Paraoxonase-2 is upregulated in triple negative breast cancer and contributes to tumor progression and chemoresistance

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1. Introduction

Breast cancer (BC) is the most common malignancy in women accounting for approximately 25% of all cancers and its incidence is constantly increasing [1]. The term BC comprises several subtypes characterized by distinctive course, prognosis, sensitivity to chemotherapy or targeted therapies, and other characteristics, due to the variety of genetic aberrations of this disease. Therefore, BC is an extremely heterogeneous neoplasm which complicates its management. Indeed, recent studies have identified several molecular subtypes of BC which differ for prognosis and response to therapies: Luminal A, Luminal B, Luminal B HER2+, HER2+ and triple negative breast cancer (TNBC) [2].

TNBC accounts for about 10-15% of all BCs and is epidemiologically associated with young women, women who are carriers of breast cancer susceptibility gene (BRCA) mutations, younger age at menarche, short duration of breast-feeding, obesity of premenopausal women, and African-American or non-Hispanic black race [3]. Despite the lower incidence compared to the other BC subtypes, TNBC displays a very aggressive behavior, associated with high histological grades, rapid proliferation and frequent involvement of regional lymph nodes. Moreover, TNBC is associated with early recurrence, high invasiveness and metastatic potential, which lead to a poor prognosis [4, 5]. Indeed, the frequency of loco-regional relapses of TNBC is similar to the HER2+ subtype, which is also considered a high aggressive subtype, and noteworthy about 50% higher than in the luminal subtypes [6].

TNBC is characterized by the lack of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2), which prevents the use of endocrine or molecular targeted therapy. Indeed, although there have been recent advances in therapeutic options for the management of other BC subtypes, chemotherapy is still the primary systemic treatment for TNBC following or prior to surgery. Furthermore, the management of TNBC is complicated by the observation that the TNBC itself is not a homogenous disease, since the genetic research has identified a significant heterogeneity which reflects into the existence of at least 6 TNBC subtypes: two basal-like, immunomodulatory, mesenchymal, mesenchymal stem, and one with androgen receptors [7].

Despite the poor prognosis and high aggressiveness of this malignancy, a significant number of TNBC patients display a particular sensitivity to chemotherapy compared with patients affected by ER-positive breast cancer. Indeed, about 30-40% of TNBC patients achieve a pathological complete response (pCR) after the neoadjuvant chemotherapy. This is particularly important since, compared with patients with residual disease, patients that reached a pCR after the neoadjuvant chemotherapy display a good prognosis since the risk of recurrence is reduced by 70% [8]. However, many patients

either display *de novo* resistance or ultimately develop resistance to chemotherapeutics, limiting their long-term utility and significantly worsening the mortality rate [9]. Despite the recent approvals of the use of immune checkpoint inhibitors (e.g. atezolizumab, sacituzumab) or the PARP inhibitors (olaparib, talazoparib) in refractory metastatic TNBC, in the last decades the overall survival of TNBC patients had a limited increase compared to other BC subtypes, which greatly benefit of novel strategies for the management of these neoplasms [9-12]. Thus, there is an urgent need to identify novel molecular targets to improve the outcome of chemotherapy given in first-line settings, as well as in patients with advanced, chemotherapy resistant TNBC. The main chemotherapeutic drugs used for BC treatment include, but are not limited to, taxanes, platinum derivatives like cisplatin (CDDP), antimetabolites such as 5-Fluorouracil (5-FU) and anthracyclines such as doxorubicin [3].

In the recent years, an increasing number of studies has shown that the enzyme paraoxonase-2 (PON2) might play a role in chemoresistance of tumor cells. PON2 belongs to the human paraoxonase (PON) gene family which also includes the enzymes paraoxonase-1 (PON1) and paraoxonase-3 (PON3); however, while PON1 and PON3 are mainly present in the serum, PON2 is expressed in various tissues, intracellularly localized upon translation and mainly associated with the nuclear envelope, endoplasmic reticulum, mitochondria, and plasma membrane [13].

PON2 plays an important role in counteracting oxidative stress due to its ability in decreasing the generation of reactive oxygen species (ROS). Indeed, the enzyme binds with high affinity to coenzyme Q10 within the inner membrane of mitochondria, thus triggering a diminution of superoxide anion release during the electron transport chain [14]. Interestingly, PON2 overexpression has been reported in various malignancies including oral, bladder, pancreatic, ovarian, gastric and skin cancer [15-17]. Therefore, the aim of this study was to evaluate the expression of the enzyme in BC specimens and subsequently evaluate the impact of PON2 downregulation on BC cells proliferation and sensitivity to the chemotherapeutics cisplatin, 5-Fluorouracil and doxorubicin, routinely used for BC treatment.

2. Material and methods

2.1 Case selection

This retrospective study examined 56 BC formalin-fixed and paraffin-embedded (FFPE) specimens, obtained from the Department of Biomedical Sciences and Public Health (Section of Pathology) of Polytechnic University of Marche. Exclusion criteria were reported as Supplementary Materials (Figure 1S). The research was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). This retrospective study was performed on formalin-fixed and paraffin-embedded tissue specimens, previously collected for diagnostic purposes. According to the Ethics Committee of the Marche region ethical approval for retrospective studies is not required. All information regarding human material was managed using anonymous numerical codes.

The specimens included 11 Luminal A, 12 Luminal B, 11 Luminal B HER2+, 10 HER2+ and 12 TNBC cases. The peritumoral healthy tissue was utilized as control. For each specimen the ductal carcinoma *in situ* (DCIS) and the ductal infiltrating carcinoma (IDC) were analyzed. Collectively samples included 56 women (median age 65; minimum age 38 years old - maximum age 94 years old). Demographic and clinico-pathologic characteristics of breast cancer cases are listed in Table 1.

2.2 Immunohistochemical analyses

Immunohistochemical analyses were performed to evaluate PON2 expression in tumor and control tissue specimens. 5 µm sections obtained from FFPE blocks were mounted on poly-L-lysine-coated glass slides, de-paraffinized in xylene, rehydrated and treated with EnVision FLEX Target Retrieval Solution High pH (Dako). Subsequently, glass slides were incubated with 3% H_2O_2 for 7 minutes and blocked with 5% Normal Goat Serum (Dako). After washing, sections were incubated with rabbit polyclonal anti human-PON2 antibody (1:1000 dilution) (Sigma-Aldrich) for 1 hour at room temperature in a humified atmosphere. After washing, glasses were treated with EnVision

FLEX/HRP (Dako) for 20 minutes and incubated with FLEX DAB+ Chromogen (Dako) for 10 minutes, after an additional washing, and finally counterstained with Mayer's haematoxylin. Samples were evaluated by two pathologists blinded to the patient group whose observations were in agreement in >95% cases. The score of the intensity of PON2 staining was classified as negative (0), moderate (1), good (2) and strong (3).

2.3 Cell Lines and culture

Human breast cancer cell lines MCF-7 (ER+), SKBR-3 (HER2+) and MDA-MB-231 (TNBC) were generously provided by Prof. Mauro Provinciali and Dr. Elisa Pierpaoli (Advanced Technology Center for Aging Research, IRCCS INRCA, Ancona, Italy). Cells were grown in Dulbecco's Modified Eagle's Medium containing 4.5 g/L glucose, 10% FBS, and 50 µg/mL gentamicin, in standard culture conditions.

2.4 shRNA-Mediated Gene Silencing of PON2

In order to achieve PON2 silencing, cells were transfected with plasmids encoding shRNA targeted to PON2. Briefly, 5.0×10^4 cells/well were seeded in 24-well plates. The day after seeding, plasmids (0.5 µg/well) encoding shRNA targeted to PON2 (pLKO.1-643 and pLKO.1-647) or empty vectors (pLKO.1-puro) were utilized to transfect cells while control cells were treated with transfection reagent only (mock). The transfection procedure was performed using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA), according to the manufacturer's instructions. 48h after the transfection procedure, the selection of cellular clones downregulating PON2 started by cultivating cells in the presence of 1µg/mL puromycin. The efficiency of PON2 gene silencing in BC cells was assessed by Real-Time PCR and Western blot analysis.

2.5 Real-Time PCR

For Real-Time PCR analysis, total RNAs were extracted and reverse transcribed as previously described [18]. Briefly, cell pellets ($1.5x10^6$) were homogenized and total RNA was isolated through the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to manufacturer's instructions. After evaluating RNA quality and quantity by nanodrop, 1µg of RNA was reverse transcribed utilizing the M-MLV Reverse Trascriptase (Promega), according to the manufacturer's instructions.

The CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) was used to perform the Real-Time PCR analyses, using as template the cDNA generated as described above. The primers used were (forward) 5'-TCGTGTATGACCCGAACAATCC-3' and (reverse) 5'-AACTGTAGTCACTGTAGGCTTCTC-3' for PON2, and (forward) 5'-TCCTTCCTGGGCA TGGAGT-3' and (reverse) 5'-AGCACTGTGTTGGCGTACAG-3' for β-actin, which was used as reference gene. SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA) was used for the Real-Time PCR, running genes in duplicate for 40 cycles at 95°C for 30 seconds and 58°C for 30 seconds.

The fluorescence produced by the EvaGreen dye, which binds to double strand DNA after every cycle, was used to monitor the direct detection of PCR product accumulation. For each sample PON2 level was expressed as ΔCt value, where ΔCt = Ct (PON2) - Ct (β-actin). Fold changes in relative gene expression were calculated by $2^{-\Delta\Delta Ct}$ method, where ΔCt = Ct (PON2) - Ct (β-actin) and Δ(ΔCt) = ΔCt (pLKO.1-puro, pLKO.1-643 and pLKO.1-647) - ΔCt (mock).

2.6 Western blot analysis

Cell pellets (2×10^6 cells) were lysed and homogenized through a 26 gauche needle of a syringe utilizing 100µl of lysis buffer (PBS 1X pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1mM sodium orthovanadate, 0.1% SDS, 1mM phenylmethylsulfonyl fluoride and 2µg/ml aprotinin).

Subsequently, the homogenate was centrifuged at 16000 x g for 10 min at 4°C and the supernatant was collected. Protein aliquots of 20μg were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes.

After standard blocking, the membranes were incubated overnight at 4°C with rabbit polyclonal antibody against human PON2 (Sigma-Aldrich, St. Louis, MO, USA) (1:500 dilution), followed by incubation (1:150000 dilution) with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA) for 1h.

PON2 protein was detected using enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The chemiluminescent signal was acquired using ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA).

2.7 Chemotherapeutic treatment

Chemotherapeutic treatment was performed as previously described [18]. Briefly, PON2-downregulating BC cells and controls were seeded into 96-well plates ($1.0x10^4$ cells/well). 24h after seeding, medium was replaced with a fresh one containing chemotherapeutic drugs cisplatin, 5-Fluorouracil and doxorubicin (Sigma-Aldrich, USA) at proper concentration.

2.8 MTT assay

BC cell viability was estimated through the colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) at consecutive time-points (0, 24, 48 and 72h) in presence or absence of cisplatin, 5-Fluorouracil and doxorubicin.

BC cells were seeded in 96-well plates at a concentration of 1.0×10^4 cells/well and were allowed to attach overnight. The cell viability was assessed by measuring the conversion of the tetrazolium salt MTT to formazan crystals. 8.4μl of MTT reagent (5 mg/ml in phosphate buffered saline) were dissolved in complete fresh medium (100μl/well) and added to each well. After an incubation for 2h at 37°C, the solution was discarded and 200μl of 2-propanol were added to each well. The reaction

product was estimated through the measurement of the absorbance at 540nm using an ELISA plate reader. Results were expressed as percentage of the control and presented as mean values ± standard deviation of three independent experiments performed in triplicate.

2.9 Statistical analysis

Results were analyzed using GraphPad Prism 8 software for Windows, Version 8.4.2 (GraphPad Software Inc, San Diego, California, USA). Differences between groups and correlations with clinicopathological parameters were determined by means of Wilcoxon signed-rank and Mann-Whitney U tests. A P-value <0.05 was considered statistically significant.

3. Results

3.1 PON2 expression in BC subtypes

The PON2 expression was evaluated in healthy and pathological *in situ* and infiltrating BC subtypes tissues by immunohistochemical analyses. No statistically significant relationship was found between protein expression level and age (p=0.37) or grade (p=0.50). Immunohistochemical analysis revealed that enzyme expression was significantly higher in tumor tissues compared to the level detected in healthy tissue margins (Fig 1). In particular, in Luminal A subtype, PON2 median staining score for the invasive breast carcinoma was 2.00 (interquartile range 2.00-3.00; min 1.00-max 3.00; p=0.0004), with a median staining score of 2.00 (interquartile range 2.00-2.00 min 1.00-max 3.00; p=0.0016) and 1.00 (interquartile range 1.00-1.00; min 1.00-max 2.00) for the DCIS and the health tissue, respectively (Fig. 1A-D). In the Luminal B subtype, the invasive breast carcinoma showed a median staining score of 1.50 (interquartile range 1.00-2.00; min 1.00-max 3.00; p=0.1411), whereas the DCIS and the health tissue showed median staining score of 2.00 (interquartile range 1.00-2.75; min 0.00-max 3.00; p=0.0915) and 1.00 (interquartile range 1.00-1.00; min 1.00-max 2.00), respectively (Fig. 1E-H). Regarding the Luminal B HER2+ subtype, the invasive breast carcinoma showed a median staining score of 1.00 (interquartile range 1.00-2.00; min 1.00-max 3.00; p=0.053), while the DCIS and the health tissue showed a staining score of 3.00 (interquartile range 1.50-3.00; min 0.00-max 3.00; p=0.0117) and 1.00 (interquartile range 1.00-1.00; min 1.00-max 1.00), respectively (Fig. 1I-N). In the HER2+ subtype, the immunohistochemistry revealed a median staining score of 2.50 (interquartile range 2.00-3.00; min 0.00-max 3.00) for the invasive breast carcinoma, 3.00 (interquartile range 2.00-3.00; min 2.00-max 3.00; p=0.0020) for the DCIS, and 1.00 (interquartile range 1.00-1.00; min 1.00-max 2.00; p=0.0015) for the health tissue (Fig. 1O-R). Finally, in TNBC subtype, the invasive breast carcinoma showed a median staining score of 2.00 (interquartile range 2.00-3.00; min 0.00-max 3.00; p=0.0177), while the median staining score for the DCIS was 2.00

(interquartile range 0.75-3.00; min 0.00-max 3.00; *p*=0.0647), with the healthy margin displaying a mean staining score of 1.00 (interquartile range 1.00-1.00; min 1.00-max 2.00) (Fig. 1S-V).

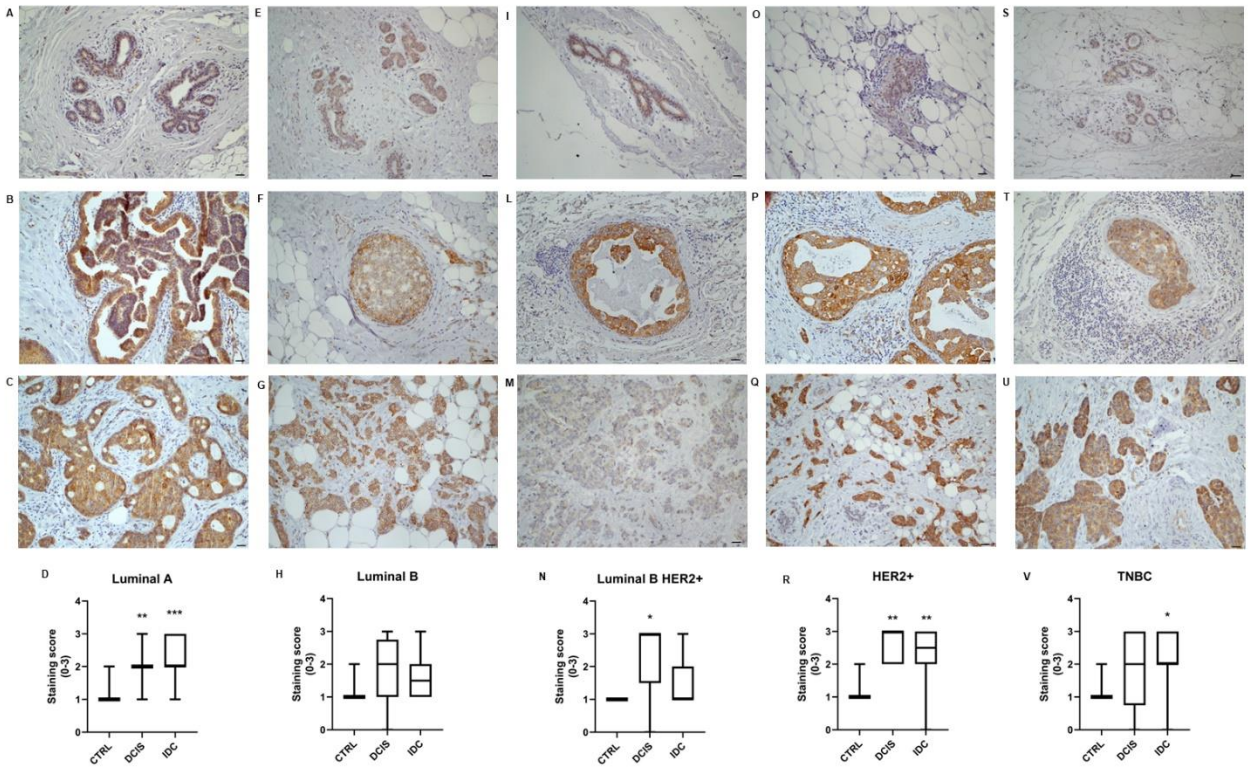

Fig 1. Immunohistochemical expression of PON2 in BC subtypes. PON2 immunopositivity was analyzed in healthy tissue, ductal carcinoma in situ (DCIS) and ductal infiltrating carcinoma (DCI). Luminal A (A – healthy; B – DCIS; C – IDC); Luminal B (E – healthy; F – DCIS; G – IDC); Luminal B HER2+ (I – healthy; L – DCIS; M – IDC); HER2+ (O – healthy; P – DCIS; Q – IDC); TNBC (S – healthy; T – DCIS; U – IDC). Bar diagrams illustrate differential PON2 immunohistochemical expression in Luminal A (D), Luminal B (H), Luminal B HER2+ (N), HER2+ (R), TNBC (V) (×200 original magnification, *p<0.05; **p<0.01; ***p<0.001). Data are showed as median and interquartile range.

3.2 Efficiency of PON2 shRNA-mediated knockdown in BC cell lines

The BC cell lines MDA-MB-231, MCF-7 and SKBR-3 were transfected as described in the Materials and Methods section. To evaluate the efficiency of PON2 knockdown, 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 transfection in all cell lines treated with pLKO.1–647 plasmids. Real-Time PCR showed a significant (p=0.041) downregulation of PON2 in MDA-MB-231 cells transfected with pLKO.1–647 plasmid (0.41 ± 0.05) compared with mock (1.00 ± 0.06) and those transfected with empty vector (0.75 ± 0.12), while cells transfected with pLKO.1–643 plasmid displayed a downregulation (p=0.063) of 0.69 ± 0.06 (Fig. 2A). In MCF-7 cell line, the downregulation of PON2 in cells transfected with pLKO.1–647 plasmid (p=0.038) was 0.29 ± 0.04 compared with mock 1.00 ± 0.07, whereas cells transfected with empty vector expressed PON2 mRNA similarly to the mock group (1.04 ± 0.14), and with the pLKO.1–643 plasmid that displayed a limited efficiency (0.70 ± 0.07) (Fig. 2B). For SKBR-3 cell line, the downregulation of PON2 (p=0.011) in cells transfected with pLKO.1–647 plasmid was 0.05 ± 0.05 compared with mock 1.00 ± 0.07 and those transfected with empty vector (1.16 ± 0.18) (Fig. 2C). As shown in figure 2D, BC cells transfected with pLKO.1–647 plasmid vector displayed a markedly decreased PON2 expression thus confirming an effective gene silencing of PON2 also at protein level.

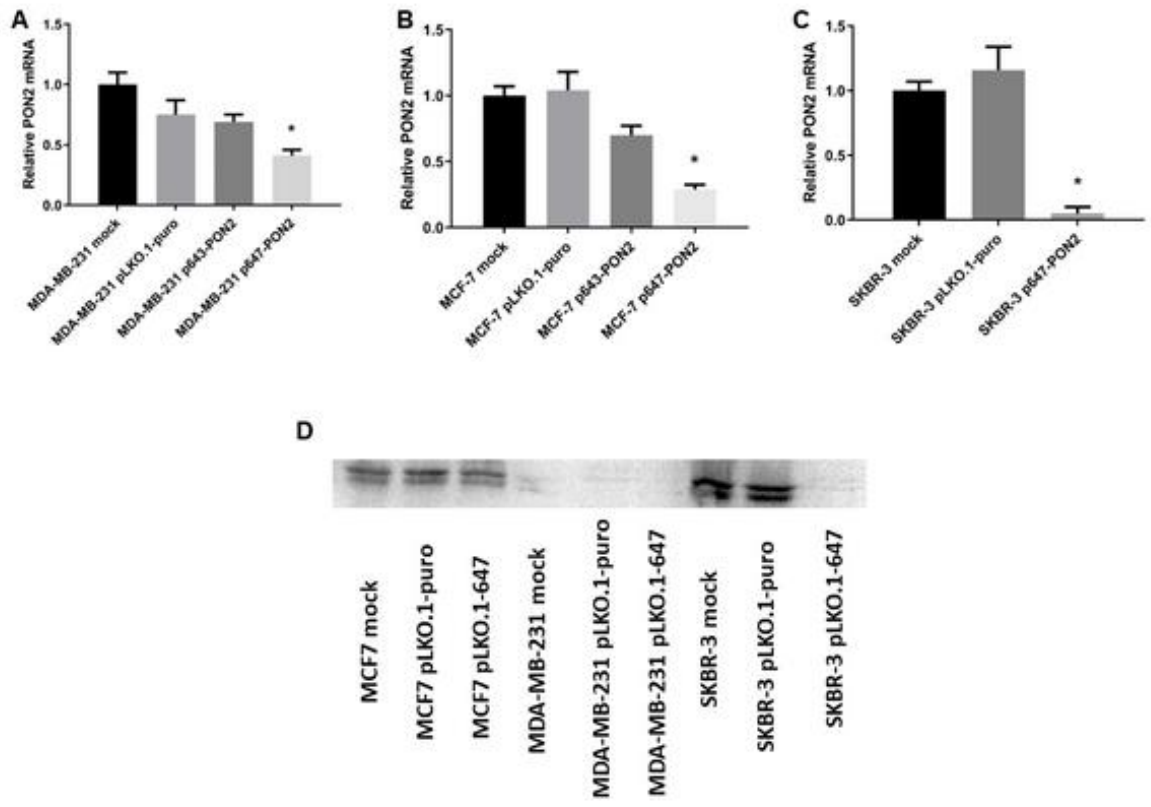

Figure 2. Evaluation of PON2 silencing. MDA-MB-231 (A), MCF-7 (B) and SKBR-3 (C) cells were treated with shRNA plasmid against PON2 (pLKO.1-643 and pLKO.1-647), with empty vector (pLKO.1-puro) or with transfection reagent only (mock). PON2 expression was evaluated at mRNA and protein level by Real-Time PCR (A-C) and Western blot (D), respectively (n = 3). Values are expressed as mean ± standard deviation (*p<0.05).

3.3 Impact of PON2 silencing on BC cell proliferation.

To investigate the role of PON2 in BC cell metabolism and examine the biological effects following the enzyme down-regulation, shRNA vectors targeting PON2 were introduced into BC cells, and the proliferation rate was assessed at different timepoints (0, 24, 48 and 72h), through the MTT colorimetric assay. The results were expressed as relative cell viability referred to control (absorbance at zero time and equal to 100%). In MDA-MB-231 cell line, enzyme downregulation resulted in significant reduced proliferation compared to control and empty vector-transfected cells, starting at timepoint 48h (p=0.039) and becoming more evident at the 72h timepoint (p=0.022) (Fig. 3A). In MCF-7 and SKBR-3 cell lines, the PON2 knockdown determined a significant (p=0.036) reduced proliferation compared to control and empty vector-transfected cells at 72h, while no significant differences were observed for the earlier timepoints (Fig. 3B and C).

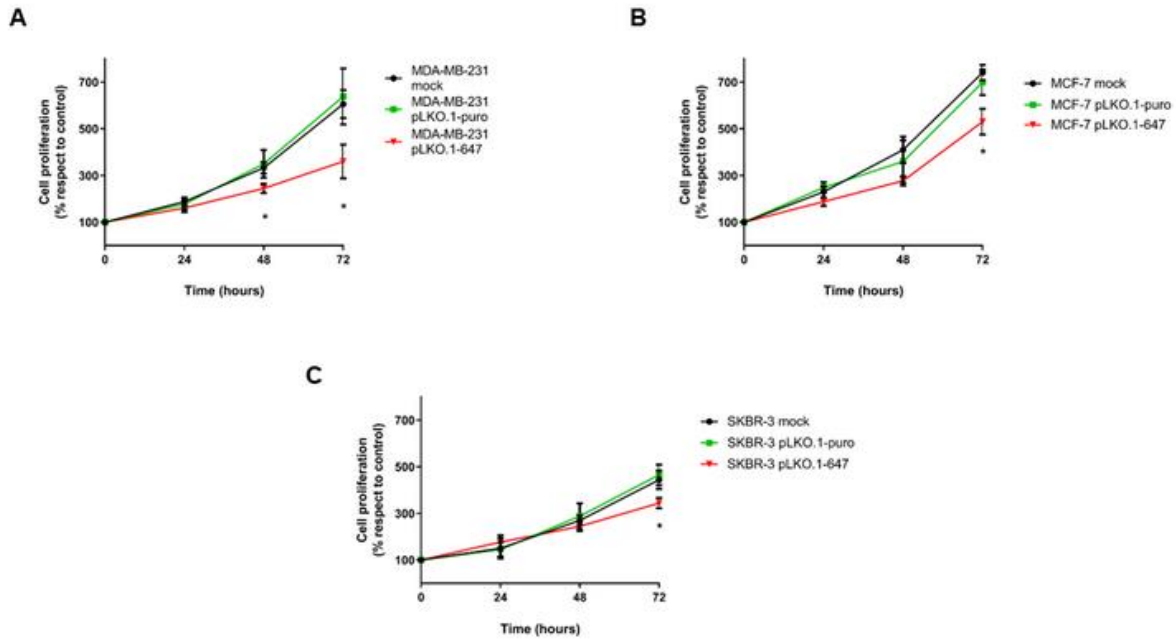

Figure 3. In vitro effect of PON2 silencing on cell proliferation. Proliferation was analyzed by through MTT assay in mock, empty vector (pLKO.1-puro) and PON2 downregulating clones (pLKO.1-647), at 0, 24, 48 and 72h, in MDA-MB-231 (A), MCF-7 (B) and SKBR-3 (C) cells (n=3). Values are expressed as mean ± standard deviation (*p<0.05).

3.4 Effect of PON2 downregulation on response to chemotherapeutic drugs

MTT assays were used to evaluate the effect of treatment with chemotherapeutic agents on the cell viability of the breast cancer cell lines downregulating PON2.

MDA-MB-231 cell line was treated with doxorubicin (0.1-10µM) and cell viability was assessed at different timepoints (0, 24, 48 and 72h). Interestingly, the decrease of cell viability upon treatment with doxorubicin 0.1µM was markedly enhanced in PON2-downregulating MDA-MB-231 cells compared with that measured in mock and empty vector transfected cells for all the timepoints tested (24h p=0.032; 48h p=0.032; 72h p=0.032) (Fig. 4A). Cell viability of PON2-silenced MDA-MB-231 cells showed an enhanced decrease upon treatment with doxorubicin 1µM for the timepoints 24 (p=0.042) and 48h (p=0.034) (Fig. 4B), whereas when doxorubicin was used at a concentration of

10µM a significant difference in cell viability between PON2-silenced cells and controls was observed only for the timepoint 24h (p=0.019) (Fig. 4C).

Analogously, the treatment with the chemotherapeutic agent 5-Fluorouracil had similar effects on PON2-downregulating MDA-MB-231 cells. In detail, the decrease of cell viability upon treatment with 5-Fluorouracil 0.5µg/ml was markedly enhanced in PON2-downregulating MDA-MB-231 cells compared with that measured in mock and empty vector transfected cells for all the timepoints tested (24h p=0.028; 48h p=0.037; 72h p=0.025) (Fig. 4D), while 5-Fluorouracil 1µg/ml resulted in differences in cell viability limitedly for the timepoint 72h (p=0.043) (Fig. 4E), and 5-Fluorouracil 5µg/ml induced a higher decrease in cell viability for the timepoints 24 (p=0.033) and 48h (p=0.036) (Fig. 4F). In addition, the treatment with the chemotherapeutic agent cisplatin 1µM was able to trigger a decrease of cell viability in PON2-downregulating MDA-MB-231 cells compared with that measured in mock and empty vector transfected cells for the timepoints 24 (p=0.026) and 48h (p=0.030) (Fig. 4G). Higher concentrations of cisplatin were also explored, but without inducing significant differences in cell viability reduction between samples, due to the excessive concentration (data not shown). Subsequently, we evaluated whether the combination of the three chemotherapeutic drugs, used at lower concentration, could display a synergic cytotoxic effect in PON2-silencing MDA-MB-231 cells. The treatment with a combination of 10nM doxorubicin, 100nM cisplatin and 50ng/ml 5-Fluorouracil was able to trigger a significant decrease of cell viability in PON2-silencing MDA-MB-231 cells compared to the controls, for the timepoints 48 (p=0.037) and 72h (p=0.039).

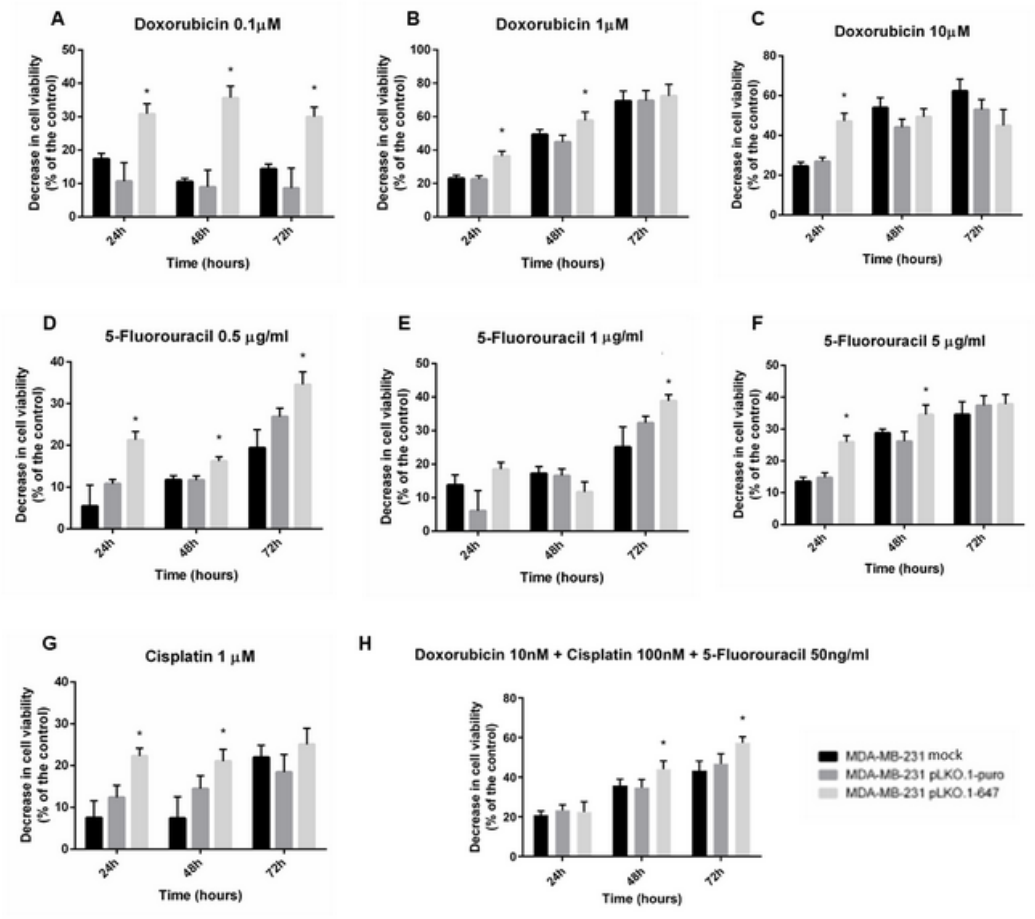

Figure 4. Effect of chemotherapeutic treatment on MDA-MB-231 TNBC cell line. MTT assay was used to evaluate the effect of doxorubicin (0.1, 1 and 10µM) (A-C), 5-Fluorouracil (0.5, 1 and 5µg/ml) (D-F), cisplatin (1µM), or a combination of the drugs (10nM doxorubicin, 100nM cisplatin and 50ng/ml 5-Fluorouracil) on cell proliferation of mock, empty vector (pLKO.1-puro) and PON2 downregulating (pLKO.1-647) cells. Measurements (n=3) were performed at different time points (0, 24, 48 and 72h). All values are expressed as mean ± standard deviation (*$p<0.05$).

4. Discussion

BC is the most common female cancer worldwide, with more than 2.1 million new diagnosis and 620,000 deaths every year. Among the BC subtypes, TNBC is the most aggressive displaying the poorest prognosis [2]. Indeed, despite its relatively low incidence, it accounts for the 5% of all-cancer-related deaths every year [19]. The current therapeutic approaches for the TNBC management include surgery, radiotherapy, and chemotherapy [20]. Due to its intrinsic molecular features, TNBC cannot benefit from therapeutical options which are available for other BC subtypes, including endocrine therapy and targeted therapy, for instance through the monoclonal antibody trastuzumab, and thus chemotherapy is still the best option for these patients. However, while surgical resection of the early primary tumor may be curative, the remaining available treatments are hardly curative and thus the malignancy rapidly progresses resulting in high morbidity and mortality. Nevertheless, until recently, the highly invasive radical mastectomy was the main surgical approach in the TNBC management, with important consequences for health (e.g. lymphedema and other postoperative complications) and psycho-emotional disorders [21].

Thus, it is essential identifying novel molecular biomarkers involved in cancer progression and resistance to chemotherapy, in order to develop efficacious therapeutic targeted strategies to improve the TNBC clinical outcome, and eventually enabling the use of a less aggressive demolitive surgery, which is particularly relevant due to the younger age of patients affected by TNBC, given the impact of the surgery on the quality of life of young women. The purpose of this experimental work was to evaluate the PON2 levels in BC subtypes, and to investigate the role played by the enzyme in BC cell metabolism, with a particular emphasis on the TNBC, focusing on the possibility of the enzyme to affect the BC cell sensitivity to the chemotherapeutic treatment. Indeed, there is growing evidence that PON2 might play a role in several solid malignancies, increasing the ability of the cancer cells to counteract the high levels of oxidative stress that characterize themselves, boosting the proliferation rate and eventually enhancing the chemoresistance.

The immunohistochemical analysis revealed that the PON2 expression levels were significantly higher in the infiltrating BC of the subtypes Luminal A, HER2+ and TNBC compared to the healthy tissue, whereas the expression levels of the *in situ* carcinomas of the same subtypes were not always significantly increased, although a clear tendency was present. In several neoplasms, PON2 was found to be upregulated and positively correlated to the aggressiveness of the neoplasm subtype. For instance, in basal cell carcinoma (BCC) PON2 expression was reported to be significantly upregulated only in infiltrating BCC, while in the less aggressive variant nodular BCC the expression levels were similar to that of controls [17]. In gastric cancer, high PON2 expression also had significantly positive association with diffuse type and tumor invasion [22]. Similarly, in another study carried out in urine samples obtained from bladder cancer patients, PON2 showed an inverse correlation with tumor stage, supporting its promising prognostic value [23]. Thus, our data are consistent with what is reported in literature, suggesting that PON2 enzyme could be involved in the first stages of the cancer progression, and thus providing this biomarker with a potential prognostic value for the BC Luminal A, HER2+ and TNBC subtypes.

Subsequently, upon generating PON2-silenced BC cell lines, we evaluated the impact of the PON2 downregulation on cell proliferation through the MTT assay. Data reported clearly demonstrated that shRNA-mediated PON2 gene silencing was associated with a significant decrease of the proliferation rate of all subtypes of BC cells tested, with a prominent effect on the TNBC cell line MDA-MB-231, thus highlighting its potential role in promoting BC tumorigenesis.

Several experimental works have reported that PON2 downregulation is able to reduce the cell proliferation in cellular cancer models including pancreatic cancer, bladder cancer and glioblastoma multiforme [16, 23-25]. In this light, our findings are in accordance with what is reported in literature, although the exact mechanism by which PON2 affect the cell proliferation has not been elucidated yet. Several studies hypothesized that the decrease of the proliferation might be the consequence of an enhanced spontaneous apoptotic rate of the cells, which affects the doubling time of the population

[26]. Notably, the ability of PON2 downregulation to reduce the proliferative capacity of TNBC cells could be particularly important, since TNBC is defined as a “fast growing tumor”, a feature that contributes to its high aggressive behavior [27].

It has been reported that resistance against chemotherapeutics or targeted therapies might be modulated also by PON2 expression levels in cancer cells [16]. Since TNBC cannot benefit from endocrine or targeted therapy, chemotherapy represents a first-line therapy for the management of this neoplasm. It is well known that platinum-based chemotherapy regimens have significant efficacy in neoadjuvant chemotherapy for TNBC, and have good safety and tolerability [28].

Despite the fact that TNBC patients display a particular sensitivity to chemotherapy compared with patients affected by other subtypes, numerous patients are prone to develop drug resistance over the time, which in turn is responsible of therapy ineffectiveness and leads to high mortality rates. Therefore, we decided to investigate whether the PON2 silencing might impact the response of TNBC cells to chemotherapeutics. For this study we utilized three chemotherapeutic agents (cisplatin, 5-Fluorouracil and doxorubicin) routinely used in the management of TNBC, which are notoriously potent inducers of massive ROS production, beside their capacity to induce single-strand and double-strand DNA damage [29]. The impact of chemotherapeutics on TNBC cells viability was significantly higher in PON2-downregulating cells compared to controls, especially for doxorubicin and cisplatin, thus suggesting that PON2 activity might exert a protective effect on cancer cells from chemotherapeutics-induced cytotoxicity. Moreover, the combination of the three drugs was able to exert important synergic cytotoxic effects on PON2-downregulating TNBC cells, allowing the use of reduced concentrations of the drugs, thus limiting their potential unpleasant side-effects. Our findings are consistent with data reported in literature. Indeed, several studies have shown that PON2 downregulation is able to sensitize cancer cells to chemotherapeutics. A recent study demonstrated that PON2 downregulation was able to increase the sensitivity of T24 bladder cancer cells to the cisplatin, while PON2 upregulation significantly counteracted the increase in cellular ROS production

in response to oxidative stress triggered by cisplatin [23]. Analogously, another study reported that PON2 gene silencing was able to increase the chemosensitivity of A375 melanoma cells to the cisplatin, a finding particularly important since melanoma is notoriously refractory to chemotherapeutics [30]. Finally, *in vitro* overexpression experiments performed utilizing the immortalized human vascular endothelial cell line EA.hy926 treated with doxorubicin revealed that the enzyme upregulation was associated with ATP reduction and inhibition of caspase-3 activation. In addition, the apoptosis induced by the treatment with staurosporine or actinomycin D was significantly decreased in EA.hy926 cells upregulating PON2 [31].

Interestingly, PON2 has been demonstrated to be involved in the resistance of some malignancies also towards non-chemotherapeutic drugs. For instance, in patients with acute lymphoblastic leukemia the levels of PON2 expression were found to be positively correlated with dexamethasone resistance, and the silencing of the enzyme significantly reduced dexamethasone resistance in acute lymphoblastic leukemia cells growth when xenografted in immunodeficient mice [32]. Furthermore, higher levels of PON2 have been reported to be associated to the development of resistance against the multikinase inhibitor imatinib in chronic myeloid leukemia [33]. The mechanisms by which PON2 contributes to the resistance of cancer cells toward chemotherapeutics is not fully elucidated. It has been proposed that the chemoresistance activity associated to the PON2 expression might be the result of an enhanced anti-apoptotic and ROS-detoxification effect. The anti-apoptotic roles of PON2 have been linked to the modulation of mitochondrial superoxide anion production and ER stress-induced apoptosis. Few studies reported that PON2 downregulation is associated with increased intracellular ROS levels, which can damage DNA, proteins and lipids [23, 30, 34]. Notably, all chemotherapeutics used in our study are able to induce massive ROS production within cell. Coupling a reduced antioxidant capacity, in our case through PON2 silencing, with the use of potent ROS-inducers, is considered one of the possible approaches for developing novel therapeutic strategies [35, 36]. The hypothesis that PON2 downregulation might induce chemosensitivity due to

enhanced ROS production is also supported by the evidence that in oral squamous cell carcinoma, the upregulation of PON2 is associated with an enhanced radioresistance [37, 38]. Indeed, the radiotherapy triggers massive cell death by inducing the activation of death signaling in tumor cells through, for a large part, the generation of ROS [39]. In the light of the above-mentioned observations, since adjuvant radiation therapy is a common component of TNBC treatment, the effect of PON2 downregulation on the response of TNBC cells to radiation would deserve to be explored.

Other mechanisms potentially involved in the anti-apoptotic role of PON2 in cancer cells involve the unfolded protein response (UPR), a signaling pathway activated during cell stress. Accordingly, it has been reported that oral squamous cell carcinoma cells overexpressing PON2 are protected from UPR-mediated cell death [37].

5. Conclusions

To the best of our knowledge, this is the first study that investigated the expression level of PON2 in molecular subtypes of breast cancer specimens, which provided evidence of an upregulation of the enzyme in association with infiltrating TNBC. We are aware that one limitation of the study is the reduced number of cases analyzed in this study. Nonetheless, the immunohistochemistry was utilized in this study as an initial approach to estimate the expression level of PON2 in the molecular subtypes of BC recognized to date, to subsequent investigate the impact of enzyme downregulation *in vitro* in cell models. In this regard, to validate a possible prognostic role of PON2 in TNBC, further analysis performed on a larger cohort of FFPE samples will be necessary, as well as a long-term follow-up of these TNBC patients to elucidate whether exists an association between enzyme expression levels and survival. Nonetheless, our *in vitro* approach clearly demonstrates that PON2 displays the potential to be a therapeutic target to improve the efficacy of the chemotherapy in TNBC patients, which, to date, still lack of targeted therapies, an occur that is partly responsible of the poor TNBC outcome.

Declarations

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Declarations of interest: The authors declare that they have no conflict of interest.

Ethics approval: This retrospective study was performed on formalin fixed and paraffin-embedded tissue specimens, previously collected for diagnostic purposes. According to the Ethics Committee of the Marche region ethical approval for retrospective studies is not required; it is sufficient to send a notification.

Informed consent: All participants gave their informed consent.

Author contributions

Roberto Campagna: conceptualization, investigation, methodology, formal analysis, writing—original draft preparation. **Valentina Pozzi**: investigation, formal analysis, visualization. **Sara Giorgini**: investigation, visualization, data curation. **Doriana Morichetti**: investigation, visualization, data curation. **Gaia Goteri**: supervision, resources. **Davide Sartini**: validation, Writing—review and editing. **Emma Nicol Serritelli**: software. **Monica Emanuelli**: supervision, resources, Writing—review and editing.

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Table 1. BC patients and clinico-pathologic findings

Cases	56
Median age	65 (min 38 – max 94)
Luminal A total cases	11
Median age	57 (mean 63; min 47 – max 84)
Histological grading	
G1	6
G2	4
G3	1
Luminal B total cases	12
Median age	61 (mean 63; min 44 – max 82)
Histological grading	
G1	2
G2	3
G3	7
Luminal B HER2+ total cases	11
Median age	66 (mean 64; min 38 – max 94)
Histological grading	
G1	0
G2	2
G3	9
HER2+ total cases	10
Median age	64 (mean 63; min 42 – max 82)
Histological grading	
G1	0
G2	0
G3	10
TNBC total cases	12
Median age	68 (mean 69; min 50 – max 90)
Histological grading	
G1	0
G2	3
G3	9