huncharek M, McGarry R, Kupelnick B, 2001].

1.1.8.2 Intravesical immunoterapy

Intravesical immunotherapy is performed by administration of Bacillus Calmette-Guérin (BCG). This microorganism is an attenuated strain of *Mycobacterium bovis* that exerts its antitumor effect through the triggering of a marked immune and inflammatory response. It is supposed that the BCG penetrate the urothelial cells and that these, by exposing specific surface glycoproteins that act as antigens, are subsequently recognized by the effectors of the immune response. It follows a massive production of pro-inflammatory cytokines such as interferon-γ, IL-2 and IL-12 and the induction of cell-mediated response by helper-1 T cells [Campbell-Walsh Urology,2012]. Intravesical immunotherapy with BCG is indicated in cases of: CIS, Ta or T1 papillary carcinoma, cases of residual tumor due to incomplete TUR or failure of adjuvant chemotherapy based on intravesical therapy [AIOM,2015]. Experimental studies have also investigated systemic therapies, such as oral bropiramine immunotherapy, but none of them was superior in effectiveness to the therapies currently in use [Campbell-Walsh Urology,2012]. Other new experimental regional therapy approaches include iontophoresis, local hyperthermia, co-administration of chemotherapy drugs and substances that increase their permeability in urothelial cells, magnetic targeting and gene therapy [Shen Z, Shen T, Wientjes M G, O'Donnell M A, and Au J L.S, 2008].

1.1.9 MIBC therapy and follow-up

Muscle-invasive bladder cancers are treated with a radical cystectomy, associated with loco-regional lymph node removal. Radical cystectomy is an invasive intervention which consists, in the male patient, in the removal of bladder, prostate and seminal vesicles; while in the woman the anterior wall of the vagina, the uterus and the urethra are removed in addition to the bladder.

Survival after radical cystectomy is closely associated with final pathological staging. Patients have a 5-year relapse-free survival of approximately 60%. Patients with pT3b N0 have 5-year survival rates between 53% and 62%, pT4 lower 50% and pN + (lymphoid infiltration) less than 35% [Hautmann RE, Gschwend JE, de Petriconi RC, et al, 2006].

1.1.9.1 Neoadjuvant chemotherapy

Neoadjuvant chemotherapy is, by definition, the administration of chemotherapy to patients with the intention of consolidating the radical cystectomy. But it is indicated only for those patients who enjoy a good state of health and maintained renal function. Systemic chemotherapy is instead mainly adopted in cases of metastatic disease (M +), where cisplatin-based treatment schemes have been the standard therapy for several years.

In standard clinical practice, for patients with metastatic disease, first-line chemotherapy is based on combinations of drugs rather than cisplatin alone, such as the combination cisplatin-gemcitabine (CG) or the combination methotrexate, vinblastine, adriamycin and cisplatin (MVAC). Response rates are 49% and 46% in patients treated with CG or MVAC, respectively. Mean survival is 14 months for GC and 15.2 months for MVAC, and 5-year survival rates are respectively 13.0% and 15.3% [Von der Maase H, Hansen S W, Roberts JT et al,2000; Von der Maase H, Sengelov L, Roberts JT et al,2005;]. Although cisplatin-based regimens are the optimal treatment for advanced-stage tumors, almost half of patients are not eligible for these therapies [Galsky MD et al, 2011]. The known side effects limit the clinical utility of cisplatin and factors such as advanced age, impaired organ function and general poor health status exclude a good part of the patients [Loehrer PJ Sr., Einhorn LH, Elson PJ et al,1992; Saxman SB, Propert KJ, Einhorn LH et al,1997]. Furthermore, although cisplatin often provides regression or stabilization of the disease, about 50% of patients show *de novo* chemoresistance or develop it in itinere [Galluzzi L, Senovilla L, Vitale I et al,2012]. The second-line drugs mainly used are: paclitaxel, docetaxel, pemetrexed and viniflunine. Paclitaxel (or taxol) and Docetaxel, being this latter a semisynthetic variant of paclitaxel, act by stabilizing the microtubules and inhibiting their depolymerization; this causes the inhibition of the normal dynamic reorganization of the microtubule structure, essential for cellular mitotic functions [de Weger V A B, Jos H, Schellens, Jan H M,2014]. Viniflunine is a fluorinated derivative of vinblastine. The mechanism of action of these substances is opposite to the taxanes: they bind the tubulin inhibiting its polymerization in microtubules. [Bellmunt J, Petrylak DP, 2012]. Pemetrexed is chemically similar to folic acid, it works by

inhibiting the formation of purine and pyrimidine nucleotide precursors, interfering with nucleic acid synthesis [Baldwin C M & Perry C M A,2009].

1.1.10 Structure and feature of cisplatin

Cisplatin, also called cisplatinum, is a metallic (platinum) coordination compound with a square planar geometry. It is a white or deep yellow to yellow-orange crystalline powder at room temperature. It is slightly soluble in water and soluble in dimethylprimanide and N-Ndimethylformamide. Cisplatin is also called "cis-diaminodichloroplatin" beacuse the prefix "cis" refers to the arrangement of atoms around the central platinum atom. In fact, the two chlorine atoms are arranged on the same side with respect to the platinum atom and the two amino groups are arranged on the other side as can be seen in figure 6.

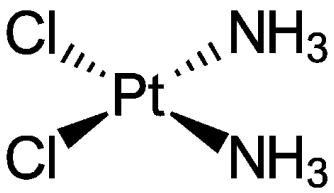


Figure 6. Chemical structure of cisplatin (Weiss et al., 1993).

Cisplatin has a molecular weight of 301.1 gm/mol, a density of 3.74 g/cm3, a melting point of 270°C, a log Kow of -2.19 and a water solubility of 2.53 g/L at 25°C [HSDB, 2009]. The biological activity of cisplatin was discovered about 125 years after the initial report of its synthesis and characterization [Peyrone,1845]. In 1965, Rosenberg and co-workers reported that the cytostatic effect induced by electric fields on cultures of *Escherichia coli* was due to the formation of cisplatin and its corresponding tetrachloroplatinum (IV) analog by electrochemical reactions on platinum electrodes [Rosenberg. B,1965]. In 1969, preclinical pharmacological studies had shown that cisplatin was endowed with antitumor properties [Rosenberg, B.; Van Camp, L.; Trosko, J.E.; Mansour, V.H, 1969].

1.1.10.1 Biochemical mechanism of action of cisplatin

Cisplatin is administered intravenously to the patients within a sterile saline solution [Polovich M, White JM, Kelleher LO, 2005] given the high concentration of chloride, part of the cisplatin remains unchanged and neutral in the blood stream [Marija Petrovic, Danijela Todorovic,2016]. The plasma proteins like albumin, transferring, cysteine can strongly bind cisplatin resulting in deactivation of large amount of applied cisplatin [N. Nagai, R. Okuda, M. Kinoshita, H. Ogata, 1996]. It is reported that 65–95% of cisplatin may bind with blood plasma protein just within 24h of administration [A.I. Ivanov, J. Christodoulou, J.A. Parkinson, K.J. Barnham, A. Tucker, J. Woodrow, P.J. Sadler,1998]. The remaining cisplatin can be transported within tumor cells by passive diffusion through plasma membrane [Rebecca A. Alderden, Matthew D. Hall, Trevor W. Hambley,2006]. Modern studies reveal that copper transporter protein CTR1 is also responsible for cisplatin uptake [S. Ishida, J. Lee, D.J. Thiele, I. Herskowitz,2002].

When the cisplatin enters the cell, it is activated by replacing one or two of the chloride ligands into water ligand. This mono and/or diaquation of cisplatin occurs because concentration of chloride in cytoplasm is relatively low (approximately 4–20 mM) and they are potent electrophile. [S. Dasari, P.B. Tchounwou,2014] because water is better leaving group than chloride [M.A. Fuertes, J. Castilla, C. Alonso, J.M. Pérez,2002].

The main toxic actions of cisplatin take place through the formation of reactive oxygen species (ROS) and subsequent apoptosis. Cisplatin induces oxidative stress by forming ROS like hydroxyl radicals, superoxide which depends on the concentration of cisplatin and time of exposure [A. Brozovic, A. Ambriović-Ristov, M. Osmak,2010]. ROS is thought to be responsible for peroxidation of lipid, depletion of sulfhydryl groups, changed different signal transduction pathways, which can cause DNA damage and consequently apoptosis of cells [Ana-Maria Florea, Dietrich Büsselberg,2011]. Cisplatin may also induce cell apoptosis from cell membrane. The release of cytochrome C and procaspase-9 that bind with cytosolic Apaf-1 (apoptotic protease activating factor 1) and ATP (adenosine triphosphate) to form an apoptosome complex which activates caspase-9 [M.A. Fuertes, C. Alonso, J.M. Perez,2003]. The activated caspase-9 interacts

with other caspases to activate caspase-3, caspase-6 and caspase-7, which lead to apoptosis through cleavage of key substrates [John C. Reed, 2002].

Another mechanism of apoptosis activation is mediated by the type II transmembrane protein Fas ligand (FasL) which, upon binding Fas receptor, induce the formation of apoptosome complex from FADD (Fas-associated death domain) and procaspase-8 [M.A. Fuertes, J. Castilla, C. Alonso, J.M. Pérez,2002]. This apoptosome complex activates caspase-8 which subsequently activates caspase-3, caspase-6 and caspase-7 that finally cleaves key substrate and leads to cell apoptosis (see Fig. 7).

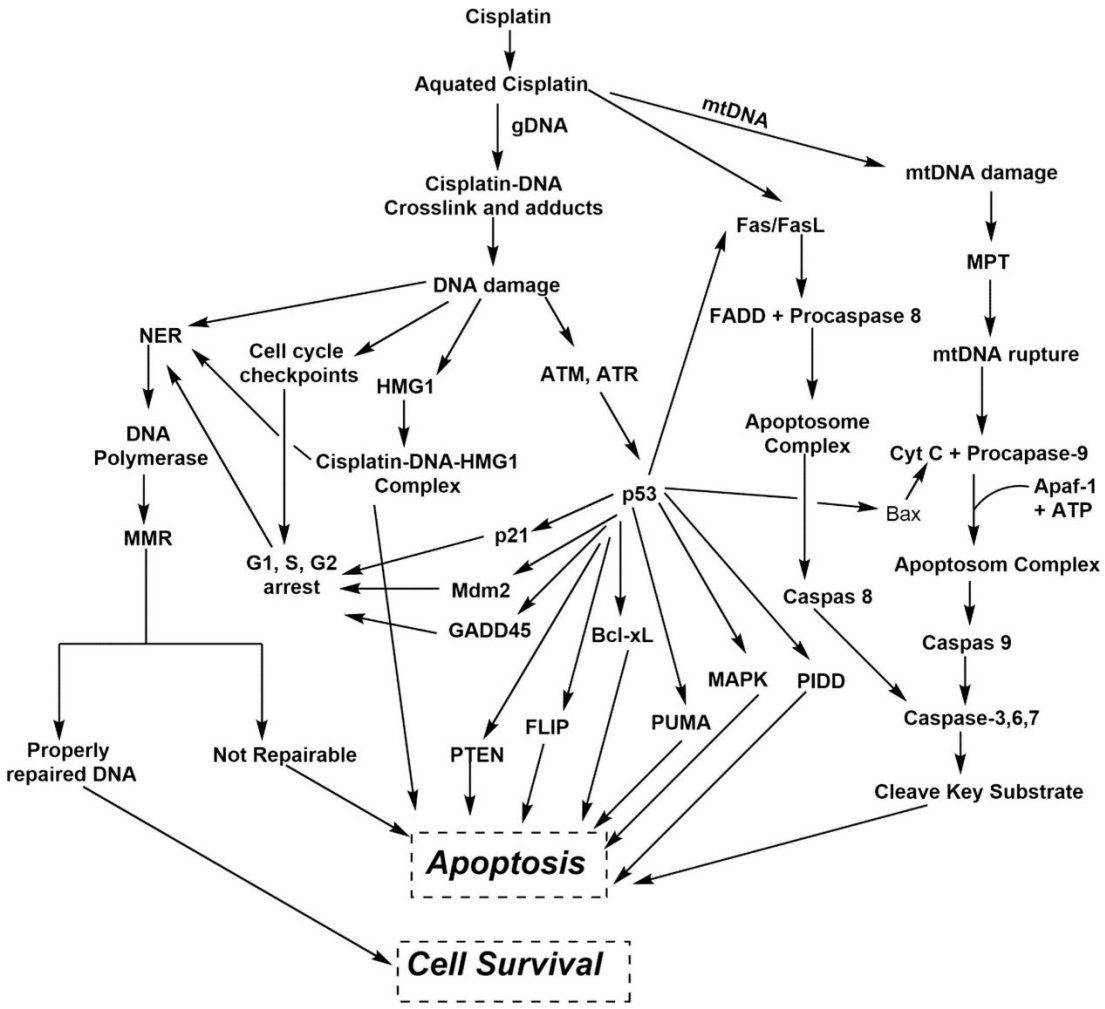


Figure 7. Mechanisms of action of cisplatin and its anticancer activity.

The main target of cisplatin is genomic DNA (gDNA), but a very little amount (~1%) of intercellular cisplatin is generally bound to gDNA [X. Lin, T. Okuda, A. Holzer, S.B.

Howell,2002]. Several proteins like HMG (high mobility group) proteins can easily recognize cisplatin-boud DNA and are able to shield and protect it from repairing through NER (nucleotide excision repair) mechanism, thus couteracting repair of DNA and cell survival [D.B. Zamble, Y. Mikata, C.H. Eng, K.E. Sandman, S.J. Lippard,2002]. The DNA repairing mechanism is one of the most important parts of cisplatin cytotoxicity. The proteins related to DNA repairing are NER and MMR (mismatch repair). The NER system contains 17 different proteins which recognizes Pt-DNA intrastrand crosslinks and then excises DNA sequences up to 20–29 base pairs [J.G. Moggs, D.E. Szymkowski, M. Yamada, P. Karran, R.D. Wood,1997]. DNA polymerase fills the remaining gaps [J.T. Reardon, A. Vaisman, S.G. Chaney, A. Sancar,1999]. The MMR would try to input correct nucleotide on the nondamaged strand opposite to the intrastrand adduct between two adjacent guanines [Alexandra Vaisman, Maria Varchenko, et al.,1998]. When it failed to repair the damages, apoptosis of the cell occurs. Cell apoptosis is also possible through cell cycle arrest (G1, S and G2 phase) caused by cisplatin [J.M. Wagner, L.M. Karnitz,2009]. The tumor suppressor protein p53 plays a central role in cisplatin induced apoptosis, since it can cause apoptosis directly by different mechanisms like: degradation of FLIP (flice-like inhibitory protein), direct binding and counteracting the antiapoptotic function of Bcl-xL (B-cell lymphoma-extra-large) and overexpression of PTEN (phosphatase and tensin homolog) [S. Dasari, P.B. Tchounwou, 2014; Alakananda Basu, Soumya Krishnamurthy,2010]. Again p53 actives PUMA [John R. Jeffers, et al.,2003], PIDD [Y. Lin, W. Ma, Benchimol S. Pidd,2000] and MAPK protein family which are responsible for cell apoptosis. It is also reported that p53 facilitates Fas/FasL which lead to apoptosis through caspase 8, caspase 3 pathway [A. Brozovic, A. Ambriović-Ristov, M. Osmak,2010].

1.1.10.2 Therapeutic uses and clinical toxicity of cisplatin

The usual dosage of cisplatin is 20mg/m^2 per day for 5 days, 20-30mg per week for 3-4 weeks or 100mg /m^2 once every 4 weeks. It is important, to prevent renal toxicity, to induce a chloride diuresis before treatment. The appropriate amount of cisplatin is then diluted in a solution of

dextrose and mannitol in physiological solution and administered intravenously over a period of 4-6 hours.

Cisplatin treatment is associated with toxic side effects including nephrotoxicity (de Jongh et al., 2003) and cardiotoxicity (Al-Majed, 2007). In many cases, various pathologies affecting the heart muscle have been reported, such as arrhythmias, myocarditis, cardiomyopathies and congestive heart failure (Yousef et al., 2009). The toxicity of cisplatin also results in a decrease in the antioxidant defense system understood as the formation of reactive oxygen species and a decrease in the activity of enzymes with antioxidant capacity (Kart et al., 2010). Considering the non-negligible side effects due to treatment with cisplatin, research efforts are currently aimed at designing structural analogues that preserve antitumor efficacy and reduce the toxicity of the patient (Mantri et al., 2006).

1.1.10.3 Resistance of cisplatin

The major problem of using cisplatin is the different sensitivity of cancer cells to it. Very sensitive tumor cells are testicular, ovarian, head and neck and small cell lung cancer, while colorectal cancer and nonsmall cell lung cancer are very resistant to cisplatin [M.A. Fuertes, C. Alonso, J.M. Perez, 2003; Franco M. Muggia, Gerrit Los,1993]. Cisplatin resistance may occurr at different moments. During drug circulation, through bloodstream, some proteins, as human serum albumin and cysteine, can bind the cisplatin [Edwin L.M. Lempers, Jan Reedijk,1991]. It has been shown that [M.A. Fuertes, C. Alonso, J.M. Perez, 2003] that reduced accumulation of cisplatin is due to the reduction in drug absorption rather than to the increase in the outflow of the drug. It has been known that the copper transport protein Ctr1, together with passive transport, is responsible for the influx of cisplatin. Cisplatin causes a decrease in Ctr1 concentration and therefore the influx of cisplatin decreases significantly, resulting in drug resistance [X. Lin, T. Okuda, A. Holzer, S.B. Howell, 2002]. Two other copper transporter ATP7A and ATP7B help to export cisplatin from cell and lead to resistance [K. Nakayama, K. Miyazaki, A. Kanzaki, M. Fukumoto, Y. Takebayashi,2001]. TMEM205, a membrane protein, is also responsible for cellular resistance to cisplatin [S. Dasari, P.B. Tchounwou,2014]. The most important mechanism of cisplatin

resistance is intracellular inactivation of cisplatin through binding with glutathione and metallothioneins [T. Ishikawa, C.D. Wright, H. Ishizuka, 1994]. Alterations of the MMR and NER systems promote cisplatin resistance. Cells with over expression with NER cause very lower sensitive to cisplatin [M. Selvakumaran, D.A. Pisarcik, R. Bao, A.T. Yeung, T.C. Hamilton, 2003]. It is well established that altered expression of oncogenes like c-fos, Hras, c-abl and c-myc and tumor suppressor gene p53 can create cellular resistance to cisplatin [M. Kartalou, J.M. Essigmann, 2001]. The resistance of cisplatin is also due to drug induced dysregulation of microRNA function, thus causing problems in cell signaling, DNA methylation and invasiveness or cell survival which result in resistance of cisplatin [Ana-Maria Florea, Dietrich Büsselberg, 2011; I.P. Pogribny, J.N. Filkowski, V.P. Tryndyak, A. Golubov, S.I. Shpyleva, O. Kovalchuk, 2010].

1.1.11 Structure and feature of gemcitabine

Gemcitabine (2'2'-difluoro-2'-deoxycytidine, dFdC) is a nucleoside analog (NA) in which the hydrogen atoms on the 2′ carbon of deoxycytidine are replaced by two fluorine atoms (Figure 8) [Hertel LW, Kroin JS, Misner JW, Tustin JM. 1988; Toschi L, Finocchiario G, Bartolini S, Gioia V, Cappuzzo F.,2005; Mini E, Nobili DS, Caciagli B, Landini I, Mazzei T.,2006; Bergman AM, Pinedo HM, Peters GJ.,2002].

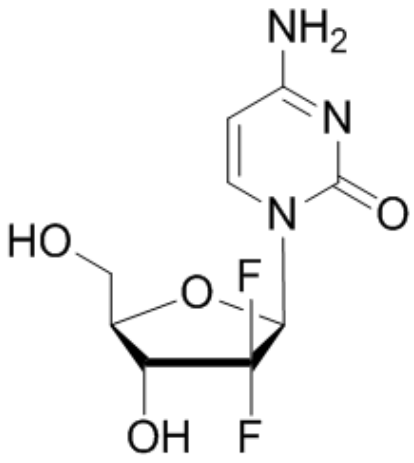


Figure 8. Structure of gemcitabine.

1.1.11.1 Biochemical mechanism of action of gemcitabine

Considering its hydrophilic nature, gemcitabine needs active transporters to be taken up in the cell, represented by both human equilibrative (hENTs) [Young JD, Yao SY, Sun L, Cass CE, Baldwin SA,2008; Johnson ZL, Lee JH, Lee K et al.,2014] and concentrative (hCNTs) [Spratlin JL, Mackey JR,2010; Hung SW, Marrache S, Cummins S et al.2015] nucleoside transporters. Deoxycytidine kinase (dCK) catalyzes the gemcitabine activation into the monophosphate form and subsequently by other nucleotide kinases to its active metabolites, gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP) [Honeywell RJ, Ruiz van Haperen VWT, Veerman G, Peters GJ,2015]. The phosphorylation of gemcitabine by dCK is the rate-limiting step in the accumulation of the active intracellular metabolite dFdCTP [Gandhi V, Plunkett W,1990; Abbruzzese JL, Grunewald R, Weeks EA et al,1991] (Figure 9).

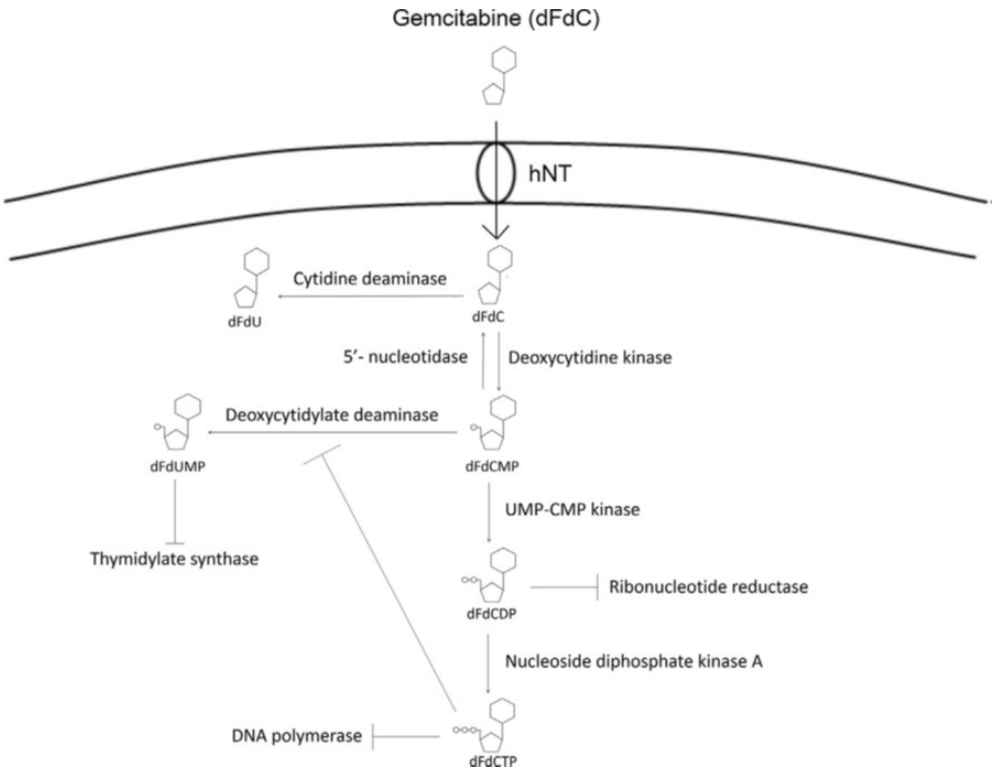


Figure 9. Cellular metabolism of gemcitabine

Cellular accumulation of its 5′-triphosphate (dFdCTP) is associated with gemcitabine cytotoxicity. The incorporation of dFdCTP into DNA, may causes the stopping of cell cycle into G0/G1 and S phase which triggers apoptosis [Huang P, Robertson LE, Wright S, Plunkett W.

1995; Garcia-Diaz M, Murray MS, Kunkel TA, Chou KM. 2010]. Inside the cells, the presence of gemcitabine decreases cellular deoxynucleotide (dNTP) pools especially deoxyadenosine triphosphate (dATP) and deoxycytidine triphosphate (dCTP) in a concentration-dependent manner [van Moorsel CJ, Smid K, Voom DA, Bergman AM, Pinedo HM. Peters GJ,2003]. The reduction in the intracellular concentrations of dNTPs enhances the incorporation of dFdCTP into DNA [Ruiz van Haperen VW, Veerman G, Vermoken JB, Peters GJ.,1993]. dFdCDP forms complexes with ribonucleotide reductase subunits and ATP causing depletion of the deoxyribonucleotide pool required for DNA synthesis. It is very important that the dFdCTP is incorporated into the DNA and, after the incorporation of another nucleotide, leads to the termination of the DNA strand polymerization [Heinemann et al., 1988]. The ability of cells to incorporate dFdCTP into DNA is critical for gemcitabine-induced apoptosis. Gemcitabine is inactivated by cytidine deaminase, an enzyme expressed both in cancer cells and in healthy cells. Preliminary evidence suggests that the response to gemcitabine in pancreatic cancer is positively correlated with a high expression of hENT [Giovanetti et al., 2006] and a low expression of ribonucleotide reductase subunits, but forms of resistance have been found in tumors with low expression of deoxycytidine kinase and high levels of the inactivating enzyme cytidine deamminase [Ohhashi, 2008]. The overexpression of the HMGA1 transcription factor in murine tumor causes resistance to gemcitabine [Liau and Whang, 2008], probably due to the effect on the Akt-dependent survival pathway. Another important mechanism of action is the induction of apoptosis through the caspase pathway [Ferreira et al., 2000; Habiro et al., 2004]. Indeed, in pancreatic cancer cells gemcitabine activates the MAPK protein kinase which induces apoptosis in response to cellular stress [Habiro et al., 2004; Kummer et al., 1997]. Kopper et al. have shown that the activity of the MK2 protein (Mitogen-Activated Protein Kinase-Activated Protein Kinase 2) is necessary for the activation of the apoptotic cascade induced by gemcitabine in cells in vitro. In fact, MK2 inhibition leads to the survival of osteosarcoma cells when treated with gemcitabine [Kopper et al., 2013]. In contrast, the activation of p38-MAPK and MK2 can induce

phosphorylation of the Hsp27 protein, a chaperone that leads to the suppression of cell growth in vitro [Nakashima et al., 2006].

1.1.11.2 Therapeutic uses and clinical toxicity of gemcitabine

Gemcitabine has modest activity if used alone in the clinical setting, with less than 50% of stable disease rate and response rates usually below 20%, so combined therapy is preferred. As previously reported, the intracellular concentration of dFdCTP strongly correlates with its incorporation into the DNA and with inhibition of synthesis of DNA [Huang P, Chubb S, et.al, 1991]. It has been hypothesized that a slow-release infusion program may be more effective in achieving higher intracellular concentrations of dFdCTP with a lower toxicity profile [Gandhi V, Plunkett W, DU M, Ayres M, Estey EH.,2002]. The increase in the intracellular concentration of dFdCTP also depends on the association with other drugs, such as cisplatin, which suggested that administration of cisplatin 24 hours before gemcitabine could lead to the highest accumulation of dFdCTP in peripheral blood mononuclear cells on patients with solid tumors [van Moorsel CJ, Pinedo HM, Veerman G et al. 1999]. The main toxicity of gemcitabine is myelosuppression and liver toxicity, excpecially with prolonged infusions. Non-hematological toxicities include a parainfluenza syndrome, asthenia and rarely a posterior leukoencephalopathy syndrome. In more than 40% of patients, a moderate reversible increase in hepatic transaminases can be observed. In the first two cycles interstitial pneumonia may develop, which sometimes progresses to acute respiratory distress syndrome, usually responsive to corticosteroids. Although very rarely, patients treated with gemcitabine for many months may develop a slow progressing uremic-hemolytic syndrome, which requires drug withdrawal [Humphreys et al., 2004]. Gemcitabine is a very potent radio sensitizer, probably as a result of inhibition of ribonucleotide reductase, dATP depletion and DNA repair inhibition [Flanagan et al., 2007] and should not be used with radiotherapy, if not in tightly controlled clinical trials.

1.2 Paraoxonase gene family

The paraoxonase (PON) gene family consists of a cluster of three distinct but structurally similar genes (PON1, PON2, PON3), located in tandem on the short arm of human chromosome 7 (7q21.3-22.1) and on murine chromosome 6 (Figure 10) [Primo-Parmo SL, Sorenson RC, J Teiber, La Du BN, 1996; SorensonRC, Primo-Parmo SL, Camper SA, La Du BN,1995].

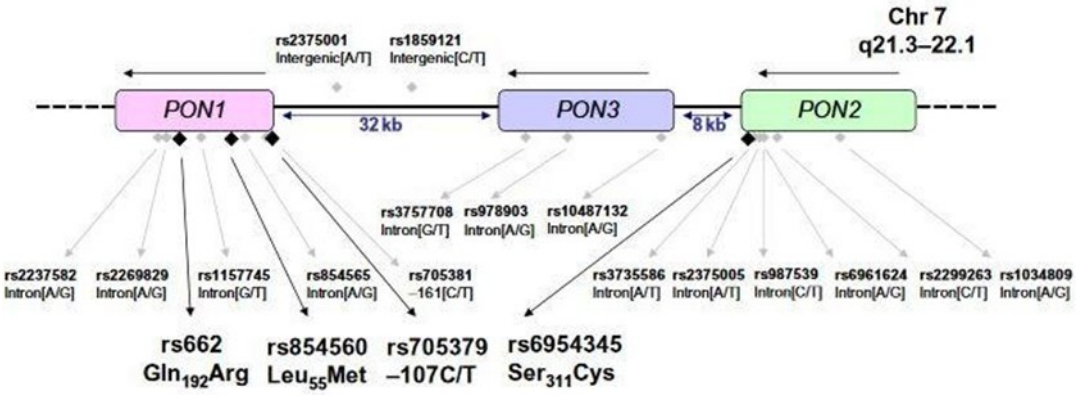


Figure 10. The gene locus of the PON family.

In humans, the three genes have a 70% homology at the nucleotide sequence level and the homonymous proteins derived from them share 60% of their amino acid sequence. The members of the PON family are also well conserved in mammals where there are values of identity ranging between 79-95% for the amino acid sequence and between 81-95% for the nucleotide sequence, between different species. Phylogenetic analyzes, based on structural homology studies, suggest that three genes originate through mechanisms of gene duplication and identify PON2 as the evolutionarily oldest member of the family, followed by PON3 and finally by PON1 [Draganov DI, Teiber JF, Speelman A, Osawa Y, Sunahara R, La Du BN, 2005]. In all three genes there are 9 exons approximately the same in length [Rajkovic MG, Rumora L, Barisic K.,2011]. The human genes PON1 and PON2 have exon-intron junctions that occur in equivalent positions. PON1 has an extra codon at position 106 (equivalent to a lysine in human PON1).

At mRNA level, PON2 has a coding sequence of 1062 nucleotides which results in the synthesis of a protein consisting of 354 amino acids, having a molecular mass of about 40 kDa. The PON2 gene has more than one site of initiation for transcription and primary transcripts may undergo several splicing events.

The nomenclature of PONs derives from the studies conducted on PON1, the first paraoxonase to be isolated and the only one, among the PON family, capable of hydrolysing the toxic metabolite of parathionyl insecticide *in vitro*: paraoxone. This substance acts as a parasympathomimetic, inhibiting acetylcholinesterase (AChE), an enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine [Ecobichon DJ,2001]. PON2 and PON3 do not share this precise enzymatic activity with PON1 but act on different substrates with variable specificities. The PONs have indeed been reclassified as lactonase.

1.2.1 Polymorphisms of PON2

There are two most studied polymorphisms of the PON2 gene: A148G and C311S. These two polymorphisms derive respectively from the replacement in position 148 of the amino acid alanine with glycine and in position 311 of the amino acid cysteine with serine. Shi J. et al, in the study published in 2004, suggest an involvement of the S311 polymorphic form in the increase of the onset of cardiovascular pathologies, diabetes, Alzheimer's disease and sporadic amyotrophic lateral sclerosis [Shi J et al., 2004]. A relationship between the A148G and C311S polymorphisms and the alterations in plasma LDL and glucose levels has been highlighted. The Ala148/Ser311 homozygous subjects had significantly higher total cholesterol and LDL cholesterol levels compared to heterozygous or homozygous subjects for the second allele [Hegele RA et al., 1999, Hegele RA et al., 1997].

1.2.2 Structure and localization of PON2

To date, PON1 remains the only well-characterized member of the PONs family. PON1 is a 45-kDa glycoprotein composed of 355 amino acids, which is synthesized in the liver and secreted into blood flow, where was found in association with high-density lipoproteins (HDL) [Hassett C, Richter RJ, Humbert R, et al., 1991]. In mice PON1 mRNA was detected in a variety of tissues including liver, kidney, heart, brain, intestine and lungs [Primo-Parmo SL, Sorenson RC, J Teiber, La Du BN,1996; Camps J, Marsillach J, Joven J,2009]. Immunohistochemistry confirmed the presence of PON1 at the protein level in various murine tissues

including most of the epithelia, brain, muscle and adipose tissue. The absence of apolipoprotein A-I (ApoA-I) supports the hypothesis that PON1 is synthesized locally and not simply transported via HDL in circulation [Marsillach J, Mackness B, Mackness M, et al.,2008]. In humans, the liver displays the highest expression of PON1 and acts as a source of this protein for its release into the circulation, a task that it is also achieved for PON3. It is interesting to note that an intracellular antioxidant role has been proposed for PON1 in the liver, a function that seems plausible given the role of liver microsomes in xenobiotic metabolism and the generation of free radicals and by-products of the oxidation of these substances [Primo-Parmo SL, Sorenson RC, J Teiber, La Du BN,1996]. PON3 is certainly the least studied of PONs. Just like PON1, PON3 is detectable in the circulation in association with HDL, while its enzymatic properties overlap both with those of PON1 and PON2.

PON2 differs from other PONs since it is not possible to trace it in serum. It was first detected in the brain, liver, kidneys and testicles [Mochizuki H, Scherer SW, Xi T, Nickle DC, Majer M, Huizenga JJ, Tsui LC, Prochazka M,1998] but it is also expressed in many other sites in which it remains confined. Ng and colleagues have decreed that the highest levels of PON2 transcripts are found in the heart, lung, and placenta. The expression of PON2 was also observed in primary and immortalized human endothelial cells and in arterial smooth muscle cells [Ng CJ, Wadleigh DJ, Gangopadhyay A, Hama S, Grijalva VR, Navab M, Fogelman AM, Reddy,2001]. PON2 has a long hydrophobic N-terminal tail which allows it to behave like a type II trans membrane protein, able to occupy the entire thickness of the phospholipid double layer (**Figure 11**). However the subcellular localization of PON2 remains ambiguous.

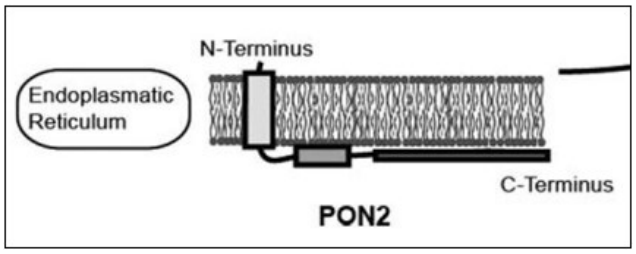


Figure 11. The model prpposed for PON2 arrangement in membranes: the N-terminal transmembrane domain acts as an anchor; the terminal C portion contains the enzymatic domain of the protein that can counteract lipid peroxidation.

Although early studies have widely demonstrated that PON2 is an intracellular protein, subsequent results obtained by confocal microscopy, cell fractionation and membrane protein digestion techniques have revealed that PON2 can also be found in the nuclear envelope, in the endoplasmic reticulum (RE), in the plasma membrane and associated with mitochondria. For example in EA.hy 926 cells (human vascular endothelial cells) it was localized in the membrane of RE and nucleus [Horke S, Witte I, Wilgenbus P, Krüger M, Strand D, Förstermann U.,2007], while in Caco-2 and HT-29 lines (human adenocarcinoma of the colon) in addition to the endoplasmic reticulum, PON2 has been traced within the plasma membrane [Shamir R, Hartman C, Karry R, Pavlotzky E, Eliakim R, Lachter J, Suissa A, Aviram M,2005; Rothem L, Hartman C, Dahan A, Lachter J, Eliakim R, Shamir R., 2007]. Other experimental works have also assumed a possible translocation mechanism of PON2 from the RE to the plasma membrane mediated by high levels of intracellular Ca^{2+} associated with oxidative damage. These conflicting results suggest that the subcellular localization is influenced by the cell type in which PON2 is expressed (**Figure 12**).

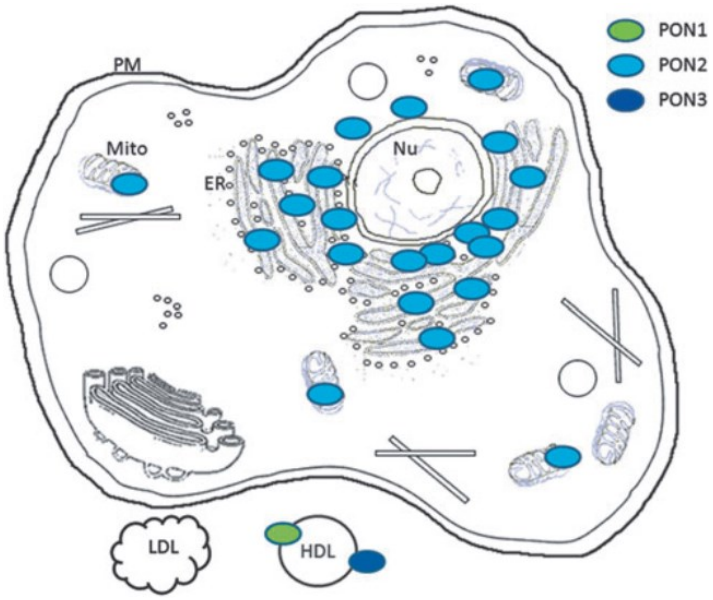


Figure 12. Subcellular localization of PON proteins.

In 2004, the tertiary structure of PON1, obtained by X-ray diffraction of the recombinant crystallized protein, was resolved and deposited in the Protein Data Bank (PDB). The three-dimensional structure of PON2 has not been solved yet, but comparative *in silico* analyzes have been carried out, aimed at extrapolating the structural and functional characteristics of PON2, starting from its primary structure.

PON1 is a "six-bladed β-propeller", characterized by 6 β sheets called "blade-shaped" arranged toroidally around a central axis. Each leaflet is typically made up of four antiparallel β filaments arranged so that the first and fourth strands are almost perpendicular to each other. The active site of the enzyme is located in the cavity formed in the center of the structure and the catalytic residues reside in the loops that connect the different β-sheets that follow one to another. In PONs the stability of the structure is also implemented by a disulfide bridge which in the PON1 occurs between Cys42 and Cys353. The disulfide bridge covalently binds the N- and the C-terminal portions of the protein. Inside the cavity there are two Ca^{2+} ions, one of which has been attributed a structural value, and to the other a catalytic role [Hare M, Aharoni A, et.al, 2004]. The results obtained, following the *in silico* comparative analysis between PON1 and PON2, allowed to generate a predictive model of the three-dimensional structure of the PON2 (**Figure 13**).

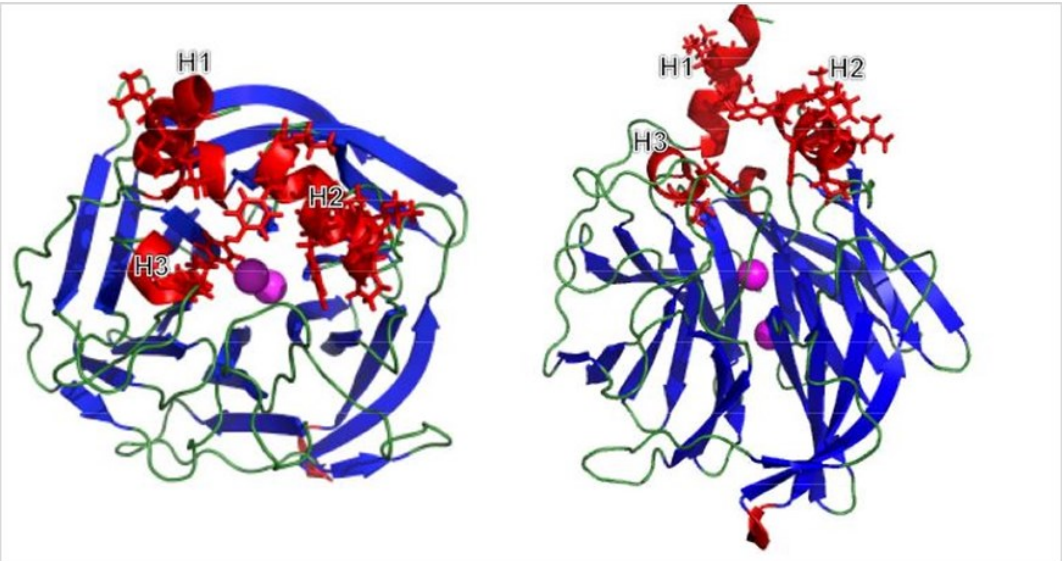


Figure 13. Top view (left) and side view (right) of the three-dimensional structure of the human PON2. The "six-bladed β-propeller" structure is indicated by the blue parts, the three projecting helices are represented in red and the hydrophobic residues inside the helices are represented in "sticks" form. In green, it is possible to observe the loops and in magenta the two Ca^{2+} ions. The structure was generated with Robetta based on the mammalian PON1 (PDB = 1V04). (Taken from Li, 2016).

Of the Ca^{2+} ions, one interacts with the aspartic acid residues at positions 168 and 54, and with the residue 116 of isoleucine, while the other is in contact with glutamic acid at position 53, aspartic acid 268 and asparagine residues at positions 269, 167 and 223. The basic folding seems to be enriched as for PON1 by the presence

of 3 α-helices that have a high content of hydrophobic residues and that, most probably, mediate the anchorage to cell membranes [Li XC, Wang C, Mulchandani A, Ge X,2016].

1.2.3 PONs substrates

PONs are lactonases with distinct substrate specificity (**Figure 14**). They are defined as pleiotropic enzymes due to their cross-intervention to different biochemical mechanisms (**Figure 15**). PON1 has three distinct catalytic activities: paraoxonase, arylesterase and lactonase.

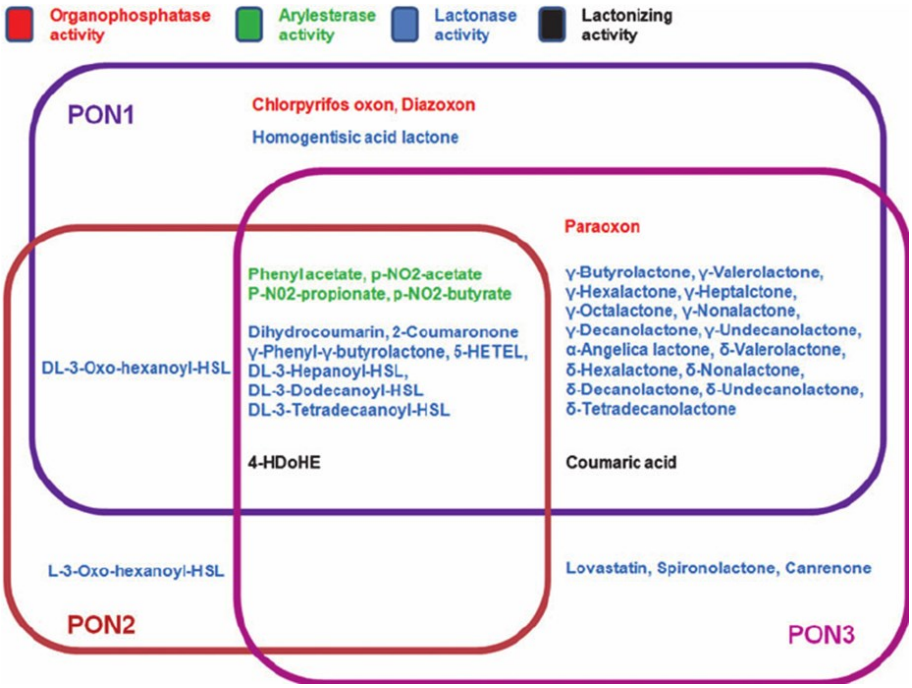


Figure 14. PONs catalytic activities: paraoxonase, arylesterase and lactonase.

Among the substrates of the PON1 enzyme, in addition to the organophosphates, which are not metabolized by PON2 and PON3, there are aromatic esters, such as phenylacetate, thiophenylacetate and 2-naphthylacetate and lactones, both aromatic (dihydrocumarine and 2-coumaronone) and aliphatic (γ-butyrolactone). PON1 can also catalyze the lactonization of hydroxycarboxylic acids, a reaction opposite to hydrolysis of lactones. Unlike PON1, PON2 and PON3 have poor paraoxonase and esterase activity, while they have a marked ability to hydrolyze cyclic and aromatic lactones. In particular, lactonase activity has been demonstrated against substrates such as dihydrocumarine (DHC), 2-coumaronone, (±) 5-hydroxy-6Z, 8Z, 11Z, 14Z- eicosatetraenoic acid (5-HETEL) and acyl-homoserine lactones (AHL) [Draganov DI, La Du BN., 2004]. PONs can hydrolyze a series of lactones including acyl-homoserin lactones, which are bacterial mediators produced by gram-

negative pathogenic microorganisms (such as *Pseudomonas aeruginosa*) that play an important role in regulating the expression of factors of virulence and inducing an inflammatory response of the host. PON2 has the highest activity against acyl-HCL compared to PON1 and PON3 [Draganov DI et al., 2005; Stoltz DA et al., 2007; Teiber JF et al., 2008; Horke S et al., 2010]. Degrading these compounds, PON2 could represent an important factor in the innate immune response. The administration of *Pseudomonas aeruginosa* inoculum in PON2-deficient mice led to a reduced bacterial clearance in the lung, liver and spleen compared to that of wild type mice [Devarajan A et al., 2013]. Furthermore, in peripheral tissues PON2 was found to play an important role in modulating sensitivity to bacterial infections thanks to the strong hydrolytic activity against acyl HCL, so PON2 could represent a pharmaceutical target for infection prevention [Precourt et al., 2011]. PON2 display arylesterase activity, although at reduced level compared to that exhibited by PON1 and PON3. It is interesting to note that some drugs commonly used in the treatment of cardiovascular diseases, such as lovastatin and spironolactone, are effectively hydrolysed by PON3. Finally, PON1 has been proposed, although requiring further confirmation, as a determinant of clopidogrel efficacy, a drug used for the prevention and treatment of cardiovascular diseases. PON1 would be able to modulate its bioactivation [Draganov DI, Teiber JF, Speelman A, Osawa Y, Sunahara R, La Du BN,2005; Bouman HJ, Scho¨mig E, van Werkum JW, et al.,2011].

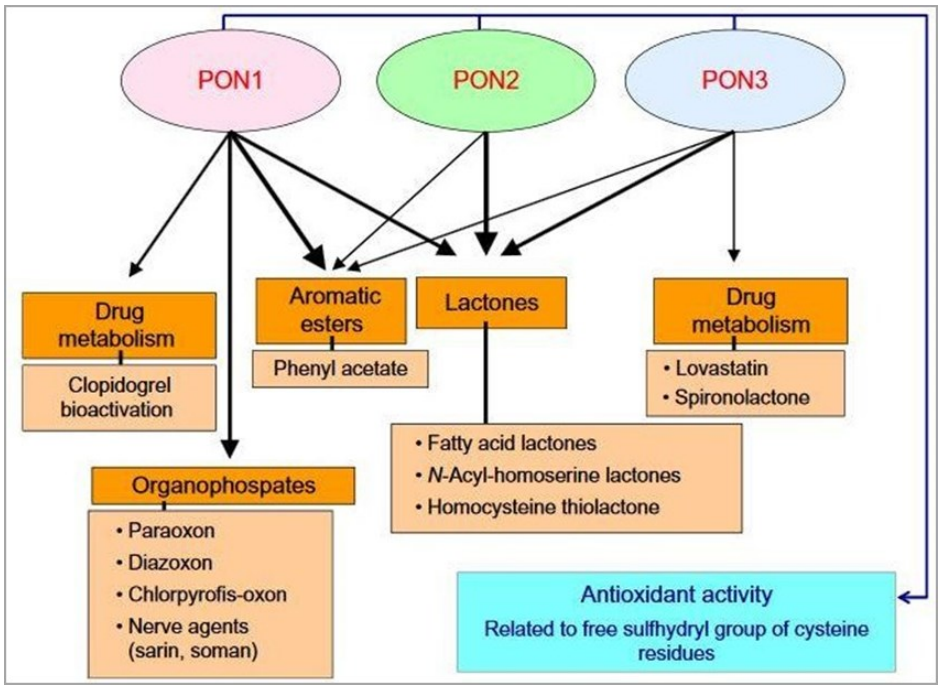


Figure 15. Pleiotropic effects of the PONs family

1.2.4 Physiological role of PON2

The physiological role of PONs has not yet been fully clarified, as well as their exact natural substrates. Despite this, today it is clear that they are involved in different physiological and pathophysiological processes.

- **Innate immunity**. Of the three PONs, PON2 has the highest hydrolytic activity towards AHL, an important molecule for mediating quorum sensing and a key regulator of bacterial virulence factors that mainly influence the inflammatory response of the host. This finding suggests a potential role for PON2 in innate immunity and defense against bacterial infections and opens up the scenario to potential therapeutic applications in this area [Li XC, Wang C, Mulchandani A, Ge X,2016].

- **Mitochondrial function**. In contrast to PON1 and PON3, PON2 has been shown to exert an antioxidant effect that is mainly expressed in the intracellular environment. It is expressed in most of the tissues examined and studies of subcellular localization suggest a preponderance of the enzyme at the level of the mitochondrial membrane. Since mitochondria are a significant source of oxidative stress, the predominant localization of PON2 in mitochondria supports a preventive role towards oxidative damage. In particular, some authors have shown that PON2 interacts with coenzyme Q10 associated with complex III in mitochondria, probably with the role of scavenging reactive oxygen species (ROS) produced physiologically during oxidative phosphorylation. To emphasize the role of PON2 in normal mitochondrial physiology, studies on murine knockout models for PON2 show that this deficit leads to severe mitochondrial dysfunctions [Devarajan A, Bourquard N, et.al, 2011].

- **Neuroprotection**. A well-explored role of PON2 is at the level of the central nervous system. Expression analysis of PON2 showed high levels of enzyme in the black substance, in the *nucleus accumbens* and in the *striatum*, while a reduced expression is found in the cerebral cortex, in the hippocampus, in the cerebellum and in the brainstem. PON2 is more expressed in astrocytes by localizing at the level of the endoplasmic reticulum and mitochondria. To investigate the role played by PON2, studies have been carried out on murine astrocyte cell lines

that have shown that the enzyme is able to counteract the effect induced by oxidizing agents, such as 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) and H_2O_2.

Also in this case, in the cells in which PON2 expression was knocked-out ($PON2^{-/-}$) a greater susceptibility to these substances has been demonstrated, both in terms of ROS content and of cell proliferation inhibition. Glutathione levels are identical in cells derived from wild type guinea pigs and from PON2 knockout guinea pigs, suggesting that the difference in susceptibility to oxidative stress toxicity is mainly due to PON2 and is not the consequence of other antioxidant metabolic pathways. An important role in the defense against oxidative stress induced by neurotoxic substances was therefore attributed to PON2 [Giordano G, Cole TB, Furlong CE, Costa LG., 2011].

1 **Inhibition of atherogenesis**. High levels of ROS are associated with numerous disorders such as cancer, cardiovascular disease, neurodegenerative diseases and diabetes. PONs have been investigated for their probable intervention in atherogenesis processes. This is especially true for the PON1 and PON3. The potential association between decreased serum PON1 activity and myocardial infarction was first proposed in 1986 by McElveen and colleagues. Unfortunately, this hypothesis was not corroborated by strong experimental evidence but later, in 1991, Mackness and colleagues showed that PON1 limits the accumulation of lipidic hydroperoxides in LDL, laying the foundations for PON1 involvement in atherogenesis and cardiovascular disease [Mackness MI, Arrol S, Durrington PN.,1991]. In fact, only five years later a gene polymorphism of PON1 was associated with greater susceptibility to cardiovascular diseases and in 1998, Shih and collaborators, provided additional and conclusive evidence demonstrating that PON1-knocked out murine models were more susceptible to the development of atherosclerosis through increased oxidation of lipoproteins [Ruiz J, Blanche´ H, James RW, et al.1995; Serrato M, Marian AJ.,1995; Shih DM, Gu L, Xia YR, et al.1998]. In the same context, a possible involvement of PON2 was also investigated. During exposure of Hela cells to hydrogen peroxide or oxidized phospholipids, the PON2 overexpression attenuates the levels of intracellular oxidative stress by reducing the levels of ROS, preventing lipid peroxidation of LDL, reversing oxidation of LDL not completely oxidized and inhibiting the ability of the latter

to induce chemotaxis of monocytes [Ng CJ, Wadleigh DJ, Gangopadhyay A, Hama S, Grijalva VR, Navab M, Fogelman AM, Reddy ST, 2001]. Interestingly, the expression of PON2 can be stimulated by the activation of endoplasmic reticulum stress pathways such as unfolded protein response (UPR) which plays an important role in cell survival/apoptosis. By reducing the effects of oxidative stress associated with UPR and blocking the activation of the caspase system, PON2 represents an endogenous defense mechanism against vascular oxidative stress involved in atherogenesis [Horke S, Witte I, Wilgenbus P, Kru¨ ger M, Strand D, Fo¨rstermann U.,2007]. In vivo animal studies have also provided similar results. PON2 has been shown to provide protection against the oxidation of HDL and LDL and to inhibit the transmigration of monocytes in response to the oxidation of LDL in murine models. In $PON2^{-/-}$ mice, an increase in number of foamy cells, an larger lipid droplets (LD) and atherosclerotic lesions were found compared with wild type counterparts [Ng CJ, Hama SY, Bourquard N, Navab M, Reddy ST, 2006]. In contrast, atherosclerotic lesions in mice overespressing PON2 (AdPON2) and with apoE deficiency ($apoE^{-/-}$) were significantly less extensive than in control mice. In macrophages, in addition to increasing cholesterol efflux, PON2 upregulation resulted in inhibiting the biosynthesis of triglycerides and the activity of microsomal diacylglycerol acyltransferase 1 (DAGT1) [Meilin E, Aviram M, Hayek, 2010; Rosenblat M, Coleman R, Reddy ST, Aviram M.,2009].

1.2.5 PON2 and cancer

Using different experimental models, it was shown that PON2 within cells plays an antioxidant and anti-apoptopic role. The expression levels of PON2 are increased in some solid tumors such as prostate cancer, renal carcinoma, hepatocellular carcinoma, bladder cancer and glioblastoma multiforme (**Figure 16**) [Witte I, Foerstermann U, Devarajan A, Reddy ST, Horke S.,2012]. In the pediatric neoplasm, upregulation of PON2 in subjects with acute lymphoblastic leukemia (ALL) has been associated with a poor prognosis [Kang H, Chen IM, Wilson CS, Bedrick EJ, Harvey RC, Atlas SR, et al.,2010].

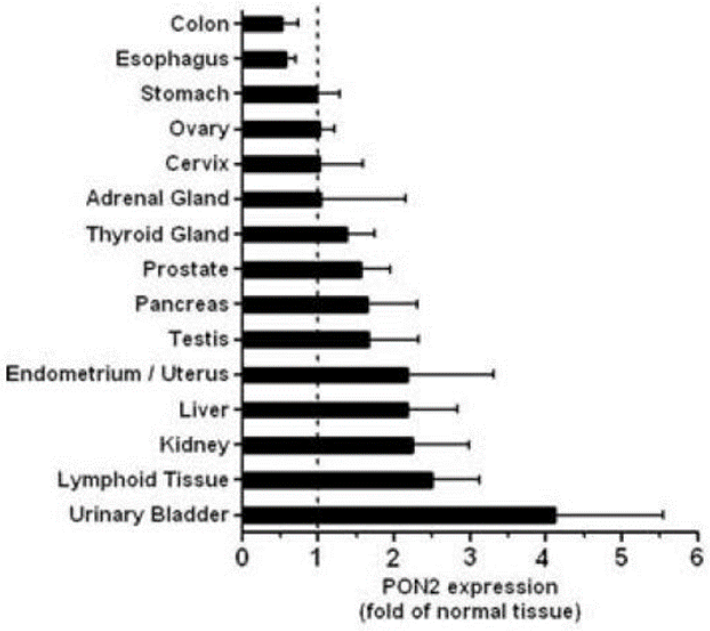


Figure 16. Expression levels of PON2 (protein) in tumors in relation to their respective healthy tissues (taken from Witte et al; 2011).

In many tumor cell lines, PON2 overexpression has shown to confer increased cell proliferation and survival indices. For example, recently, the overexpression of PON2 in the human urinary bladder cancer cell line T24 has led to a significant increase of resistance to oxidative stress triggered by tert-butylhydroperoxide and of tumor cell proliferation [Bacchetti T, Sartini D, Pozzi V, Cacciamani T, Ferretti G, Emanuelli M.,2017]. In contrast, PON2 deficiency causes apoptosis of some tumor cells, without further stimulation, helping to support "the oncogenic hypothesis" for this enzyme [Altenho¨ fer S, Witte I, Teiber JF, et al.,2010]. Looking further into this aspect, PON2 seems to intervene mainly by modulating apoptotic phenomena. The avoidance of the apoptotic system is confined only to the intrinsic mechanism, triggered by the release of cytochrome C from the intermembran space of the mitochondria in the cytosol, that leads to the activation of the caspase cascade. The activity of PON2 associated with Q10, leading to a reduction in mitochondrial levels of O_2^-, is able to counteract the peroxidation of cardiolipin and prevent the triggering of the apoptotic process (**Figure 17**).

Subsequent investigations, carried out in endothelial cells, have shown that PON2 is also able to counteract the pro-apoptotic stimuli generated following the oxidative damage caused to the endoplasmic reticulum,

known as UPR. At the molecular level, high levels of the enzyme inhibit the production of the c-Jun N-terminal kinases protein (JNK). Normally the kinase is responsible for the activation by phosphorylation of the transcription factor c-Jun, which thus becomes able to promote the transcription of CHOP, a powerful pro-apoptotic factor. In fact, even in this case the PON2 upregulation would result in the inhibition of programmed cell death (**Figure 17**) [Witte I, Altenhöfer S, Wilgenbus P, Amort J, Clement AM, Pautz A, Li H, Förstermann U, Horke S.,2011].

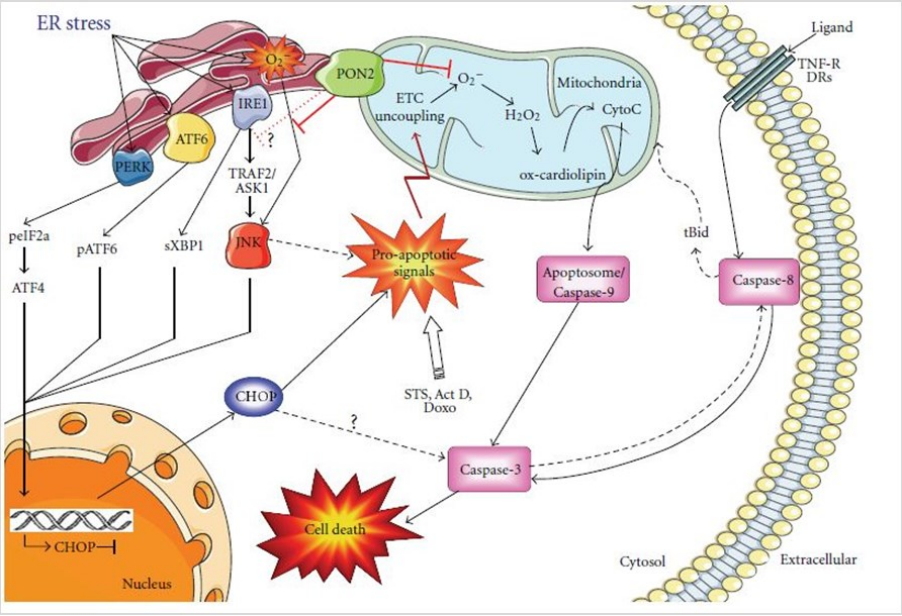


Figure 17. Schematic presentation of the suggested antiapoptotic mechanism for PON2. The ability to prevent the formation of O_2^- impacts on both apoptotic pathways, the one induced by RE stress (through the action on JNK and CHOP) and that one which acts on proapoptotic mitochondrial signaling, such as cardiolipin peroxidation and the release of cytochrome C.

Other authors have shown that tumor cell resistance to chemotherapy can also be modulated by PON2. For example, higher levels of PON2 correlate with resistance to the imatinib tyrosine kinase inhibitor used in the treatment of chronic myelogenous leukemia (CML) and ALL [Frank O, Brors B, Fabarius A, Li L, Haak M., Merk S, et al.,2006].

An interesting phenomenon has been highlighted by array studies on tumor tissues and confirmed in other more specific studies for the enzyme. When the expression of PON2 is evaluated taking into account the tumor stage, the greater expression of PON2 appears to belong to the tissues deriving from neoplasms that are in

early stages (**Figure 18**) [Witte I, Foerstermann U, Devarajan A, Reddy ST, Horke S ,2012; Bacchetti T, Sartini D, Pozzi V, Cacciamani T, Ferretti G, Emanuelli M., 2017].

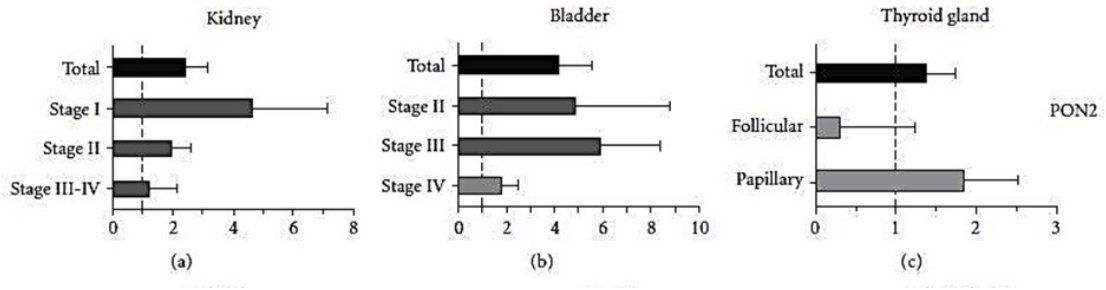


Figure 18. PON2 is more expressed in the early stages of neoplastic development than in late ones. The graphs are a processing of recent array data of PON2 cDNA levels (normalized through GAPDH) and expressed in relation to the levels of the relative healthy controls (taken from Witte et al; 2012).

This could indicate that, especially in the early stages of tumor formation, the antioxidant and antiapoptotic function of PON2 is an important platform for malignant neoplasms transformation.

Another hallmark of cancer cells is the reprogramming of glucose metabolism in order to respond to the high energy demand associated with the rapid development of neoplastic cells. A recent study reports that in pancreatic cancer cells (PANC1 and AsPC-1) PON2 is able to promote tumor growth by increasing glucose absorption levels by interacting with and modulating the activity of GLUT1 transporter. Furthermore, using murine xenograft and orthologous transplant models, the authors demonstrated that PON2 knockdown inhibits the development of this tumor. Resistance to the phenomenon of anoikis is a key feature of malignant transformation and metastasis, as it promotes cell growth after detachment from the extracellular matrix and the survival of circulating tumor cells [Paoli P, Giannoni E and Chiarugi P.,2013]. Nagarajan and his group therefore assessed in vivo whether PON2 expression played a role in the metastasis of pancreatic cancer at sites where it usually occurs most frequently: lungs and liver. Compared to controls, PON2 knockdown tumors demonstrated to significantly reduce the number of metastases indicating that PON2 facilitates the growth and multi-organ metastasization of pancreatic cancer.

At the conclusion of this important study the authors identified the molecular mechanism that would lead to the over-expression of PON2 in pancreatic cancer cells and that could underlie its disregulation in many other

types of neoplasms. The expression of PON2 under normal conditions is modulated by p53 which acts as a repressor of transcription by physically binding to the promoter site of the enzyme. The mutations affecting the TP53 gene are among the most widespread in cancer, with over half of all tumors presenting this alteration [Nagarajan A, Dogra SK, Sun L, Gandotra N, Ho T, Cai G, et al., 2017].

1.2.6 Modulation of PON2 expression levels

Most studies regarding the induction of PON2 expression focused on the effects of oxidative stress due to the intracellular antioxidant role it plays. The basic idea is that high levels of oxidative stress can induce a cellular compensation mechanism that would lead to an increase in the expression of PON2 and/or its activity, in order to counteract the harmful effects of such stress [Shiner M, Fuhrman B, Aviram M.,2006]. In vitro and in vivo studies have shown that expression and enzymatic activity increase in response to oxidative stress in different cell types (HepG2 cells and macrophages), in animal models (mice fed with diets high in fat and apoE knockout mice), and in hypercholesterolemic patients [Shih DM, Gu L, Hama S, Xia Y-R, Navab M, Fogelman AM, et al,1996; Shih DM, Gu LY, Xia R, Navab M, Li W, Hama S, et al.,1998; Forte TM, Subbanagounder G, Berliner JA, Blanche PJ, Clermont AO, Jia Z, et al.,2002; Rosenblat M, Draganov D, Watson CE, Bisgaier CL, La Du BN, Aviram M.,2003; Rosenblat M, Hayek T, Hussein K, Aviram M,2004; Rosenblat M, Oren R, Aviram M.,2006]. For example, in murine peritoneal macrophages (MPM) treated with various agents that induce oxidative stress, an increase in the expression of PON2 and its lactonase activity has been demonstrated [Rosenblat M, Draganov D, Watson CE, Bisgaier CL, La Du BN, Aviram M.,2003]. The expression of PON2 has also been studied in the context of common pathological conditions that are characterized by an increase in oxidative stress, such as metabolic disorders due to high circulating levels of cholesterol or glucose. It has been observed that in murine models, chronic exposure to high doses of cholesterol leads to an increase in PON2 hepatic mRNA levels [Forte TM, Subbanagounder G, Berliner JA, Blanche PJ, Clermont AO, Jia Z, et al, 2002]. Similar results were obtained by treating murine macrophages J774A.1 with lysophosphatidylcholine (LPC) [Rosenblat M, Oren R, Aviram M., 2006]. In another in vivo study it was observed that human macrophages derived from monocytes (HMDM) from hypercholesterolemic patients,

subjected to oxidative stress, presented less than half of the levels of PON2 expression detected in HMDM isolated from control patients [Rosenblat M, Hayek T, Hussein K, Aviram M., 2004]. A similar result was obtained in diabetic mice, demonstrating that the up-regulation of cellular PON2 in macrophages is associated with an increase in oxidative stress [Hayek T, Kaplan M, Kerry R, Aviram M.,2007]. Furthermore, an increased expression of PON2 was observed in response to various pharmaceutical compounds, such as the hypolipidemic agent atorvastatin and the antidiabetic drug rosiglitazone. Atorvastatin and rosiglitazone in HMDM and in murine macrophages led to high activity of PON2 which correlates with a reduction in cellular oxidative stress [Rosenblat M, Hayek T, Hussein K, Aviram M.,2004; Shiner M, Fuhrman B, Aviram M.,2007]. In contrast, some proinflammatory agents, such as lipopolysaccharide (LPS), appear to reduce the expression of PON2 in Caco-2/15 cells and in the human intestine [Precourt LP, Seidman E, Delvin E, Amre D, Deslandres C, Dominguez M, et al.,2009]. In 2006 Shiner and his colleagues investigated in macrophages the effects induced on the PON2 lactonase activity by a considerable series of substances considered pro-oxidants (eg copper sulfate) and anti-oxidants (eg Vitamin E). This in-depth analysis revealed that within certain ranges of concentrations both types of stimuli enhanced the enzymatic activity of PON2. Of particular interest is the fact that antioxidant substances have a biphasic effect on PON2: concentrations that leave the cell in a state of stress unchanged with respect to control or slightly decreased do not seem to affect the expression and activity of PON2. However, exceeding a certain threshold the process is reversed enhancing the activity of this enzyme.

2. AIM OF THE STUDY

In this work, the attention was focused on the enzyme PON2. The main function exerted by the enzyme within cell is mainly related with its antioxidant activity. Indeed, Ng et al. demonstrated that PON2 contributes, together with other intracellular enzymes and systems, to protect cells from oxidative stress [Ng CJ, Wadleigh DJ, Gangopadhyay A, Hama S, Grijalva VR, Navab M, Fogelman AM, Reddy ST., 2001]. Considering its intracellular localization and antioxidant function, PON2 was reported to display an anti-apoptotic role, with potential consequences on tumor cell behavior [Witte I, Foerstermann U, Devarajan A, Reddy ST, Horke S., 2012]. PON2 overexpression has been reported in a variety of tumors, including pancreatic cancer [Nagarajan A, Dogra SK, Sun L, Gandotra N, Ho T, Cai G, et al., 2017], glioblastoma multiforme, and recently BC but the functional significance of alterations in enzyme expression associated with disease remains partly undisclosed.

Concerning the role of PON2 in bladder cancer, in our previous study we demonstrated that enzyme levels were significantly higher in tumor compared with adjacent normal looking tissue samples from BC patients. Moreover, preliminary results obtained from analyses performed on bladder cancer cell line seemed to suggest that PON2 is able to promote cell proliferation and resistance to oxidative stress [Bacchetti T, Sartini D, Pozzi V, Cacciamani T, Ferretti G, Emanuelli M,. 2017]. In this light, the major aim of this project was to explore the role of the enzyme in cancer cell metabolism. Enzyme silencing and overexpression were induced in T24 bladder cancer cell line. Real-Time PCR and Western blot were used to evaluate enzyme dysregulation. Subsequently, T24 cell proliferation, migration and susceptibility to oxidative stress were mesured, before and after treatment with cisplatin and gemcitabine to evaluate the resistance to chemotherapeutic agents. Further analyses were performed to explore the potential involvement of the enzyme in cellular pathways, such as apoptosis, evaluating the activity levels of both caspase-3 and caspase-8, as key regulators of the apoptotic response.

3. MATERIALS AND METHODS

3.1 PON2 SILENCING AND OVEREXPRESSION IN T24 BLADDER CANCER CELL LINE

Cell lines and culture conditions

The human bladder cancer cell line T24, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), was maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum and 50µg/ml of gentamicin, at 37°C with 5% CO_2 air-humidified atmosphere.

Cloning

The plasmid vector pLKO.1-647 containing stem-loop cassette encoding short hairpin RNA (shRNA) targeted to human PON2 (Sigma-Aldrich, St. Louis, MO) was used for PON2 gene silencing.

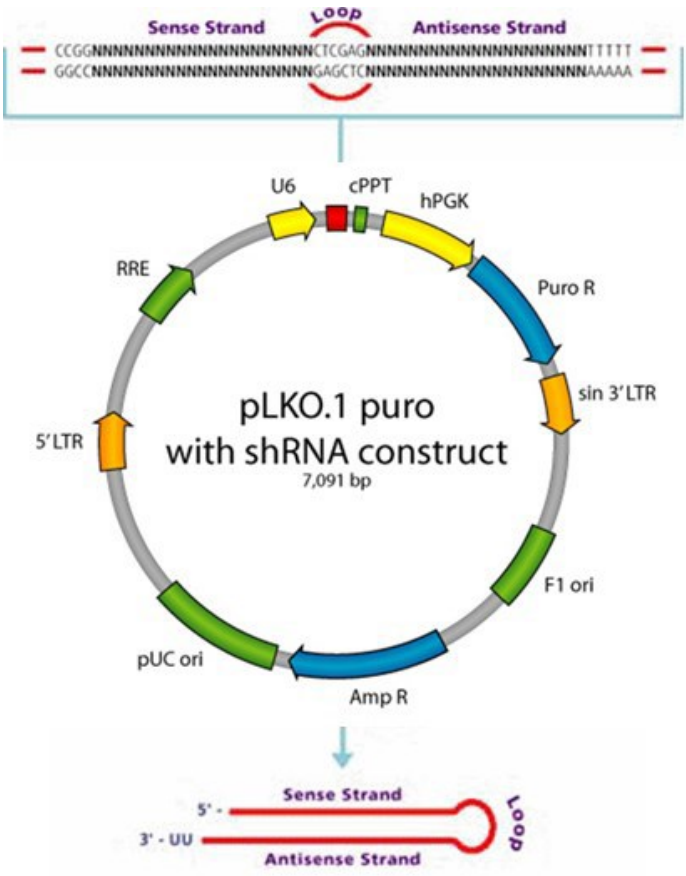


Figure 19. pLKO.1-puro vector. Expression of shRNAs are driven by the human U6 promoter. The vector contains the puromycin resistance (PuroR) gene for selection in mammalian cells.

For the induction of PON2 overexpression, total RNA was isolated from T24 cells ($1x10^5$) using RNeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The concentration of total RNA was determined spectrophotometrically, by measuring the absorbance at 260nm. The purity of RNA was assessed by calculating the ratios between absorbance at 260 and 280nm, as well as absorbance at 260 and 230 nm. The integrity of the purified RNA was confirmed by electrophoresis on denaturing 1% agarose gel. Total RNA (2μg) was reverse transcribed in a total volume of 25 μl for 60 minutes at 37°C with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), using random primers. 1 μl of the reaction mixture was then subjected to PCR with KOD Hot Start DNA Polymerase (Novagen, Darmstadt, Germany) in a total volume 50μl, using the primers 5'-TCCGGATCCATGGGGCGGCTGGT-3' (forward) and 5'-TTACTCGAGTTAGAGTTCACAAT-3' (reverse) to amplify the human PON2 open reading frame (ORF) and to insert BamHI and XhoI restriction sites. PCR was performed in a thermal cycler using the cycling conditions as follows: initial denaturation step at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds (denaturation step), 58°C for 30 seconds (primers annealing step), and 72°C for 1 minute (extension step). The amplification process was completed with a final extension step at 72°C for 5 minutes. The amplified PCR product was subjected to electrophoresis on 1% agarose gel in Tris-acetate-EDTA (TAE) buffer. The DNA PCR product was isolated and purified from the agarose gel, using the NucleoSpin Extract II Kit (Macherey-Nagel, Duren, Germany), according to manufacturer's protocol. Subsequently, the amplified product as well as pcDNA3 plasmid vector (Life Technologies, Carlsbad, CA, USA) (**Figure 20**) were digested and hen sujected to ligation, in order to obtain the recombinant plasmid construct pcDNA3-PON2.

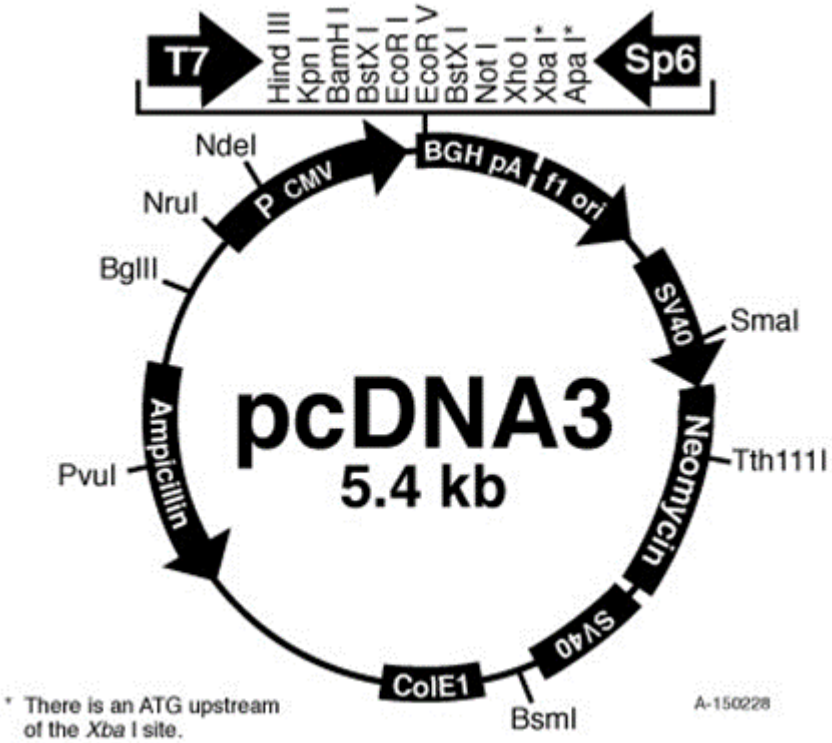


Figure 20. pcDNA3 expression vector. pcDNA3 vector contains the strong CMV enhancer-promoter for high level expression of recombinant proteins in mammalian cells.

In details, cloning procedure was achieved as follows. 1µg of both insert and vector DNA was digested by using BamHI and XhoI restriction enzymes (Promega), in two separate reaction mixtures. After incubation at 37°C for 1 hour, the digestion products were subjected to electrophoresis on 1% agarose gel in TAE buffer, and the corresponding bands were purified through the NucleoSpin Extract II Kit. The concentrations of the purified products were determined by spectrophotometer, and insert and vector DNA were subsequently subjected to ligation reaction, following a 3:1 insert:vector DNA molar ratio. The amount of insert to use for the ligation reaction was calculated according to the following formula:

$$\text{ng insert} = \frac{\text{ng vector x kb of insert}}{\text{kb of vector}} \text{x } \frac{3}{1}$$

Considering the sizes of insert (1,1 kb) and vector (5,4 kb), the amount of the insert DNA to use, for ligation with 100 ng of pcDNA3 vector, resulted 60 ng. Ligation mixture (10µl) contained 2X Rapid Ligation Buffer,

0.3 Weiss unit of T4 DNA Ligase (Promega) as well as insert and vector DNA, and was incubated for 3 hours at room temperature (Figure 21).

Recombinant plasmid (pcDNA3-PON2) was used to transformation of *E. coli* JM109 competent cells, as described below. Briefly, ligation mixture (10 µl) was added to a 200µl aliquot of bacterial cells and treated as follows:

- incubation for 30 minutes on ice,
- 40-50 seconds at 42°C (heat-shock),
- 5 minutes on ice.

After adding 800 µl of sterile Luria-Bertani (LB) medium, the transformation mixture was incubated at 37°C for 1-2 hours in a shaking incubator. Subsequently, 200µl of the bacterial culture were seeded onto a LB-agar plate containing 0.1 mg/ml ampicillin and incubated overnight at 37°C (Figure 21). The day after, the presence of the recombinant plasmid into JM109 cells was confirmed by the presence of bacterial clones, some of which were subjected to colony-PCR. Reaction mixture (25 µl), prepared according to manufacturer's instructions, contained Green GoTaq Flexi Buffer (Promega), $MgCl_2$, dNTPs, GoTaq DNA Polymerase (Promega), and primers used for PON2 cloning. PCR was performed in a thermal cycler using the following conditions: initial denaturation at 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds (denaturation step) and 58°C for 30 seconds (primers annealing and extension step). Amplified products were subjected to electrophoresis on 1% agarose gel in TAE buffer.

Clones harboring recombinant vector were grown in 3 ml of LB media, containing 0.1 mg/ml ampicillin and incubated overnight at 37 °C in a shaking incubator. The day after, recombinant plasmids were isolated from bacterial pellets using the NucleoSpin Plasmid (Macherey-Nagel, Duren, Germany), and subjected to the analysis of nucleotide sequence.

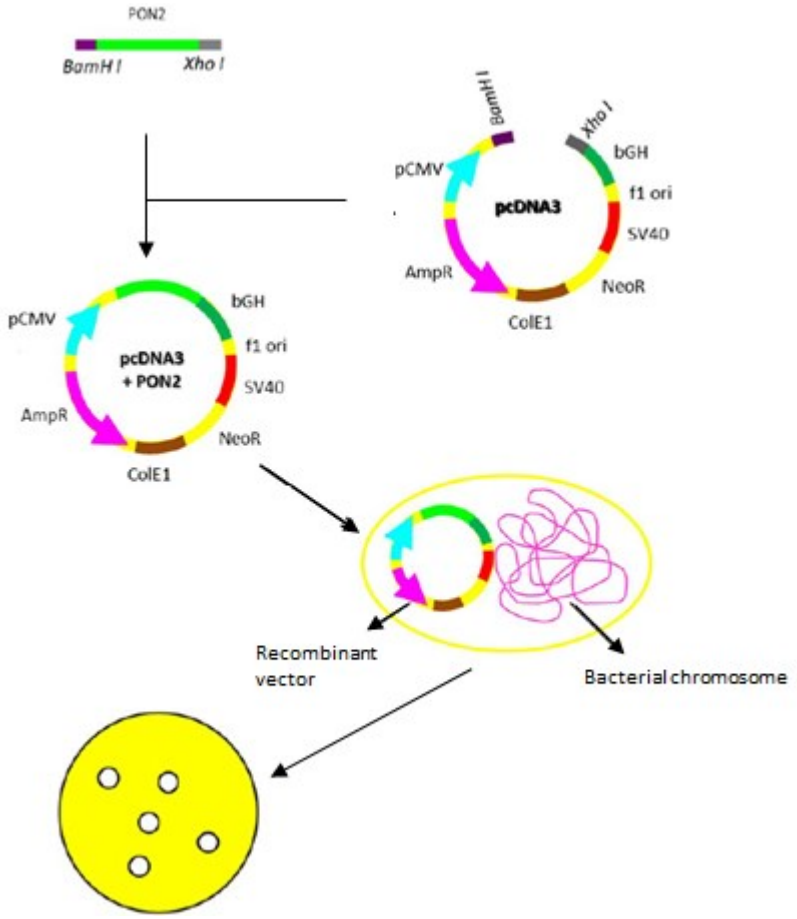


Figure 21. Molecular cloning of PON2 coding sequence into pcDNA3: amplification of PON2 fragment, digestion of both the amplification product and pcDNA3 with BamHI and XhoI, ligation reaction, bacterial cells transformation with pcDNA3-PON2 and growth on LB-agar plates.

Transfection

To achieve PON2 silencing, T24 cells were seeded in 24-well plates (4×10^4cells/well) the day before transfection. The plasmid (0.5µg/well) against PON2 (pLKO.1-647) or empty vector (pLKO.1-puro) was used to transfect 80% confluent cells. Control cells were treated with transfection reagent only (mock).

To induce the overexpression of PON2, cells were seeded in 6-well plates ($2.4x10^5$ cells/well) the day before transfection and were then transfected with pcDNA3-PON2 plasmid vector (3µg/well). Control cells were transfected with the empty vector (pcDNA3) or treated with transfection reagent only (mock).

Both procedures were performed using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA), following the manufacturer's instructions and keeping constant a 3:1 FuGENE HD Transfection Reagent:DNA ratio.

Forty-eight hours from the beginning of the transfection, culture medium was discarded and replaced with new and complete one, containing puromycin (1μg/ml) or geneticin (800 μg/ml), in order to select cellular clones downregulating or overexpressing PON2, respectively. For all subsequent experiments, puromycin and geneticin resistant cells were maintained in complete selection medium.

3.2 EFFICIENCY OF PON2 SILENCING AND OVEREXPRESSION

Total RNA extraction and cDNA synthesis

Cell pellets ($1x10^6$ cells) were homogenized in lysis buffer, and total RNA was isolated through the SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's protocol.

The quantity and quality of RNA were assessed spectrophotometrically at 260 nm and 280 nm, and confirmed by electrophoresis, as previously described. Total RNA (2 μg) was reverse transcribed in a total volume of 25 μl for 60 minutes at 37°C with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) using random primers. cDNA samples were used to perform subsequent Real-Time PCR analysis.

Real-Time PCR

A Real-Time PCR assay was setup to evaluate PON2 mRNA levels, using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and cDNA, obtained as described above. To avoid false-positive results caused by amplification of contaminating genomic DNA in the cDNA preparation, all primers were selected to flank an intron, and PCR efficiency was tested for both primer pairs and found to be close to 1. The primers used were 5'-TCGTGTATGACCCGAACAATCC-3' (forward) and 5'-AACTGTAGTCACTGTAGGCTTCTC-3' (reverse) for PON2, 5'-TCCTTCCTGGGCATGGAGT-3' and (reverse) 5'-

AGCACTGTGTTGGCGTACAG-3'for β-actin. Both genes were run in duplicate for 40 cycles at 94°C for 30 seconds and 58°C for 30 seconds, using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA). All samples were tested in triplicate with the reference gene β-actin for data normalization to correct for variations in RNA quality and quantity.

Direct detection of PCR products was monitored by measuring the fluorescence produced by EvaGreen dye binding to double strand DNA after every cycle. These measurements were then plotted against cycle numbers. The parameter threshold cycle (Ct) was defined as the cycle number at which the first detectable increase above the threshold in fluorescence was observed. Upon threshold cycle (Ct) determination, PON2 knockdown/overexpression in T24 cells line was evaluated by $2^{-\Delta\Delta Ct}$, where ΔCt = Ct (PON2) - Ct (β-actin), and ΔΔCt = ΔCt (pLKO.1-647/pcDNA3-PON2 or empty vector) - ΔCt (mock).

Western blot analysis

Cell pellets ($2x10^6$ cells) were suspended in 200 µl lysis buffer (phosphate buffered saline containing 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1mM phenylmethylsulfonyl fluoride and 2µg/ml aprotinin) and homogenized by passing 3-5 times through a 30 gauche needle attached to a 1ml syringe. After centrifugation at 16000 xg for 10 minutes at 4°C, the supernatant containing the protein extract was collected.

Samples containing 40µg protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli method [Laemmli UK, 1970.], using a 12,5% polyacrylamide running gel. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 250 mA for 40 minutes, using a wet transfer method. PVDF membranes were blocked overnight at 4°C in 1X tris-buffered saline (TBS) solution containing 2% bovine serum albumin (BSA), and 1% tween-20. After washing three times with 1X TBS containing 1% tween-20, the membranes were incubated with rabbit polyclonal antibody against PON2 (Sigma-Aldrich, St. Louis, MO, USA) (1:500 dilution) for 1 hour, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA) (1:15000 dilution) for 1 hour. PON2 protein was visualized using enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The chemiluminescent signal of PON2

protein detected in blots was acquired using ChemiDoc XRS + System (Bio-Rad Laboratories, Hercules, CA, USA).

Monolayer Wound Healing Assay

To evaluate the migration capacity, T24 cells were seeded into 6-well plate ($6x10^5$ cells/well) and allowed to attach and grow up to 90-100% confluence. Cell monolayers were scratched by using a sterile 200µl pipette tip to make a vertical wound. Wounded monolayers were then washed three times with phosphate-buffered saline (PBS) to remove cell debris and then incubated in medium containing 0.5% FBS. Control cells were incubated with culture medium only. Upon medium replacement, cells were monitored under a microscope equipped with a camera (Deiss) and photographed at 6h, 12h and 24h. The images of the monolayer of cells with dysregulated PON2 were compared with those of the monolayer generated by controls, to evaluate the effects of PON2 on cell wound healing capacity. The percentage healing area was calculated using ImageJ software. Each experiment was performed in triplicate and independently repeated three times.

3.3 EFFECT PON2 DISREGULATION ON SENSITIVITY OF T24 CELLS TO CHEMOTHERAPEUTIC TREATMENT

Chemotherapeutic treatment

T24 cells downregulating and overexpressing PON2, as well as controls, were seeded in 96-well plates ($3x10^3$ cells/well). The day after seeding, cells were treated with cisplatin and gemcitabine, as previously reported [Da Silva GN, de Castro Marcondes JP, de Camargo EA, da Silva Passos Júnior GA, Sakamoto-Hojo ET, Salvadori DM, 2010*;* Da Silva GN, Filoni LT, Salvadori MC, Salvadori DMF, 2018]. In particular, cells were incubated with cisplatin (20 µmol/L), gemcitabine (6.25 µmol/L) or a combination of both drugs (1.0 µmol/L cisplatin and 1.56 µmol/L gemcitabine) for 24h. After incubation with drugs, cells were washed PBS and complete fresh medium was added.

MTT assay

Cell proliferation was determined using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). This assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzyme activity of the intact mitochondria of living cells.

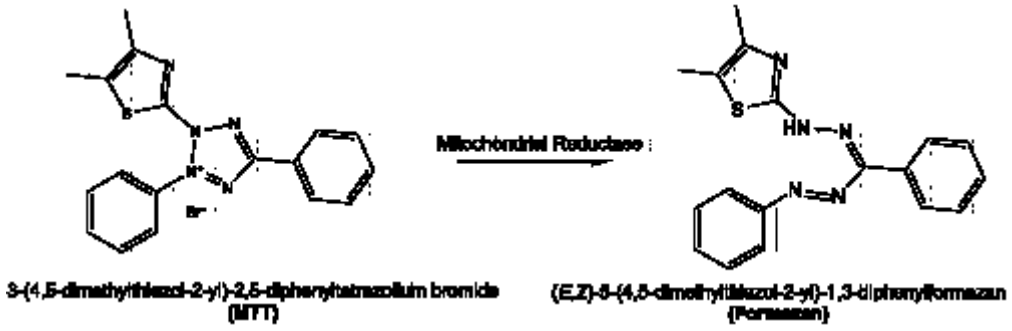


Figure 22. Conversion of MTT to formazan salt.

After transfection, T24 cells were seeded in 96-well plates ($3x10^3$ cells/well). Cells were allowed to attach overnight, and cell proliferation was evaluated in untreated cells (0h) and at different time points (12h, 24h, 36h, 48h and 72h) after starting and independently from treatment with drugs. Briefly, cells were incubated with 100µl of medium containing 10µl of MTT reagent (5 mg/ml in phosphate buffered saline) for 2h at 37°C. The medium was removed and 100 µl of dimethyl sulfoxide (DMSO) were added. The amount of formazan crystals formed directly correlated with the number of viable cells. The reaction product was quantified by measuring the absorbance at 570 nm using an ELISA plate reader. Experiments were repeated three times. Results were analyzed and expressed as percentage of the control (control equals 100% and corresponds to the absorbance value of each sample at time zero), and presented as mean values ± standard deviation of three independent experiments performed in triplicate.

Detection of intracellular oxidative stress

To evaluate the intracellular oxidative stress, the oxidation of 2',7'-dichlorodihydrofluorescein diacetate H_2DCF-DA (Sigma-Aldrich, St. Louis, MO, USA) was measured. H_2DCF-DA is a non-fluorescent probe readily taken up by cell thanks to its lipophilic characteristics. Inside the cell the H_2DCF-DA is subsequently

de-esterified to 2',7'-dichlorodihydrofluorescein (H_2DCF) by cellular esterases (which they remove acetate groups), and later oxidized by ROS into the fluorescent molecule 2',7'-dichlorofluorescein (DCF).

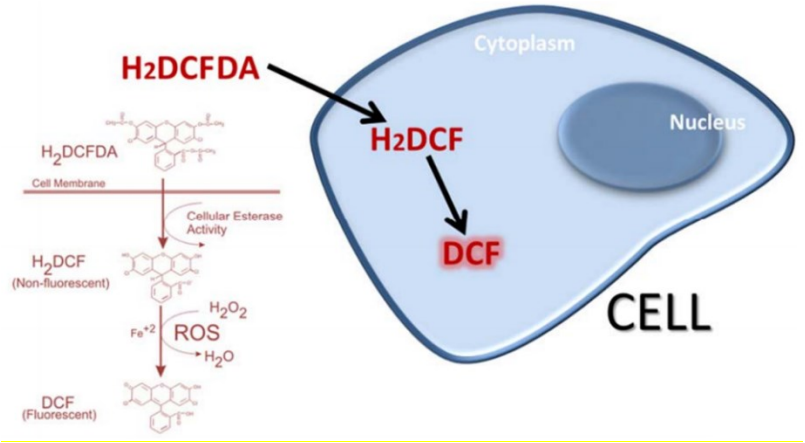


Figure 23. ROS-induced formation of fluorescent DCF compound.

Cells were seeded on 96-well black plates ($3x10^3$ cells/well), provided with clear bottom wells, and allowed to adhere overnight. The day after, cells started to be treated with chemotherapeutic drugs, following what above reported. Oxidative stress induced by chemotherapeutic compounds was determined in untreated cells (0h) and at different time points (12h, 24h, 36h, 48h and 72h) after starting treatment with drugs, by measuring the DCF produced. Cells were treated with 50µM H_2DCF-DA for 45 min at 37°C in the dark. After washing with PBS for extracellular H_2DCF-DA removal, the fluorescence was measured by using a plate reader at Ex/Em= 485nm/535nm. Each experiment was performed in triplicate and independently repeated three times.

Determination of caspase-3 and caspase- 8 activity

In cells after 24h treatment with chemotherapeutic drugs or with 1µM Dexamethasone (positive control), caspase-3 and caspase-8 activities were determined, using the Caspase-3/CPP32 and Caspase-8/FLICE Colorimetric Assay Kits (Biovision, Milpitas, CA, USA), respectively, The day before starting treatment, cells were seeded in T25 falsks ($1x10^6$). After 24h of incubation with drugs, cells were trypsinized, centrifuged at 500xg for 5 min at 4°C, resuspended in 50µl lysis buffer provided by each kit and incubated on ice for 10 min. Cell lysates were then centrifuged at 10000xg for 1 min at 4°C and supernatant were used for further analysis.

After determination of protein concentration, 150µg protein extract were transferred to a 96-plate well and incubated with 50µl 2x reaction buffer containing 10mmol/L DTT, and 4mmol/L DEVD-p-nitroanilide substrate (for caspase-3) or IETD-p-nitroanilide substrate (for caspase-8), followed by 1h incubation at 37°C. Cells tretated without substrates represented negative control samples. Optical density (OD) for each specimen was determined at 405nm using microtiter plate reader. Each experiment was performed in triplicate and independently repeated three times.

Statistical analysis

Data were analysed using GraphPad Prism software version 8.00 for Windows (GraphPad Software, San Diego, CA, USA). Differences between groups were determined using the one-way analysis of variance (ANOVA). A p-value < 0.05 was accepted as statistically significant.

4. RESULTS

4.1 EFFICIENCY OF PON2 shRNA-MEDIATED KNOCKDOWN AND OVEREXPRESSION IN T24 CELLS

In order to modulate PON2 expression for functional assays, T24 cell line was transfected with plasmid encoding shRNA targeting a specific region of PON2 mRNA (pLKO.1-647) or with vector for PON2 overexpression (pcDNA3-PON2). Control cells were treated with empty vectors (pLKO.1-puro or pcDNA3) or with transfection reagent only (mock). 48h after transfection, cells started to be treated with puromycin or geneticin, until complete selection. To evaluate the efficiency of treatment aimed to induce enzyme downregulation or overexpression, PON2 mRNA and protein levels were evaluated by Real-Time PCR and Western blot analysis, respectively.

Compared with mock and empty vector, PON2 expression levels were significantly reduced upon treatment with pLKO.1–647. Real-Time PCR showed a significant ($p<0.05$) downregulation of PON2 in cells transfected with pLKO.1–647 plasmid (0.32 ± 0.05) compared with mock (1.00 ± 0.09) and those transfected with empty vector (0.99 ± 0.08) (Figure 24, panel A). On the contrary, T24 cells transfected with pcDNA3-PON2 displayed a significant ($p<0.05$) enzyme upregulation (2.49 ± 0.09) with respect to cells treated with pcDNA3 (1.20 ± 0.08) and mock (1.00 ± 0.07) (Figure 24, panel B).

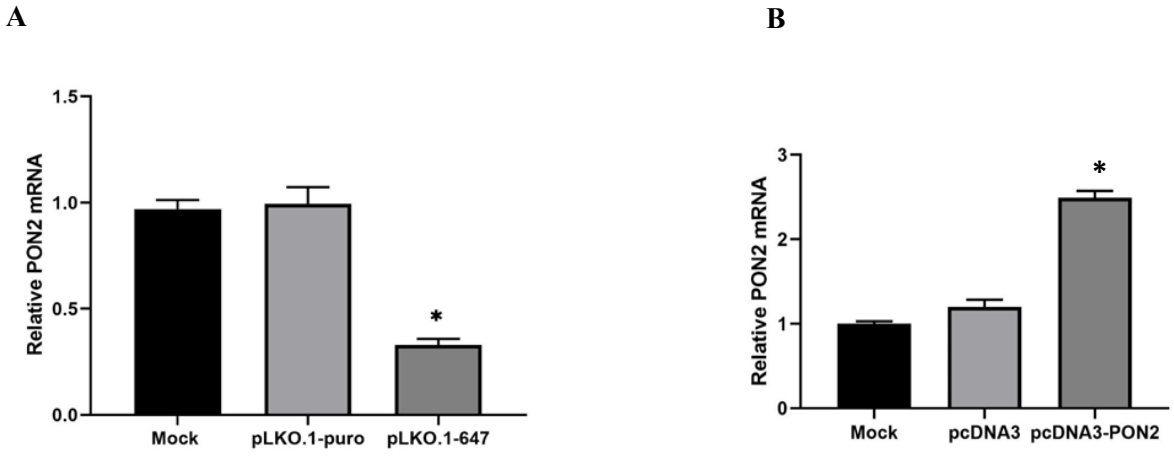
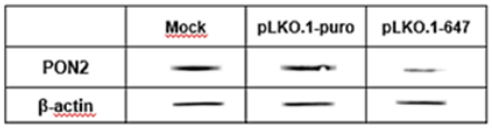


Figure 24. PON2 mRNA levels in T24 cells. In order to induce PON2 silencing or overexpression, T24 cells were transfected with pLKO.1-647 or pcDNA3-PON2, respectively. Control cells were treated with empty vectors (pLKO.1-puro or pcDNA3) or with transfection reagent only (mock). Real-Time PCR was used to evaluate PON2 mRNA levels in

cells transfected with plasmids inducing enzyme down- (panel A) or upregulation (panel B) compared with control. All reported values are expressed as mean ± standard deviation (*p<0.05).

To confirm the results obtained by quantitative Real-time PCR, PON2 expression was also detected at protein level by Western blot analysis. Results obtained showed that, compared with controls, cells transfected with pLKO.1–647 or pcDNA3-PON2 plasmid vectors displayed markedly decreased or increased PON2 levels, respectively (Figure 25).

A



B

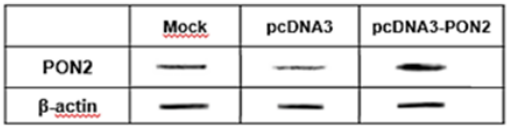


Figure 25. PON2 protein levels in T24 cells. Lysates obtained from T24 cells were analyzed by Western blot to evaluate PON2 protein levels upon enzyme knockdown (panel A) or overexpression (panel B).

4.2 EFFECT OF PON2 KNOCKDOWN AND OVEREXPRESSION ON T24 CELL PROLIFERATION AND MIGRATION

To examine the role of PON2 in tumor cell metabolism, and analyze the biological effect associated with enzyme knockdown or overexpression, cell viability and migration capacity were analyzed at different time points in T24 cells.

The effect of PON2 knockdown and overexpression on cell proliferation was evaluated by MTT assay. As shown in Figure 26, panel A, enzyme knockdown led to a significant ($p<0.05$) decrease in cell growth of T24 cells starting from 24 h time-point with respect to control cells (mock) as well as those treated with empty vector (pLKO.1-puro). On the other side, enzyme upregulation was associated with a significant ($p<0.05$) increase in cell proliferation of T24 cells starting from 48h time-point (Figure 26, panel B).

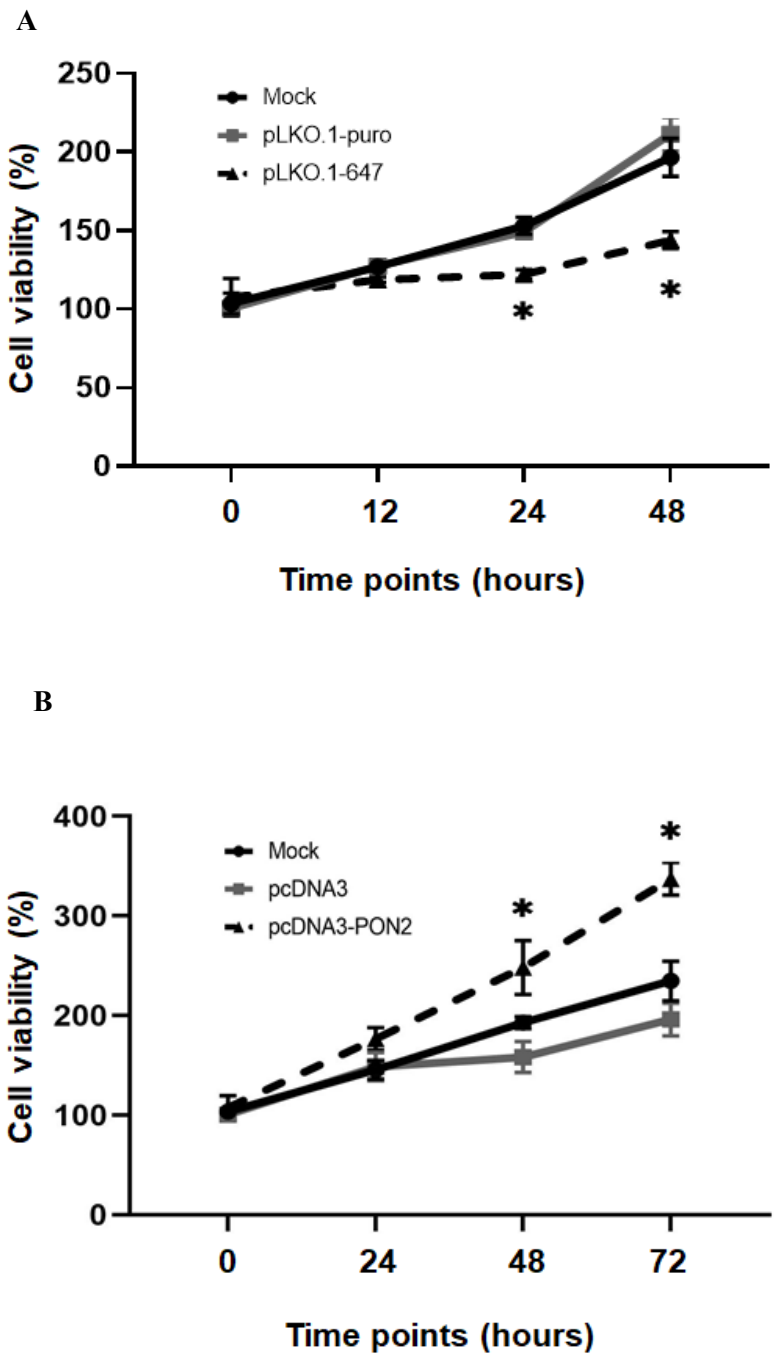


Figure 26. Evaluation of T24 cell proliferation. The effect of PON2 silencing (panel A) and overexpression (panel B) on cell proliferation was assessed by MTT assay. Cell viability was evaluated in mock and cells treated with plasmids at different time points, between 0h and 72h. All values are expressed as mean ± standard deviation (*$p<0.05$).

To assess the biological influence of PON2 on cell migration, T24 cell line transfected with pLKO.1–647 or pcDNA3-PON2 plasmids was subjected to monolayer wound healing assay. Compared with mock, the migration ability of T24 cells significantly ($p<0.05$) decreased at 6h (32% reduction) and 12h (50% reduction)

time-points after PON2 silencing (Figure 27, panels A and B). On the contrary, PON2 overexpression led to a significant ($p<0.05$) promotion of the migration ability of T24 cells at 12h (13% increase) and 24h (25% increase) time points with respect to the control (Figure 27, panels C and D).

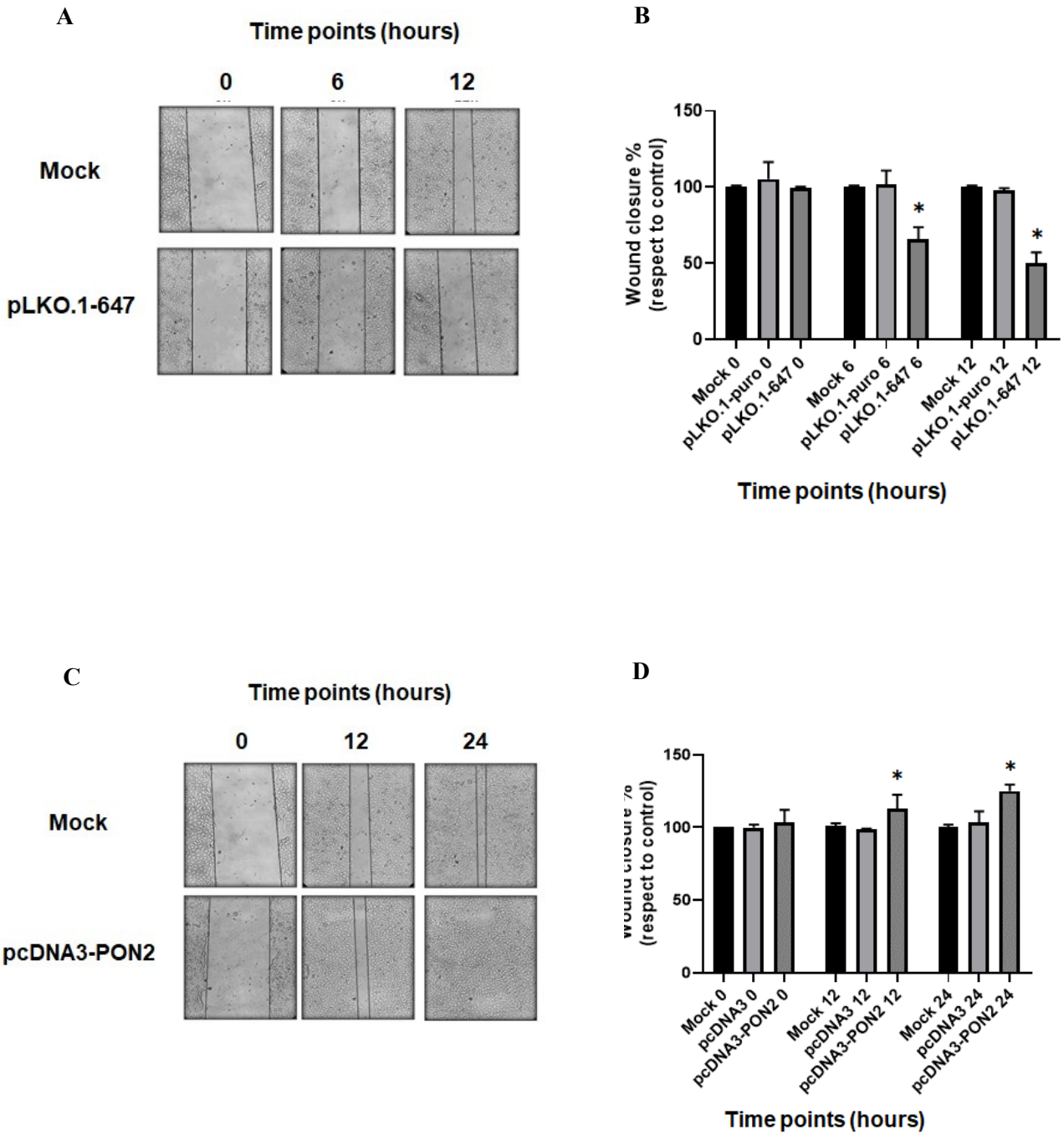


Figure 27. Migration ability of T24 cells. Wound healing assay was used to asses the impact of PON2 expression on cell migration. T24 cells transfected with plasmid aimed to induce enzyme knockdown (panels A and B) or overexpression

(panels C and D) were photographed immediately (0h) after wounding by a pipette tip and at different time points, ranging between 6h and 24h. All values are expressed as mean ± standard deviation (*$p<0.05$).

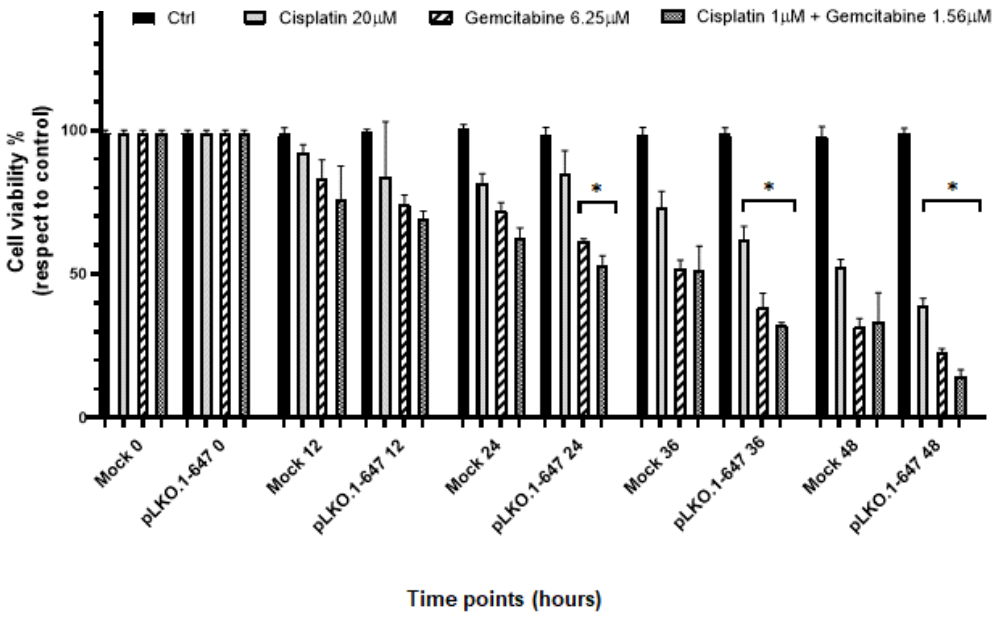
4.3 PON2 INFLUENCE ON SENSITIVITY OF T24 CELLS TO TREATMENT WITH CHEMOTHERAPEUTIC DRUGS

The effect on cell viability of administration of cisplatin (20 μmol/L), gemcitabine (6.25 μmol/L) or the combination of both drugs (1.0 μmol/L cisplatin and 1.56 μmol/L gemcitabine) was evaluated by MTT colorimetric assay. In agreement with previously reported results, chemotherapeutic drugs significantly reduced cell viability of T24 control cells (Figure 28).

Treatment with cisplatin alone led to a significant ($p<0.05$) decrease of proliferative capacity of cells transfected with pLKO.1-647 compared with that of mock, after 36h (15% reduction) and 48h (25% reduction). Treatment with gemcitabine alone or with both drugs was significantly ($p<0.05$) associated with a reduction of viability of PON2 downregulating cells (pLKO.1-647) with respect to that of mock at 24h (14% reduction for gemcitabine and 16% reduction for both drugs), 36h (27% reduction for gemcitabine and 37% reduction for both drugs) and 48h (28% reduction for gemcitabine and 59% reduction for both drugs) time points (Figure 28, panel A).

Conversely, the induction of PON2 overexpression led to a significant ($p<0.05$) enhancement in proliferative capacity of T24 cells (pcDNA3-PON2) compared with controls (mock) at 24h time-point (21% increase for cisplatin, 28% increase for gemcitabine and 36% reduction for both drugs). However, this effect seemed to be lost at higher time points (Figure 28, panel B).

A



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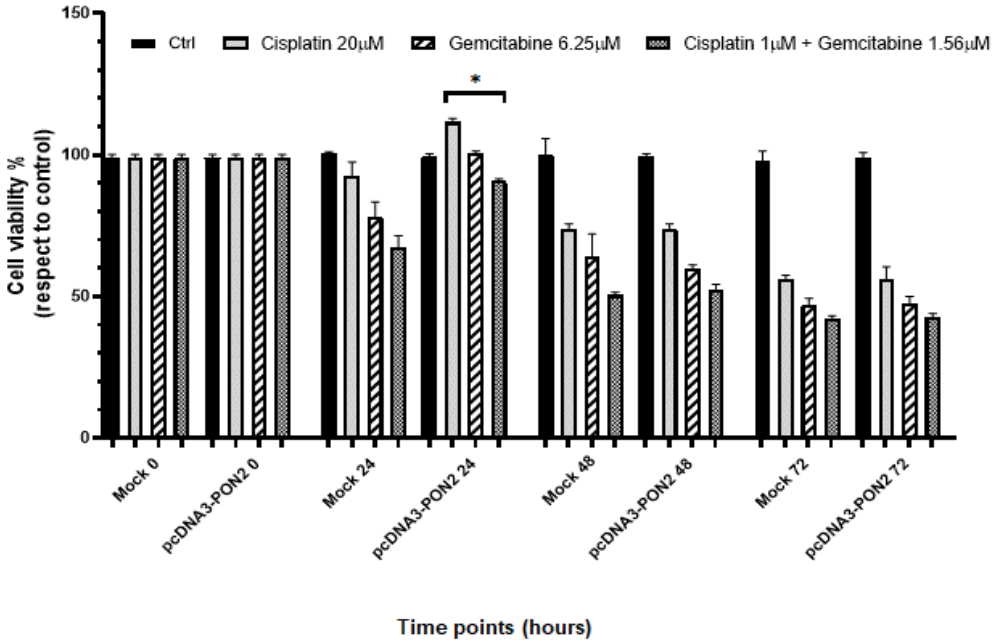


Figure 28. Effect of chemotherapeutic drugs on proliferation of T24 cells. MTT assay was performed to evaluate the influence of treatment with cisplatin (20μM), gemcitabine (6.25μM) or both compounds (cisplatin 1μM + gemcitabine 1.56μM) on viability of cells transfected with pLKO.1-647 (panel A) or pcDNA3-PON2 (panel B) compared with controls (mock). Determination of proliferative rate was carried out at different time points (0h, 12h, 24h, 36h, 48h and 72h). All values are expressed as mean ± standard deviation (*$p<0.05$).

4.4 EFFECT OF PON2 EXPRESSION ON ROS PRODUCTION OF T24 CELLS TREATED WITH CHEMOTHERAPEUTIC DRUGS

To assess the effect of the induction of PON2 knockdown and overexpression on oxidative stress, intracellular ROS levels were evaluated in T24 cells after incubation with cisplatin (20μmol/L), gemcitabine (6.25μmol/L) or both drugs (1.0μmol/L +1.56μmol/L for cisplatin and gemcitabine, respectively), as well as in untreated cells. Chemotherapeutic treatment led to a significant increase of ROS production in T24 control cells (Figure 29).

As shown in Figure 29, panel A, upon treatment with cisplatin, ROS production was significantly ($p<0.05$) higher in PON2 downregulating cells (pLKO.1-647) compared with mock at 48h time point only (1.56-fold increase). Incubation with gemcitabine alone or with combined drugs led to significantly ($p<0.05$) higher ROS levels in T24 cells transfected with pLKO.1-647 compared with mock, both at 36h (1.30-fold increase for gemcitabine and 1.61-fold increase for both drugs) and 48h (1.85-fold increase for gemcitabine and 2.55-fold increase for both drugs) time points.

On the contrary, upon treatment with both drugs used alone or combination, intracellular ROS production was significantly ($p<0.05$) lower in PON2 overexpressing cells compared with mock at 48h (1.37-fold decrease for cisplatin, 1.96-fold decrease for gemcitabine and 3.03-fold decrease for both drugs) and 72h (2.00-fold decrease for cisplatin, 2.78-fold decrease for gemcitabine and 4.00-fold decrease for both drugs) time points, while cisplatin alone had no significant effect at 24h (1.26-fold decrease for gemcitabine and 1.69-fold decrease for both drugs) (Figure 29, panel B).

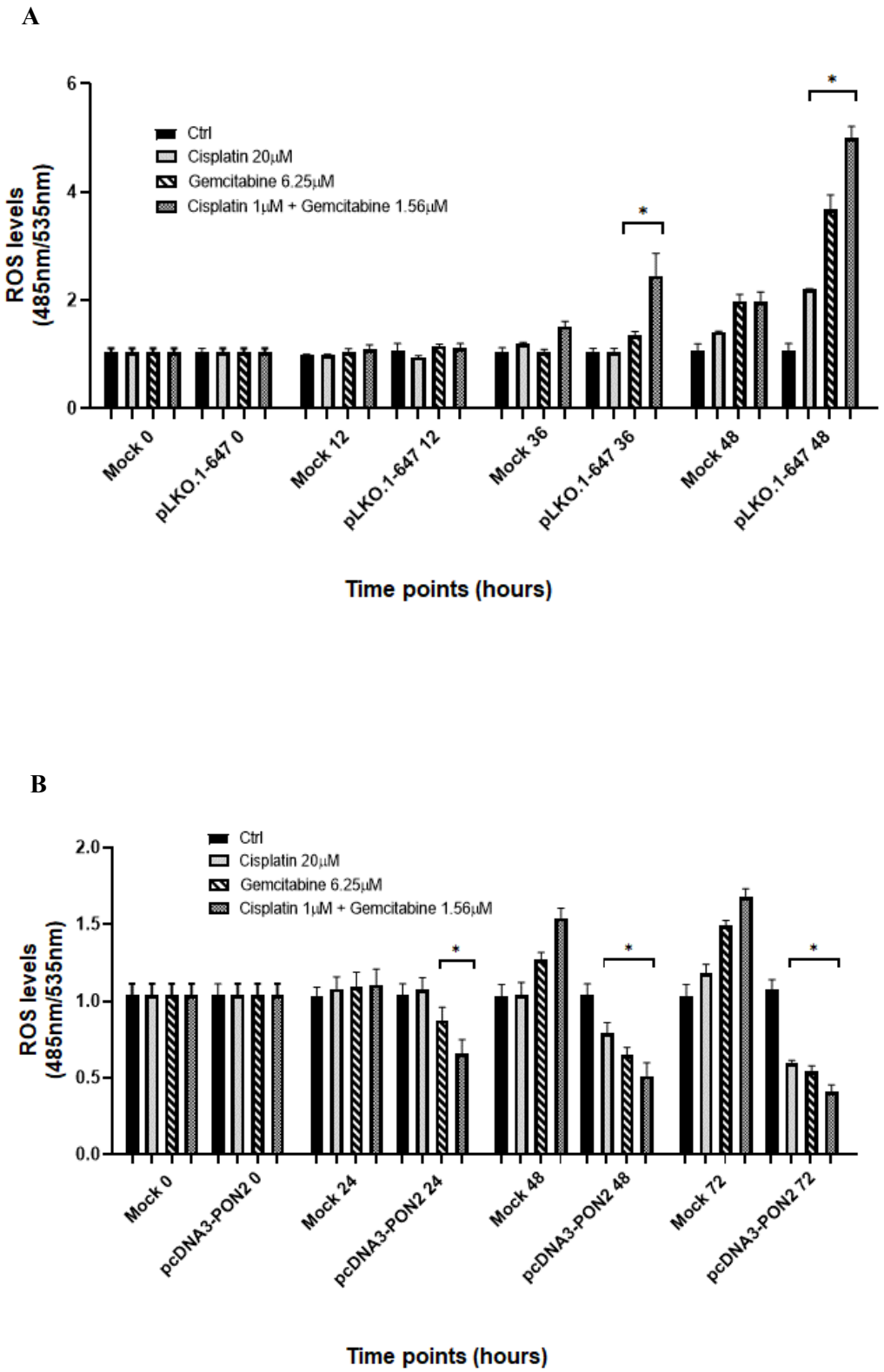


Figure 29. Intracellular ROS levels in T24 cells upon treatment with drugs. In vitro effect of PON2 silencing (panel A) and overexpression (panel B) on intracellular ROS production upon treatment with cisplatin (20μM), gemcitabine (6.25μM) or both compounds (cisplatin 1μM + gemcitabine 1.56μM). ROS levels were determined at different time points (0h, 12h, 24h, 36h, 48h and 72h). All values are expressed as mean ± standard deviation (*$p<0.05$).

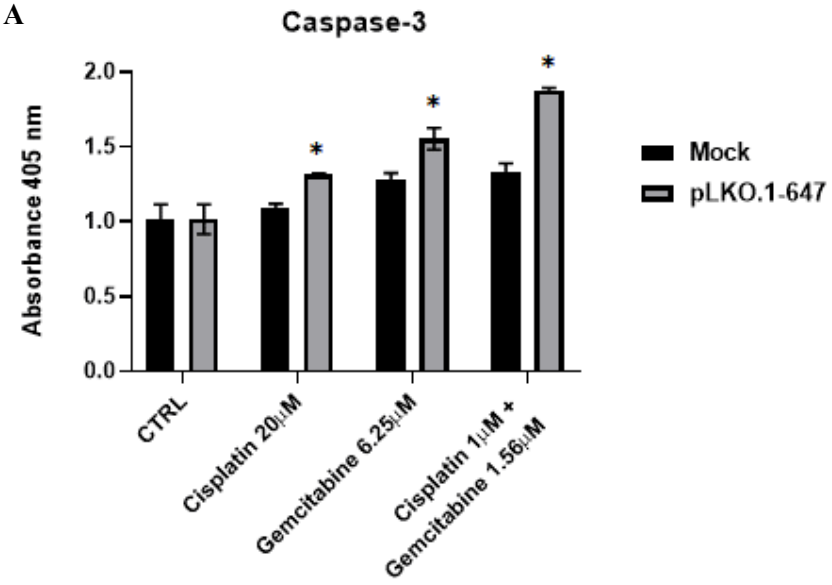
4.5 PON2 PROTECTS T24 CELLS AGAINST APOPTOSIS THROUGH CASPASE-3 AND CASPASE-8 ACTIVATION

In order to investigate the contribution of enzyme to BC tumorigenesis by promoting apoptotic escape, caspase-3 and caspase-8 activities were evaluated in T24 cells downregulating and overexpressing PON2, as well as corresponding controls, upon treatment for 24h with cisplatin (20μmol/L), gemcitabine (6.25μmol/L) or both drugs (1.0μmol/L +1.56μmol/L for cisplatin and gemcitabine, respectively).

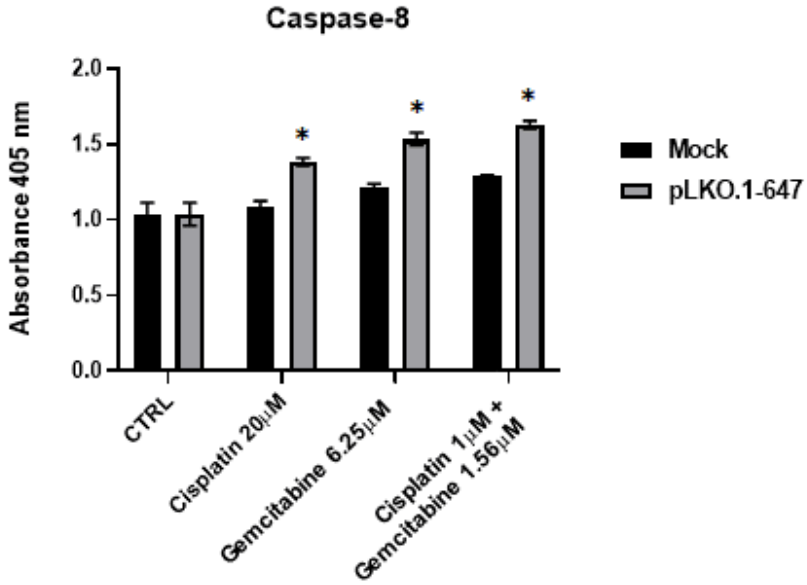
Treatment with chemotherapeutic drugs was able to induce a significant ($p<0.05$) activation of both caspase-3 and caspase-8 in T24 cells transfected with pLKO.1-647 compared with mock. In particular, caspase-3 activation was higher in PON2 downregulating cells treated with both compounds (1.41-fold increase) than in T24 cells upon incubation with cisplatin (1.20-fold increase) or gemcitabine (1.21-fold increase) used alone. On the contrary, the activation of caspase-8 did not seem to be significantly different (pLKO.1-647 versus mock) among treatments (1.28-fold increase for cisplatin, 1.26-fold increase for gemcitabine and 1.23-fold increase for both drugs) (Figure 30, panels A and B).

On the other side, T24 cells overexpressing PON2 showed significantly ($p<0.05$) lower levels of caspase-3 and caspase-8 activity than those determined in mock. Concerning caspase-3, the reduction of activity levels was enhanced in PON2 overexpressing cells that underwent combined treatment (1.35-fold reduction) compared with that observed in T24 cells incubated with cisplatin (1.18-fold reduction) or gemcitabine (1.19-fold reduction). Conversely, the decrease of caspase-8 activity detected in T24 cells upon enzyme overexpression (pcDNA3-PON2) did not seem to be significantly altered based on treatment with different compounds or drug combination (1.25-fold reduction for cisplatin, 1.30-fold decrease for gemcitabine and 1.28-fold decrease for both drugs) (Figure 30, panels C and D).

A


Caspase-3
Absorbance 405 nm
2.0
1.5
1.0
0.5
0.0
*
*
*
CTRL
Cisplatin 20μM
Gemcitabine 6.25μM
Cisplatin 1μM + Gemcitabine 1.56μM
Mock
pLKO.1-647

B


Caspase-8
Absorbance 405 nm
2.0
1.5
1.0
0.5
0.0
*
*
*
CTRL
Cisplatin 20μM
Gemcitabine 6.25μM
Cisplatin 1μM + Gemcitabine 1.56μM
Mock
pLKO.1-647

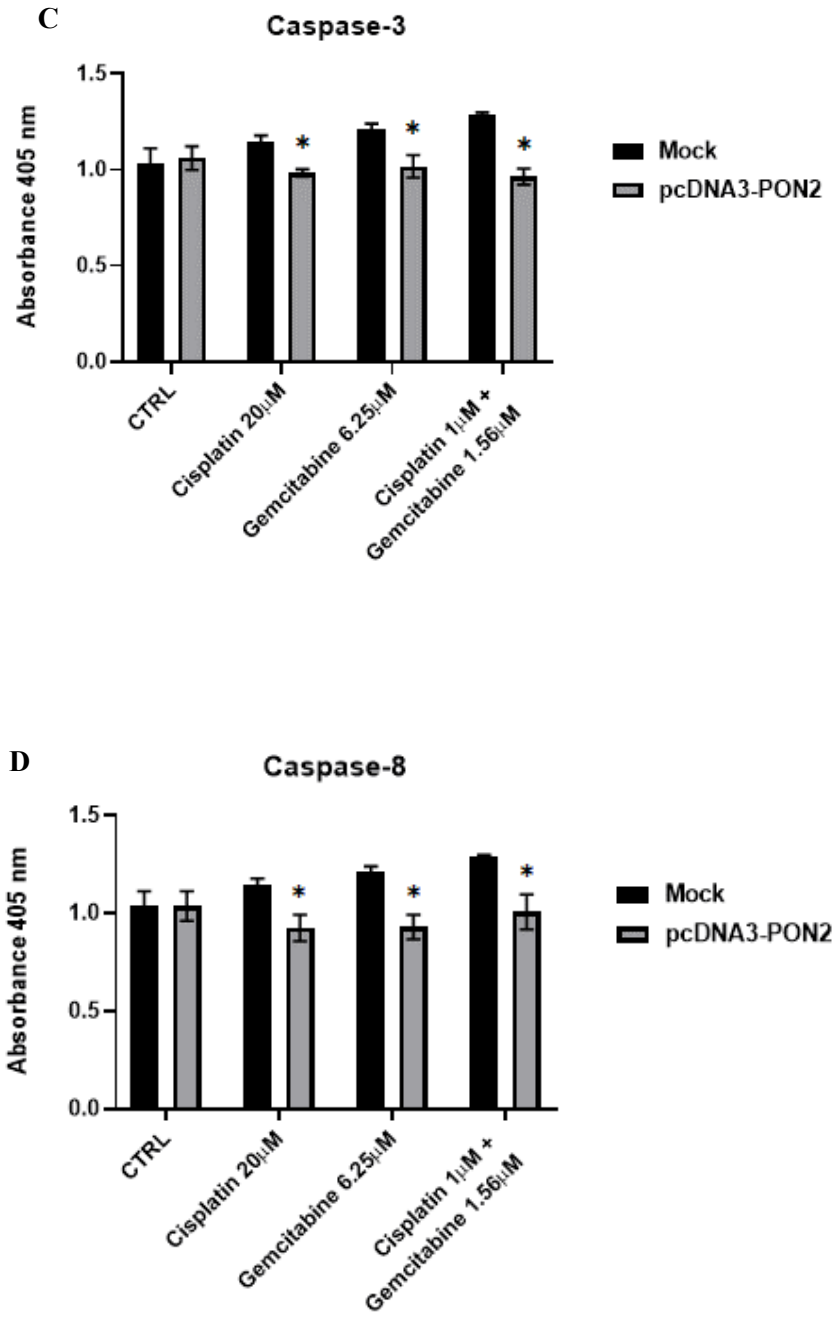


Figure 30. Caspase-3 and caspase-8 activity in T24 cells treated with chemotherapeutic compounds. Apoptosis induction in PON2 downregulating (panels A and B) and overexpressing (panels C and D) cells was determined by evaluating the activity levels of both caspase-3 and caspase-8 after 24h treatment with cisplatin (20µM), gemcitabine (6.25µM) or both compounds (cisplatin 1µM + gemcitabine 1.56µM). All values are expressed as mean ± standard deviation (*$p<0.05$).

5. DISCUSSION AND CONCLUSIONS

Bladder cancer is the 9th most common malignancy in the world. Epidemiological data indicate that it represents about 3.1% of all cancers [Ferlay J. et al, 2012] and is much more common in males than in females (the ratio is about 3.5:1), with estimate 260,000 new cases occurring each year in men and 76,000 in women. In both sexes, the highest rates of bladder cancer incidence were observed in Western Europe, North America and Australia. There are several potential risk factors for bladder cancer: cigarette smoking and occupational exposure to aromatic amines are the most important factors [IARC, 2004; Dietrich H et al, 2001]. It is estimated that the risk in smokers is 2-6 times greater than in non-smokers. The probability of survival of this tumor increases significantly if the tumor is discovered in the early stages. In North Africa and East Asia, most bladder carcinomas are caused by schistosomiasis, responsible for about 10% of bladder carcinomas in developing countries and 3% of global cases [Parkin DM et al, 2006]. A large-scale study has identified some genes predisposing to the development of urothelial carcinoma in the genetic make-up of European populations [Rafnar T et al, 2011]. In general, genetic factors are responsible for 7% of bladder tumors. Therefore, the study of the molecular mechanisms underlying tumor onset and progression, and the search for markers that can be used for diagnose cancer in the early stages represents an important and interesting research area.

In industrialized countries, transitional cell carcinoma is the predominant histological BC form, accounting for 93–95% of cases. Generally, 75–80% of cases are diagnosed as superficial BC, being the remaining muscle-invasive neoplasms [Eble JN, Sauter G, Epstein JI, Sesterhenn IA. World Health Organization, 2004; Adami HO, Hunter D, Trichopoulos D., 2008].

Since many years, chemotherapy represents the standard clinical practice, for patients with metastatic disease. Rather than use of single drugs, such as cisplatin, effective chemotherapy is based on combined regimens, such as cisplatin-gemcitabine (CG) or methotrexate, vinblastine, adriamycin and cisplatin (MVAC). Response rates are 49% and 46% in patients treated with CG or MVAC, respectively, mean survival is 14 months for GC and 15.2 months for MVAC and 5-year survival rates are respectively by 13.0% and 15.3% [Von der Maase H, Hansen S W, Roberts JT et al,2000; Von der Maase H, Sengelov L, Roberts JT et al,2005;]. Although cisplatin-based regimens are the optimal treatment for advanced-stage tumors, almost half of patients are not eligible

for these therapies [Galsky MD et al, 2011]. However, tumor cells gradually develop chemoresistance, thus allowing neoplasm to recur and metastasize [von der Maase H, Sengelov L, Roberts JT, Ricci S, Dogliotti L, Oliver T, Moore MJ, Zimmermann A, Arning M, 2005; von der Maase H, Hansen SW, Roberts JT, Dogliotti L, Oliver T, Moore MJ, Bodrogi I, Albers P, Knuth A, Lippert CM, Kerbrat P, Sanchez Rovira P, Wersall P, Cleall SP, Roychowdhury DF, Tomlin I, Visseren-Grul CM, Conte PF, 2000]. The plasma membrane events involved in cisplatin-induced apoptosis remain still unknown and could constitute new targets to improve therapeutic strategies in order to limit cytotoxicity, or to prevent the development of chemoresistance.

In recent years, great efforts have been made to identify molecules that are involved in BC tumorigenesis and/or are able to reflect changes in neoplastic tissue, thus acting as potential biomarkers for early and noninvasive BC detection [de Martino M, Shariat SF, Hofbauer SL, Lucca I, Taus C, Wiener HG, Haitel A, Susani M, Klatte T, 2015].

There is a growing scientific consensus that recognizes to PON2 a possible role in the physiopathology of cancer. In fact, several studies reported the upregulation of PON2 in different human cancers [Witte I, Foerstermann U, Devarajan A, Reddy ST, Horke S, 2012; Witte I, Altenhöfer S, Wilgenbus P, Amort J, Clement AM, Pautz A, Li H, Förstermann U, Horke S, 2011]. Furthermore, it has been shown that PON2 overexpression is involved in the survival and proliferation of tumor cells such as glioblastoma cells, lung carcinoma, leukemia, HELA cells and colon cancer [Witte I et al 2011; Witte I et al 2012; Tseng JH et al 2017; Horke S et al 2010].

Our recent study demonstrated that PON2 was significantly upregulated in BC compared to adjacent normal looking tissue. We also investigated enzyme expression in urothelial cells collected from urine specimens of subjects affected by BC and healthy controls. The obtained results showed that PON2 mRNA levels in pathological samples were inversely related with clinical parameter pT, which considers the size and the extent of primary tumor, thus suggesting a potential role for the enzyme in the early stages of tumor. Furthermore, in T24 cells, PON2 overexpression was able to promote cell proliferation and increase resistance to oxidative stress [Bacchetti T, et al, 2017]. These data suggest that the increased expression of PON2 observed in tumor cells, including those of the bladder, may represent an important strategy of tumor cells to resist cell death and

escape the intrinsic ROS-induced apoptotic program that appears to be one of the main obstacles to formation of cancer.

Based on this evidence, the purpose of this work was to further investigate the role of PON2 in the development and progression of bladder cancer. In particular, the objective was to verify the possible role of PON2 in chemoresistance.

The potential role of PON2 on *in vitro* tumorigenicity of BC cell was investigated. To achieve this goal, enzyme knockdown and overexpression were induced in T24 human bladder cancer cells which were further treated with chemotherapeutic drugs. Subsequent analyses were carried out to evaluate the effect of modulation of PON2 expression on several phenotypic aspects, including cell proliferation, migration, response to oxidative stress and apoptosis.

Reported data demonstrated that PON2 expression exerted a positive influence on T24 cell proliferation and migration, thus highlighting its potential role in promoting bladder tumorigenesis. In particular, enzyme overexpression led to an increase of cell viability of T24 cells treated with chemotherapeutic drugs while PON2 downregulation was associated with a significant reduction of their proliferative capacity. These results are in agreement with those reported in previous studies, demonstrating that PON2 is involved in survival and proliferation of cancer cells [Bacchetti T, Ferretti G, Sahebkar A, 2019].

In addition, Bacchetti T. et al, in 2017, observed high levels of MIB-1 (also known as Ki-67) in T24 cells overexpressing PON2 than those detected in the control cells. Ki-67 is known to be present in cell nuclei in G1, S, even the G2 phases as well as in mitosis, while it is not expressed in quiescent or resting cells (phase G0). From this point of view, MIB-1 upregulation in T24 cells overexpressing PON2 could demonstrate at molecular level the ability of the enzyme to promote proliferative capacity of T24 cells [Gerdes J, 1984].

Recent data suggested that, among mechanisms underlying the cytotoxic effect exerted by cisplatin and gemcitabine, there is ROS formation [Brozovic et al., 2010]. In fact, cisplatin and gemcitabine have been reported to cause an increase in oxidative damage to the mitochondrial membrane, with consequent impairment of mitochondrial function, increase in ROS formation and activation of pathways leading to cell death [Saad et al., 2004].

The antioxidant and cytoprotective effect of PON2 had been previously demonstrated in other cellular models. PON2 is an intracellular and ubiquitous protein present in almost all tissues, mainly located in the membrane of the mitochondrion, of the endoplasmic reticulum as well in plasma membrane. PON2 has been shown to play an important role in mitochondrial activity. In the inner mitochondrial membrane PON2 interacts with components of the electron transport chain, such as semiquinone, preventing the formation of reactive oxygen species. In fact, different studies demonstrated that enzyme activity protected macrophages, vascular and other cells against oxidative stress, whereas PON2 downregulation reversed these effects [Horke S, Witte I, Wilgenbus P, Krüger M, Strand D, Förstermann U, 2007; Ng CJ, Wadleigh DJ, Gangopadhyay A, Hama S, Grijalva VR, Navab M, Fogelman AM, Reddy ST, 2001].

In particular, we demonstrated that PON2 upregulation significantly counteracted the increase in cellular ROS production in response to oxidative stress triggered by chemotherapeutic compounds. On the contrary, enzyme downregulation led to an increase of ROS levels in treated T24 bladder cancer cells. Our data showed that PON2 plays an important role in modulating sensitivity to drugs used in chemotherapy. Therefore, it is possible to suggest that PON2 is able to reduce cell death caused by chemotherapy agents, by reducing the apoptotic thrust following ROS production and release.

Results obtained from further *in vitro* experiments demonstrated the influence exerted by PON2 expression on drug-induced apoptosis. After treatment with chemotherapeutic drugs, the colorimetric assay revealed an increase in caspase activity upon PON2 silencing in T24 cells, while a reduction was determined in PON2-overexpressing cells.

There is accumulating evidence that cancer cell response to apoptotic insults is significantly affected by the cellular redox status. On the other hand, it is also well established that oxidative stress is closely linked to cell death and cancer [Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB, 2010; Panieri E, Santoro MM, 2016]. The anti-apoptotic effect exerted by PON2 is reasonably strictly related with its intracellular localization, mainly within the membranous systems of endoplasmic reticulum (ER) and mitochondria. Altenhofer et al. recognized to PON2 the ability to prevent mitochondria-derived superoxide formation which is responsible for the activation of the apoptotic pathway in response to cardiolipin peroxidation and subsequent cytochrome c

release [Altenhöfer S, Witte I, Teiber JF, Wilgenbus P, Pautz A, Li H, Daiber A, Witan H, Clement AM, Förstermann U, Horke S, 2010].

In this field of research, many studies have reported results that allowed to get an insight into the anti-apoptotic role played by PON2. The activation of executioner caspase-3 and subsequent induction of apoptosis can arise from a longer-lasting ER stress which triggers the unfolded protein response (UPR) pathway [Zhang K, Kaufman RJ, 2006]. Horke et al. demonstrated that human umbilical vein endothelial cells EA.hy 926, stably overexpressing PON2, displayed a significant reduction of caspase 3/7 activation, although treated with the UPR-inducing reagent tunicamycin. Conversely, in PON2-silenced EA.hy 926 cells, treatment with tunicamycin led to an opposite effect, thus determining an increase of caspase 3/7 activation status [Horke S, Witte I, Wilgenbus P, Krüger M, Strand D, Förstermann U, 2007]. Further results demonstrated that PON2 could confer protection against ER-stress-induced caspase-3 activation, by modulation of calcium homeostasis [Horke S, Witte I, Wilgenbus P, Altenhöfer S, Krüger M, Li H, Förstermann U, 2008].

Data reported in the present *in vitro* study demonstrated, partly confirming what previously described, that PON2 contribute to bladder tumorigenesis by promoting both cell proliferation and migration. Moreover, the enzyme was found to play a significant role in BC cell resistance to chemotherapeutic treatment with cisplatin and gemcitabine. In this light, PON2 overexpression detected in association with BC could represent an adaptive mechanism of tumor cell to escape/survive cell death and apoptosis induced by chemotherapy.

As discussed in the previous paragraphs, to date, PON1 remains the only well-characterized member of the PONs family. Harel M, in 2004 first reported and described the crystal structure of a variant of PON1 family member obtained by directed evolution, thus allowing to provide with a detailed description of PON1's active site and catalytic mechanism.

Considering PON2 contribution to bladder tumorigenesis, the resolution of 3D structure of the protein could represent an important goal. Indeed, once obtained the 3D structure of the protein, specific and effective inhibitors of its catalytic activity could be designed, synthesized and assayed. The effective reduction of PON2 activity induced by the presence of these inhibitors could represent an interesting starting point for the development of new molecules for effective pharmacological treatment of bladder cancer.

Although further analyses will be required to deeply investigate the molecular mechanisms by which PON2 could participate to bladder cancerogenesis, our study clearly demonstrated for the first time the impact of the enzyme on tumor progression and susceptibility of bladder tumor cell to chemotherapeutics, suggesting a potential use of PON2 as an interesting molecular target for BC therapy.

In conclusion, results obtained highlighted that PON2 plays an important role in proliferation and tumorigenic capacity of BC cells, representing a potential molecular target for an effective anti-cancer therapy. Despite experimental evidences demonstrated that PON2 was involved in tumorigenesis, further studies are required to deeply understand the molecular mechanisms affected by the enzyme in cancer cells. Data obtained also showed a possible correlation between PON2 and caspases expression levels, suggesting a key role of the enzyme in the regulation of apoptosis.

6. LIST OF ABBREVIATIONS

BC Bladder Cancer

HPV Papilloma virus

EMA European Medicines Agency

TCC Transitional cell carcinoma

WHO World Health Organization

ICCR International Cancer Control Partnership

UAE European Urology Association

NMIBC Non-muscle-invasive bladder cancer

MIBC Muscle-Invasive Bladder Cancer

CIS situ transitional cell carcinoma

TNM tumor, node, metastasis classification

UICC Union for International Cancer Control

FGFR3 receptor 3 fibroblast growth factor

RB retinoblastoma

Uro-CT urographic scans

TUR transurethral resection

MR magnetic resonance

FDG Fluoro-Desoxy-Glucose

PDD Fluorescence urethrocystoscopy

HAL hexaminolevulinic acid

NBI Narrow-band imaging

EORTC European Organisation for Research and Treatment of Cancer

Re-TUR second transurethral resection

BCG Bacillus Calmette-Guérin

CG combination cisplatin-gemcitabine

MVAC combination methotrexate, vinblastine, adriamycin and cisplatin

Apaf-1 apoptotic protease activating factor 1

ATP adenosine triphosphate

FasL Fas ligand

FADD Fas-associated death domain

gDNA genomic DNA

HMG high mobility group

NER nucleotide excision repair

MMR mismatch repair

FLIP flice-like inhibitory protein

Bcl-xL B-cell lymphoma-extra-large

PTEN phosphatase and tensin homolog

dFdC 2'2'-difluoro-2'-deoxycytidine

NA nucleoside analog

dFdCDP gemcitabine diphosphate

dFdCTP gemcitabine triphosphate

dCK Deoxycytidine kinase

dNTP deoxynucleotide

dATP deoxyadenosine triphosphate

dCTP deoxycytidine triphosphate

MK2 protein Mitogen-Activated Protein Kinase-Activated Protein Kinase 2

PONs paraoxonase gene family

HDL high-density lipoproteins

ApoA-I apolipoprotein A-I

RE endoplasmic reticulum

DHC dihydrocumarine

AHL acyl-homoserine lactones

ROS reactive oxygen species

DMNQ 2,3-dimethoxy-1,4-naphthoquinone

LDL low-density lipoproteins

UPR unfolded protein response

LD lipid droplets

DAGT1 diacylglycerol acyltransferase 1

ALL acute lymphoblastic leukemia

JNK c-Jun N-terminal kinases protein

CML chronic myelogenous leukemia

MPM murine peritoneal macrophages

LPC lysophosphatidylcholine

HMDM human macrophages derived from monocytes

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

DMSO dimethyl sulfoxide

H_2DCF-DA 2',7'-dichlorodihydrofluorescein diacetate

PUNLMP papillary urothelial neoplasm of low malignant potential

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