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THE LONG PENTRAXIN 3 CONTRIBUTES TO TRIPLE NEGATIVE BREAST CANCER STEMNESS AND TUMORIGENICITY

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RIASSUNTO DELLA TESI

Il tumore della mammella è uno dei tumori più diffusi a livello mondiale, e rappresenta la prima causa di morte per cancro nelle donne. Negli ultimi anni, le terapie a bersaglio molecolare contro il recettore degli estrogeni, il recettore del progesterone e il recettore 2 per il fattore di crescita epiteliale hanno generato dei risultati eccezionali nel trattamento del cancro della mammella. Ciononostante, una percentuale dei tumori della mammella non esprime questi recettori, questo è il caso dei tumori tripli negativi (Triple negative breast cancers, TNBCs) rendendo inutile queste terapie a bersaglio molecolare. Questi tumori sono particolarmente aggressivi e i pazienti tendono ad avere una prognosi infausta e una sopravvivenza globale ridotta a causa della carenza di terapie efficaci.

La Pentraxina lunga 3 (PTX3) è una molecola facente parte dell'immunità innata che svolge un ruolo controverso nel cancro. Numerosi studi riportano che questa proteina agisce come un fattore sia anti- che pro-tumorale, dipendentemente del contesto. PTX3 è overespressa nel cancro della mammella e in questo tipo tumore sembra favorire la crescita tumorale.

In questo studio, abbiamo analizzato il ruolo di PTX3 nella progressione del TNBC. Le osservazioni preliminari *in vitro* e *in silico* mostrano che PTX3 è overespressa nei tumori tripli negativi e che è positivamente correlata con il grado del tumore. Le analisi di espressione genica e i risultati in vitro e in vivo dimostrano che l'espressione di PTX3 correla positivamente con la proliferazione tumorale, il potenziale tumorigenico e la staminalità nelle cellule di TNBC. Oltre a ciò, abbiamo osservato che il silenziamento di PTX3 porta alla riduzione dell'attività di pathways importanti nella progressione tumorale, quali Akt/ NF-κB e JNK/c-Jun.

In conclusione, i nostri risultati dimostrano che PTX3 svolge un ruolo non ridondante nei tumori di mammella tripli negativi e che PTX3 o le vie di segnale modulate da PTX3 possono rappresentare nuovi bersagli molecolari nel trattamento del TNBC.

ABSTRACT

Breast cancer is one of the most frequent malignancies worldwide, being the first cause of cancer-related death in women around the globe. In the last decades targeted therapies against oestrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 have yielded excellent results in breast cancer treatment. However, triple negative breast cancer (TNBC) patients do not benefit from these therapies since they do not express these targetable markers. These tumours exhibit a marked aggressive behaviour, bad prognosis and reduced overall survival in patients, due to the lack of efficient therapeutical strategies. The long Pentraxin 3 (PTX3) is a pattern recognition molecule that plays a contradictory role in cancer. Several studies report that this protein exhibits both cancer promoting and suppressing roles depending on the tumour context. PTX3 appears upregulated in breast cancer and it has been reported that, in this context, it contributes to tumour development.

In the present study, we investigate the role of PTX3 in TNBC progression. *In vitro* and *in silico* preliminary observation showed that PTX3 is upregulated in triple negative tumours and it is positively correlated with tumour grade. Gene expression analysis and *in vitro* and *in vivo* experimental results demonstrate that PTX3 is associated with tumour cell proliferation, tumorigenic ability and stem cell-like phenotype in TNBC cells. Accordingly, we report that PTX3 silencing results in the downregulation of the Akt/NF-κB and JNK/c-Jun tumorigenic signalling pathways.

In conclusion, our results demonstrate that PTX3 plays a non-redundant role in TNBC and PTX3 itself, or PTX3-modulated pathways, might represent novel target(s) for TNBC.

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1. INTRODUCTION.

1.1. Breast Cancer.

1.1.1. Breast anatomy and histology.

The breast is a complex organ that goes through multiple modifications in a woman's life, since it is responsible for milk production during infant lactation [1]. It is located on the anterior thoracic wall, from the second to the sixth rib. Broadly, the breast is composed by an epithelial and a stromal fraction [2]. The epithelial compartment, responsible for milk production, accounts for 10-15% of total breast volume and it is formed by 15 to 20 lobes, each one composed by 20 to 40 lobules. In turn, each lobule contains 10 to 100 alveoli. The lobes drain into lactiferous ducts which converge on the nipple (**Fig**. 1A). The remaining volume of the breast is formed by stroma, adipose and connective tissue, plus blood supply network and lymphatic drainage [3].

The epithelial component of the breast consists in a luminal layer of epithelial cells lined by an outer stratum of myoepithelial contractile cells. These cells are in contact with the interlobular stroma through the basal membrane (**Fig.** 1B). A population of mammary stem cells (MaSCs), also called suprabasal cells, lays in the basal layer of milk ducts [4]. This self-renewing population is responsible for the maintenance of both lineages of epithelial breast cells, *i.e.* luminal and myoepithelial [5]. The luminal progenitors can further subdivide in alveolar or ductal luminal cells. In fact, Shackleton and colleagues have described that a single sorted murine MaSC can form a fully functional mammary gland, however some reports indicate that this cannot be accomplished under physiological conditions [6]. Efforts to characterise the progenitor cells in murine breast epithelia revealed a set of specific stemness markers, $CD49f^{high}/CD29+/CD24^{low}$, that seem to identify a bipotent MaSC population, although other markers have also been reported [7, 8]. In turn, markers for the differentiated breast epithelial cells have been described: Cytokeratin 19 (CK19) for ductal luminal cells, Cytokeratin 18 (CK18) for alveolar luminal cells and Cytokeratin 14 (CK14) and α – smooth actin muscle (α-SMA) for myoepithelial cells [9].

Although minimal remodelling of the breast occurs with each menstrual cycle, it is during pregnancy and lactation that the breast is deeply altered. Due to exposure to oestrogen, progesterone and prolactin, the epithelial fraction of the breast proliferates, creating new ductal branches and inducing secretory cell differentiation [10]. In the same way, placental hormones, such as lactogen, cause breast growth through MaSC proliferation [11]. When infant lactation

ceases the breast undergoes an involution process. Another involution process takes place during menopause due to ovarian decay, resulting in reduced lobe and duct numbers and epithelial fraction shrinkage. Thus, after menopause the breast consists mainly in stroma and fat that will further reduce with time, resulting in volume and contour loss [1].

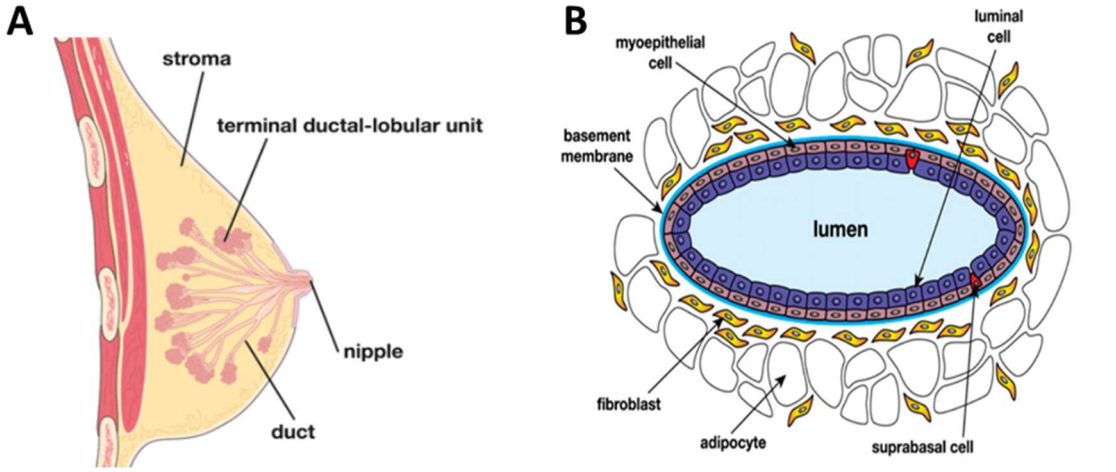

Figure 1. A) Anatomy of the breast and **B)** structure of the milk duct. Luminal epithelia sit on the basal myoepithelial layer, separated from the stroma by the basal membrane. Suprabasal cells found embedded in the myoepithelial layer [5].

Literature data suggest that normal interlobular stroma plays a key role in breast development. The paracrine interactions between epithelium derived amphiregulin and stromal epidermal growth factor receptor (EGFR) are necessary for normal breast development as reported by Luetteke and colleagues [12]. Similarly, stromal expression of the growth hormone is necessary to attain normal ductal elongation [13]. On the other hand, intralobular stroma is composed by mesenchymal cells that show high responsiveness to hormonal cues and it has been connected to development in normal breast [14].

1.1.2. Breast cancer statistics and risk factors.

According to the most recent global cancer report, GLOBOCAN 2018, breast cancer is the first malignancy by incidence and mortality in women worldwide, with 2.088 million women diagnosed and 626,679 cancer related deaths in 2018, respectively [15, 16]. Therefore, breast cancer has become a heavy burden in public health systems around the globe. However, due to improvement in treatment and early detection through population-wide screening campaigns, breast cancer mortality rates have remained stable or have decreased since the late 90s in the US and Europe [17]. In the EU for example, breast cancer patient mortality rate has suffered a steep decrease since the middle 90s, although due to population ageing the absolute number of deaths has not changed significantly in the last decade (92,000 in 2014 and 92,800 in 2019) [18].

Breast cancer is a challenging disease where each case shows specific genetic background and molecular alterations. However, thanks to epidemiologic and cohort studies, several risk factors have been well defined, as reviewed by Feng ad colleagues [19]. Breast cancer occurs more frequently in older women; thus, age is described as a risk factor [20]. Mutations in key genes such as BRCA1 and 2, p53 and PTEN greatly augment breast cancer risk [21]. For example, in women bearing a BRCA1 mutation the risk of developing breast cancer is increased by 55-65%, although only about 5 to 10% of all breast cancers are due to inherited mutations [19, 21]. Family history of benign breast proliferative lesions and tumours also increases the risk of breast cancer appearance [22, 23]. Reproductive history, as well as hormonal factors have a significant impact as well. Early age at menarche and late age at menopause increase the risk of developing breast cancer while parity and multiparity are protective factors [24]. Few lifestyle factors have been linked to an increase in breast cancer development. Obesity for example has been reported to increase the risk in postmenstrual, but not in premenstrual women [25]. Obese breast cancer patients show increased risk of tumour relapse when compared to non-obese patients [26]. Alcohol consumption has also been listed as a risk factor as reviewed by Shield and colleagues [27]. Moderate ethanol consumption can alter serum levels of oestrogen, which in turn increases oestrogen receptor (ER) dependent expression in ER positive tumours, resulting in hyperproliferation [28, 29]. Indeed, ethanol metabolism can result in the weakening of the antioxidant system *via* acetaldehyde, indirectly modifying histone and DNA methylation [30]. Breastfeeding has also been described as a protective factor, although the topic remains controversial [31, 32]. Oral contraceptives augment breast cancer risk, but the risk diminishes with time after usage [33]. In the same way, there is an increased risk in women using menopausal hormone therapy (MHT), particularly in those using oestrogen-progestogen treatment rather than in those using oestrogen alone, and the risk of developing breast cancer increases with duration of treatment [34].

1.1.3. Breast cancer progression.

Most breast cancers can be histologically classified as *in situ* or invasive (infiltrating) carcinomas. *In situ* carcinomas can be further classified in ductal and lobular carcinoma *in situ* (DCIS and LCIS, respectively), the first being far more frequent. These tumours progress and proliferate without breaking free from the basal membrane or disrupting the architectural integrity of the duct or lobe. DCIS can be subcategorised in comedo, cribriform, micropapillary, papillary and solid, depending on architectural features of the tumour, whereas LCIS shows little histological variation [35, 36].

In situ breast carcinomas are considered as preinvasive tumours that can infiltrate the neighbouring tissue if they are not monitored and treated, thus resulting in invasive breast carcinomas. The development of these tumours results in the disaggregation of the myoepithelial layer and basal membrane. Tumour microenvironment cells such as tumour associated macrophages (TAMs) and cancer associated fibroblasts (CAFs) contribute to this process. In this way, tumours invade breast stroma forming a tumour associated vasculature, that can eventually allow metastasis to distant organs, death cause of 90% of cancer patients [37, 38]. Invasive breast carcinomas can be classified in ductal (IDC) and lobular invasive carcinomas (ILC) depending on the site of the lesion. IDC is the most frequent form of breast cancer, detected in 80% of all cases. This cancer can be further subdivided in tubular, medullary, mucinous, ductal lobular and infiltrating ductal [19, 39]. In addition to these subdivisions, IDC is often classified based on prognostic markers. The presence of ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu) is often reported in breast tumours and their expression is exploited for therapeutic treatment, such as tamoxifen for ER+/PR+ or trastuzumab for HER2/neu expressing tumours [36].

Breast cancer progression is driven by a handful of signalling pathways. These pathways are involved in normal breast development and homeostasis, but they are often highjacked during tumour progression. Due to genetic and epigenetic modifications, the expression of members belonging to these pathways is altered, resulting in overactivation and/or loss of inhibition of several cellular processes such as proliferation, migration and survival [19]. Mutations of signalling pathways that result in a selective growth advantage for the tumour are termed “driver mutations”. These mutations might either activate proto-oncogenes or inactivate oncosupressors [40]. In breast cancer, several altered signalling pathways have been described.

Oestrogen receptor α (ERα) plays a key role in the pathogenesis of nearly 75% of breast tumours [41]. ERα is found in the cytoplasm and upon ligand binding, it is translocated to the nucleus inducing the expression of target genes. One of the best described roles for ERα is the induction of cyclin D1 expression [42]. In turn, this protein activates cyclin dependent kinase 4 and 6, responsible for G1 to S phase transition. In the same way, cyclin D1 can bind ERα in the absence of its ligand, inducing the transcription of its target genes, thus forming a positive feedback loop [43]. Additionally, cyclin D1 is overexpressed in 60% of breast cancers resulting in an amplification of the proliferative effect of ERα [44].

Proliferation related pathways are very frequently mutated in breast cancer. HER2 is a tyrosine kinase receptor that upon stimulation leads to the activation of several signalling pathways, such as PI3K/Akt/mTOR, JAK/Stat and

Ras/Raf/MAPK. These pathways control several key cellular processes in tumorigenesis [19]. HER2 associated signalling is hyperactivated in 15 to 20% of breast cancers due to the amplification and overexpression of the HER2 gene [26]. In turn, these pathways can also be deregulated independently from HER2. Hyperactivation of the PI3K/Akt/mTOR pathway is very common in breast cancer, due to the relatively high mutation frequency in PI3K, resulting in a constitutively active form of the protein. Additionally, inactivating mutations in the negative regulators of this pathways, such as PTEN, results in the hyperactivation of the PI3K/Akt pathway [45].

Wnt/β-catenin is another signalling pathway that is often hyperactivated in breast cancer. This pathway is activated upon the stimulation by Wnt ligands, resulting in the inhibition of GSK-3β, a protein responsible for β-catenin degradation. As a result, β-catenin accumulates and translocates to the nucleus as a cofactor for the transcription machinery, inducing the expression of proliferative genes such as CCND1 (cyclin D1) and MYC [46]. Wnt signalling is constitutively active in breast cancer, due partly to the loss of Wnt inhibitors [47]. This signalling pathway has been involved in resistance to treatment and apoptosis [48].

Other signalling pathways are also implicated in breast cancer progression. The Notch pathway for example, is involved in stem cell renewal and hypoxia survival, while Sonic Hedgehog signalling has been described as a marker of post operatory relapse and breast cancer invasiveness [49, 50]. These deregulated signalling pathways are essential for tumour development and growth. Therefore, therapeutic targeting of these and other key members might significantly improve patient survival.

1.1.4. Classification of breast cancer.

Breast cancer is a particularly heterogeneous disease and several classification systems have been put in place to determine different subsets of breast cancer with common traits and behaviour. In this way, specific treatments can be designed for each subtype, increasing the possibility of success.

One of the most employed classification systems is the histological grade. It is a prognostic factor defined by morphological features of the tumour such as tubule formation, nuclear pleomorphism and mitotic count. The scale goes from 1 (differentiated cells, good prognosis) to 3 (least differentiated cells poor prognosis). High grade tumours tend to show higher recurrence rate, while low grade ones show good clinical outcome [51].

Apart from the very frequent IDC and ILC several uncommon non-epithelial tumours have been described as well, representing less than 2.5% of all cases

[52]. Sarcomas develop from the stromal fraction of the breast and can be subclassified in fibrosarcoma and leiomyosarcoma of the breast, both with good prognosis [53]. On the other hand, lymphomas in breast are very unusual, mainly being fast-growing B cell lymphomas. They can be treated with chemotherapy, radiotherapy and surgery and they show an overall 5-year survival of 53% [54]. Finally, the phyllodes tumour is equivalent to fibroadenomas, with mesenchymal and epithelial components. Most of these tumours are benign but there are some malignant cases, even if the mortality rate is very low [55].

Epithelial rare breast tumours show more subdivisions than non-epithelial ones. Solid papillary tumours for example, make up around 1.7% of all breast tumours and are formed by round nodules formed by ductal cells, normally of low grade. If not invasive, these tumours present excellent prognosis and can be excised surgically [56]. Apocrine carcinomas range from 0.3% to 4% of all breast cancer and are identified as invasive ductal carcinomas, bearing the androgen receptor (AR) which makes them suitable for anti-AR therapy. Several other rare epithelial tumours have been described, the majority with favourable prognosis [52].

The lack of specific molecular markers in previous classification systems (DCIS *vs* IDC, histological grade, etc.) limits the ability to predict adequate identification and treatment for the different tumour types. In the year 2000, Perou and colleagues analysed gene expression patterns in 65 tumours obtaining five different molecular portraits that grouped all specimens of breast cancers: Luminal A, Luminal B, HER2-enriched, basal-like and normal-like [57]. Later, another molecular subtype was defined, the claudin-low, thus completing the current molecular classification of breast cancer (**Fig.** 2). Additionally, a set of 50 genes has been described, PAM50, for the efficient classification of breast cancers in intrinsic subtypes [58]. All subtypes have been skilfully reviewed by Eroles and co-workers [26, 59].

Luminal A. This subtype represents 40 to 50% of all breast tumours. The tumour cells are originated from luminal epithelial cells. These tumours are characterised by positive expression of ER and/or PR, CK8/18, and negative expression of HER2, as well as high expression of ER controlled genes. Luminal A tumours show low proliferative rates and as a result, low Ki67 staining by immunohistochemistry (IHC), and low tumour grade [60]. In the same way, all LCIS and most ILC are classified as Luminal A. These tumours present good prognosis and show relatively low relapse rates in comparison with other subtypes. Due to the expression of ER, Luminal A cancers benefit both from aromatase inhibitors (AI; they hinder oestrogen synthesis), hormonal therapy and selective oestrogen modulators such as tamoxifen, acting through ER blockade. Due to their low proliferation rate, these tumours show low sensitivity to chemotherapy [61].

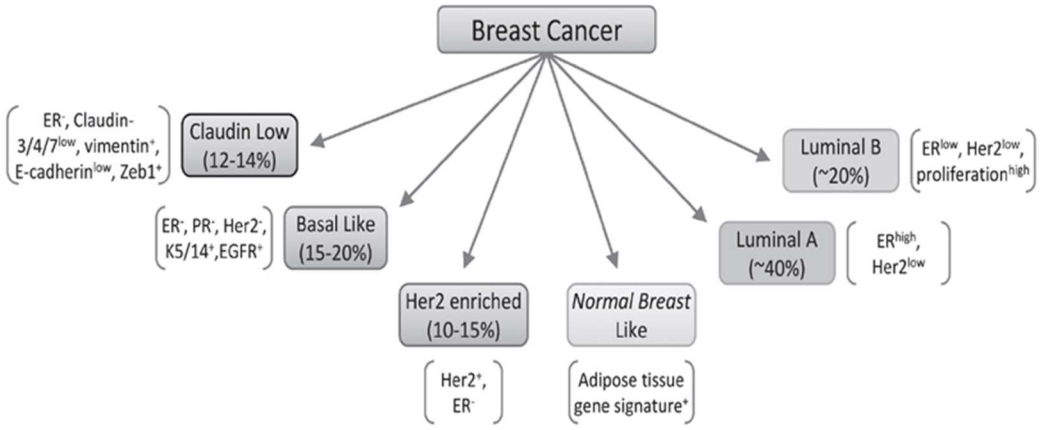

Figure 2. Molecular subtypes of breast cancer, markers and incidence [36].

Luminal B. They represent 10-20% of all breast cancers. Like Luminal A, these tumours are histologically derived from luminal epithelial cells, although Luminal B cancers show a more aggressive behaviour, worse prognosis, higher tumour grade and proliferation rate. These tumours have been labelled as ER+/HER2-, but a subset of ER+/HER2+ exists, although they show lower levels of ER and PR compared to Luminal A [62]. As a result of increased proliferation, Ki67 and cyclin B1 proteins are overexpressed, as well as growth controlling pathways such as PI3K-Akt-mTOR. [63]. Luminal B cancers show worse prognosis than Luminal A when treated with AI and tamoxifen, nonetheless, these tumours show a better response when treated with neoadjuvant chemotherapy. Several clinical trials seek to determine whether the inhibition of PI3K-Akt-mTOR pathway can be useful in the treatment of Luminal B cancers [26].

HER2 enriched. Around 15 to 20% of breast cancer cases belong to this subtype. The major characteristic of these tumours is the overexpression of HER2 and other related genes, due to an amplification in the q12 region of the chromosome 17. These tumours are of luminal origin although the expression of luminal markers is often lower than in the luminal subtypes. HER2-enriched cancers are highly proliferative and of high histological grade. In the same way, at least 40% of cases present mutations in p53. The ER-/HER2+ IHC profile does not always fit in this subtype, as HER2 overexpression detected by microarray is not always detected by IHC [64, 65]. These tumours are highly sensitive to neoadjuvant chemotherapy and they have successfully been treated with anti-HER2 targeted therapy using the HER2 blocking antibody Trastuzumab [66].

Normal-like. It is a relatively uncommon type of breast cancer, accounting for 5 to 10% of all cases. They present both epithelial and mesenchymal characteristics, as well as adipose tissue related gene profiles. Normal-like breast cancers are similar to Luminal A tumours although with worse prognosis. In general, they lack ER, PR and HER2 expression complicating the design of targeted therapies, but

some ER/PR+ HER2– cases have been reported. Some studies claim that this subtype is a technical artefact derived from normal breast tissue cross contamination [26, 57].

Claudin-low. It is the most recently described subtype, representing 12-14% of all breast cancers, owing its name to the low expression of tight-junction related proteins, such as, occludin, E-cadherin and claudins -3, -4, and -7 [59]. These tumours express low levels of luminal genes and increased expression of immune system related genes, due to the elevated number of immune infiltrates. Although they are normally negative for ER, PR and HER2, some cases express one or more hormone receptors. Histologically, claudin-low tumours are high grade invasive ductal carcinomas, with poor long-term survival. These cancers do not show appropriate response to chemotherapy [67].

Basal-like. Accounting for 15 to 20% of all breast tumours, basal-like is one of the most aggressive subtypes. These tumours are originated from myoepithelial basal cells; therefore, they express myoepithelial markers such as heavy weight cytokeratins CK5 and CK17, P-cadherin and CD44. Most basal-like tumours are negative for ER, PR and HER2, and they are often classified as triple negative (TN) in the clinics. However, about 30% of basal-like cancers are not triple negative so the term basal-like and triple negative are not interchangeable. A set of five markers (Basal Core Group) has been selected to identify basal-like cancers: ER, PR, HER2, EGFR and CK5/6 [68-70]. These tumours show poorer prognosis than luminal cancers with an increased relapse rate even though basal-like cancers respond to chemotherapy. Due to the lack of hormone receptors, no targeted therapy is available for these tumours. However, a significant fraction of basal-like cancers shows mutations in BRCA1, which is critical for DNA repair by homologous recombination, the most accurate DNA repair mechanism. Lack of proper DNA repair mechanisms leads to genetic instability when errors accumulate resulting in cell death. This feature has been used in the development of DNA damage agents such as platinum salts. Finally, the application of poly-ADP ribosepolymerase-1 (PARP-1; responsible for single strand break repair) inhibitors is showing encouraging results in BRCA1 mutated basal-like tumours [71].

1.2. Triple Negative Breast Cancer.

1.2.1 Triple negative breast cancer features and classification.

The key feature of triple negative breast cancer (TNBC) is the lack of ER, PR and HER2 expression. This breast cancer category is extremely heterogeneous as different patients show very diverse prognosis, response to treatment and overall survival. However, it is accepted that TNBCs tend to be more aggressive and present higher relapse rates than other breast tumours. They account for 10-20% of all invasive breast cancers and usually present high histological grade. TNBC is more common in premenstrual women of African and Hispanic descent, in comparison with other ethnic groups [72]. In contrast to other cancer subtypes, parity and multiparity seem to have an adverse effect and are considered as risk factor in TNBC development [73]. TN breast tumours show an increased mitotic index, necrosis, pushing borders, and patients bearing these tumours show shorter relapse free survival rates. Metastases in TNBC are more aggressive and visceral sites such as liver, brain and lungs are preferred, whereas bone metastases are less frequent [74]. The main reason for low survival in TNBC is the lack of therapeutic alternatives such as tamoxifen and trastuzumab, directed against ER and HER2, respectively. Thus, the pursuit for new pharmacological objectives has been relentless in the last decade [75].

Although the definition of the molecular subtypes by Perou and colleagues has been proven very useful, the TN signature is often preferred in clinics due to its technical simplicity, since it only requires IHC, a tool available to most pathologists. In contrast, molecular subtype definition demands microarray technology which is less affordable and thus prohibitive in many cases. The great heterogeneity and variability found in TNBC may thus respond to the existence of molecularly diverse tumours. Hormone receptor expression was one of the most relevant features used in the description of Perou's molecular subtypes and according to this classification three out of six subtypes lack ER, PR and HER2 expression to some extent [57, 59]. Thus, these tumour subtypes, namely claudin-low, normal-like and basal-like, are encompassed in the TNBC category [75].

Basal-like breast cancers are the most frequent molecular subtype in TNBC, making up about 70% of all TN tumours. This has led to the use of TNBC and basal-like breast cancer as synonymous, but at least 30% of basal-like cancers express ER, PR or HER2. Conversely, not all TNBCs are of basal type and tumours with specific features not found in basal-like tumours have been described [76]. In fact, as reported by Badve and colleagues, up to 29% of TN tumours do not show basal-like protein markers (such as CK5/6 or EGFR) [77]. Although the majority of TNBCs are invasive ductal tumours other histological types have been described [78].

Considering the heterogeneity of this cancer, Lehman and colleagues proposed a molecular subclassification in an attempt to categorise TN tumours on the basis of specifically enriched gene signatures. They analysed gene expression profiles from several breast cancer datasets, defining 6 subgroups with unique gene expression ontologies [79].

Two basal-like subtypes were classified, basal-like 1 and basal-like 2 (BL1 and BL2, respectively). The former subtype is enriched in cell cycle and cell division related genes, such as DNA replication genes, RNA polymerases and G_1 to S phase regulating genes. Genes linked to proliferation were also upregulated, such as AURKA, MYC and NRAS. This fact agrees with the high Ki67 nuclear staining by IHC in BL1 tumours. Interestingly, DNA damage response genes such as ATG and BRCA are overexpressed in BL1 tumours. Accordingly, several studies have shown that a significant part of germline $BRCA1^{mut}$ breast cancers show features that are shared with TN tumours, and in turn, a considerable portion of sporadic basal-like and TNBCs show BRCA1 mutations [80]. Consequently, the question as to whether BRCA1 malfunction induces tumorigenesis in basal-like cancers has arisen although no sufficient proof has been gathered yet. The BL2 subtype is in turn enriched in growth factor signalling pathways, such as EGF, Wnt/β-catenin, and IGF1R pathways. In addition, genes involved in glycolysis and gluconeogenesis are also enriched in accordance with the implication of IGF1R signalling. BL2 tumours also show high levels of growth factor receptors like EGFR and MET [79].

The Immunomodulatory (IM) TNBC subtype is enriched in immune cell signalling pathways such as TH1/TH2, Natural killer and B cell. As expected, cytokine signalling pathways such as the IL-7 pathway, are also enriched since they are key in the tumour-immune system interactions. In fact, IL-7 pathway has been reported to induce cell growth and proliferation in TNBC cell lines [81]. The IM tumour signature shows a significant overlap with medullary breast tumours, a rare epithelial tumour. Although the authors did originally specify that the IM signature was unique to tumour cells, in a subsequent report, they concluded that IM gene ontologies were significantly derived from immune infiltrates in the tumours. In fact, they showed that IM subtype specificity was dependent on lymphocytic infiltration. Thus, the authors discarded the IM category due to low cellularity [79, 82].

The Mesenchymal (M) subtype is heavily enriched in motility regulating pathways (actin rearrangement and Rho family genes), ECM receptors and differentiation pathways, such as Wnt and TGF-β. This gene ontology enrichment agrees with the mesenchymal phenotype present in invasive breast tumours. In fact, the TGF-β pathway has been reported to induce epithelial to mesenchymal transition

(EMT) and to clear the path for metastasis. In agreement with this, EMT related genes, such as TWIST1 and SNAI2 are also enriched in M tumours. The M subtype has been connected to the claudin-low intrinsic cancer subtype as well as to metaplastic breast cancer, a rare tumour with mesenchymal features. Another subtype connected to the mesenchymal phenotype is the so-called mesenchymal stem-like subtype (MSL). These tumours resemble the M subtype, but they present decreased expression of proliferation genes while stemness-related genes such as ALDH and BCL2 are increased. Genes involve in angiogenesis are also overexpressed in MSL. Similar to IM tumours, the MSL subtype was later disproved due to contamination of tumour samples with neighbouring stroma [79, 82].

Finally, a luminal-like TNBC subtype was described. Luminal androgen receptor (LAR) tumours are relatively isolated among TNBCs because they are the only tumours expressing luminal markers. This subtype is enriched in hormone-associated pathways, such as, steroid and androgen metabolism. Both AR mRNA and protein levels are increased tenfold when comparing with other TNBC subtypes. AR downstream proteins are also overexpressed, suggesting a strong activation of the pathway. LAR tumours have been correlated with the molecular apocrine type, a rare AR-driven breast cancer [79].

At present TNBC remains the most lethal type of breast cancer and virtually all patients bearing these tumours will die as a consequence of the disease.TN tumours are highly metastatic, and almost half of the patients will develop distant secondary tumours with the median survival time after metastasis detection being around 13 months. The relapse rate after surgical intervention is as high as 26% [74]. In fact, average time for relapse in TNBC is very low compared to non-TNBC, 19-40 *vs.* 35-67 months, respectively. Due to the aggressiveness of TN tumours and the lack of available pharmacological targets, the research for new anti-tumoral therapies is of outmost importance [83].

1.2.2. Triple negative breast cancer treatment.

Chemotherapy treatment in either adjuvant or neoadjuvant settings has proved to be more effective in TN than in hormone receptor positive tumours. TNBCs treated with taxanes and anthracyclines have shown high pathological remission rates [84]. Even though these tumours are responsive to chemotherapy, the mortality rate within the first 5 years after diagnosis is 40%. Optimisation of both classical chemotherapy and new targeted therapies is of outmost importance to increase overall survival in TNBC patients. In the same way, the unrevealing of the molecular drivers in these tumours will provide new possible targets, particularly for patients that do not benefit from the already existing therapies. At the present a limited set of chemotherapy and targeted therapies are employed in the

treatment of TNBC, and a preferred chemotherapy regime has not yet been approved [83].

Taxanes act through the inhibition of microtubule depolymerisation preventing mitosis. Additionally, taxol induces apoptosis on tumour cells. Docetaxel shares the mechanism of action with taxol but has twice the anti-microtubule effect and has a broader antitumoral spectrum [85]. Albumin-bound paclitaxel can shorten treatment period and shows improved delivery on endothelial cells. Both BL1 and BL2 tumours show increased sensitivity to anti-mitotic drugs. When treated with a taxane based therapy, the basal-like subtypes showed fourfold remission rate compared to LAR tumours [83, 86].

Anthracyclines are the most broadly employed antitumoral drugs. They are intercalating agents and act through nuclear topoisomerase II poisoning, resulting in cell death [87]. According to some reports, the use of an epirubicin based regime reduced risk of relapse and breast cancer related death [88]. In TNBC treatment, anthracycline-taxane regimes are frequent, but not all subtypes benefit equally. BL1 tumours show higher rate of pathological complete response, while BL2 tumours are not sensitive to the anthracycline-taxane combination [83].

Cyclophosphamide has alkylating properties, inducing cell death in tumours. Cyclophosphamide is frequently employed in HER2 negative cancers. As reported by Nakatsukada and colleagues, the pathological complete response rate obtained in TNBC by cyclophosphamide treatment was threefold that of Luminal breast cancers. In another study, 130 TNBC patients were treated with neoadjuvant taxane/cyclophosphamide chemotherapy. The best responding were BL1 tumours, while BL2 and LAR showed the lowest response rates [89].

5-Fluoroacil (5-Fu) can be converted by orate phosphoribosyl-transferase into fluorouridine monophosphate and fluorodeoxyuridine, metabolites with cytotoxic activity. Due to their similarity with uracil, they are incorporated in RNA biosynthesis disrupting the process and resulting in RNA and DNA damage [90]. Capecitabine for example, is converted to 5-Fu in the body and subsequently acts on tumour cells. It is employed in breast cancer patients that have developed resistance to anthracycline and taxanes. A phase II clinical trial on the use of capecitabine and cisplatin on metastatic TNBC patients has yielded promising results [83].

Platinum salts, such as cisplatin and carboplatin, are DNA damaging agents. In a phase II clinical trial, a combined cisplatin and gemcitabine therapy was administered to patients bearing TNBC metastasis. The combination treatment had a more profitable effect particularly in basal-like tumours. The addition of

carboplatin to a conventional taxol and anthracycline therapy resulted in increased pathological complete response in TNBC patients [91]. As DNA damaging agents, platinum salts can be employed in tumours with malfunctioning DNA-repairing systems, such as those harbouring BRCA1/2 mutations. These mutations confer increased sensitivity for DNA damaging agents and thus can more easily go into DNA damage-induced cell death [92].

Despite the good responsiveness to chemotherapy, TNBC is still an incurable disease. Therefore, several studies have investigated the expression of additional targetable markers in these tumours. Around 70% of TNBCs show high expression of EGFR as part of the proliferative EGFR pathway [93]. Cetuximab is a monoclonal antibody that blocks EGFR activation. In a phase II clinical trial 120 TNBC patients were administered with either cetuximab alone or cetuximab/carboplatin. Unfortunately, response rates were very low in cetuximab alone treated patients and low (17%) in the combined treatment group, thus suggesting that other pathways can overcome the blockade [94].

Poly ADP Ribose Polymerase (PARP) inhibitors have been profusely studied during the last decade. PARP-1 is a DNA repair enzyme involved in the maintenance of genomic stability and in reparation of single strand breaks. This enzyme is of vital importance, particularly when other DNA repair mechanisms, such as BRCA1/2, are not functional. Therefore, it was hypothesised than in $BRCA^{mut}$ TN tumours (up to 19.5% of all TN tumours) PARP inhibition would result in high rates of pathological complete response *via* DNA damage-induced cell death. Unfortunately, recent clinical studies showed that treatment with the PARP inhibitor olaparib did not result in significant differences between $BRCA^{mut}$ and $BRCA^{WT}$ tumours [95]. Some studies have theorised that inhibition of PI3K would be beneficial. PI3K stabilises double strand breaks interacting with the DNA repair machinery proteins, among which BRCA. Thus, PI3K inhibition would result in a $BRCA^{mut}$-like phenotype even in BRCA proficient tumours, which could improve PARP inhibitor efficacy [96].

AR is present in 10 to 15% of TNBCs (mainly the LAR subtype), consequently anti-AR therapy has been suggested as a potential treatment option for these tumours. Enzalutamide blocks AR interaction thus inhibiting internalization of the receptor and further signalling. Although it is mainly administered in prostate cancer, its use in breast cancer has been tested in a phase II clinical trial. The use of enzalutamide resulted in 25% clinical benefit rate [97]. Interestingly, LAR tumours frequently express an activating mutation in PIK3CA together with AR and they show high sensitivity for PI3K inhibitors. Preclinical data shows that combination of anti-AR and anti-PIK3CA drugs are beneficial in LAR treatment, creating a synergistic cytotoxic effect [83].

1.3. Long Pentraxin 3.

1.3.1 Pentraxin superfamily.

Long pentraxin 3 (PTX3) is a fluid phase pattern recognition molecule (PRM) involved in the host defence against pathogens. It belongs to the pentraxin superfamily, an evolutionary conserved group of proteins with essential roles in the recognition of self and nonself antigens. This superfamily is a phylogenetically conserved group encompassed in the humoral arm of the innate immune system. These PRMs show antibody-like features acting as ancestors of antibodies. They have been found from arachnids to mammals, which reflects their evolutionary importance. The main shared feature in the pentraxin superfamily is a 200 amino acid long pentraxin domain in the C terminus. Within this domain lays the specific 8 amino acid long pentraxin signature, consisting in HxCxS/TWxS where "x" is any amino acid [98].

The pentraxin superfamily is divided in short and long pentraxins (**Fig.** 3). C reactive protein (CRP) was the first described member of the family and the prototypical member of short pentraxins. It was discovered in the 1930s due to its hundredfold concentration increase in plasma from patients with inflammatory conditions. Therefore, it was labelled as an acute phase protein in humans [99]. Serum amyloid P component (SAP) is the other member of short pentraxins. Similar to CRP in humans, SAP plasma levels are also significantly increased in inflammatory contexts in mice. Both short pentraxins are formed by five tightly arranged protomers, each protomer being around 25KDa [100]. They are expressed in the liver after stimulation by inflammatory cytokines such as IL-6 and IL-1β. Short pentraxins bind ligands in a calcium dependent manner. Microbial moieties such as phosphorylcholine and lipopolysaccharide (LPS) are bound by CRP and SAP, respectively. In addition, both short pentraxins interact with the complement system, enhancing opsonisation of pathogens and subsequent clearance by immune cells [101].

The long pentraxins were first described in the 1990s. PTX3 was the first discovered member of this subfamily, and it was identified as a secreted protein in fibroblasts and endothelial cells stimulated by IL-1 and TNF, respectively. PTX3 is composed by a 203 amino acid long pentraxin domain in the C-terminal region and an unrelated N-terminal domain with no similarities to any known protein. The C-terminal region shares 57% of its amino acidic sequence with short pentraxins. After the discovery of PTX3, several novel proteins were classified as long pentraxins due to the structural similarities with PTX3, *i.e.* the pentraxin domain in the C-terminal region and an unrelated N-terminal domain [98, 102]. Neuronal pentraxins are an important subset of the long pentraxins. Neuronal pentraxin 1 (NP1/NPTX1) and neuronal pentraxin 2 (NP2/NPTX2/NARP) are

involved in postnatal brain development by refining synaptic sites. In addition, NPTX2 plays a protective role against Alzheimer's disease. In the same way, neuronal pentraxin receptor (NPR), the only known pentraxin with a transmembrane domain, has also been reported to have a protective role in Alzheimer's [103]. Guinea pig apexin, another member of this family, is located in the acrosome of mature sperm. It has been linked to fertility, but its role is undefined [104]. A new long pentraxin has been described recently, pentraxin 4 (PTX4), although little is known about its function [105].

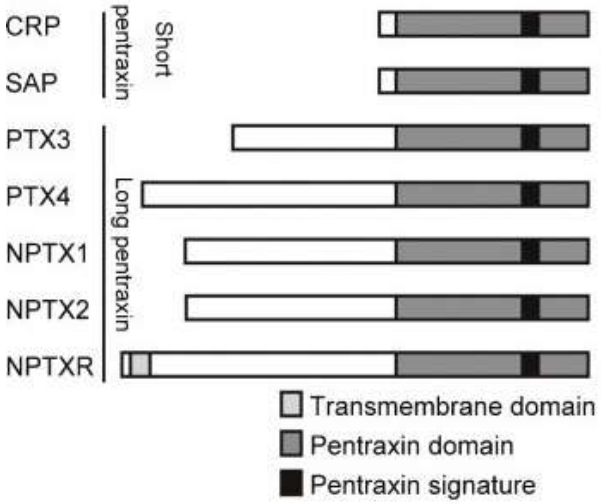

Figure 3. Pentraxin superfamily members and simplified structure [106].

1.3.2. PTX3 structure.

The human PTX3 gene is located in chromosome 3q25 and it is organised in three exons. The first two encode the leader peptide and the N-terminal domain, while the third exon codes for the C-terminal region. The proximal promoter of PTX3 presents various binding elements for transcription factors involved in different processes, such as NF-κB and AP-1. In contrast to short pentraxins, PTX3 is mainly expressed locally at the site of inflammation [107].

As a component to the innate immunity, PTX3 expression is mainly induced by pro-inflammatory agents, such as cytokines IL-1β and TNF-α, Toll-like receptor (TLR) agonists and microbial moieties like LPS and lipoarabinomannan. These molecules act on immune cells, including macrophages, neutrophiles and dendritic cells, enhancing the expression of PTX3. In both macrophages and neutrophiles, IL-10 and TLR engagement by LPS result in PTX3 expression and secretion [108, 109]. In contrast, PTX3 expression is inhibited by IFN-γ and IL-4 in macrophages. In neutrophiles, PTX3 is constitutively stored in specific granules that can be rapidly secreted in response to TLR activation. PTX3 is also expressed in non-immune cells, such as fibroblasts, chondrocytes and epithelial cells. For

example, high density lipoproteins (HDL) and oxidised low density lipoproteins (ox-LDL) can also induce PTX3 expression in endothelial and vascular smooth muscle cells [110].

Structurally, PTX3 is a 381 amino acid long protein composed by a 178 residue N-terminal (leader peptide included) and a 203 amino acid long C-terminal domain. Although PTX3 was first described as a decameric protein, Inforzato and colleagues demonstrated that it forms an octamer stabilised both by covalent and non-covalent interactions [111]. A network of cysteine disulphide bridges and non-covalent bonds permits the association of monomers into higher order oligomers. The secondary structures of both terminal domains are essential for the formation of the PTX3 octamer. Due to the homology between the C-terminal domain and short pentraxins, a CRP based structural model has been proposed. The C-terminus of PTX3 is composed by two anti-parallel β-sheets organised in a β-jelly roll topology forming a hydrophobic core. A single α-helix is located in the protein surface and an intrachain disulphide bridge between Cys^{210} and Cys^{271} stabilises the two β-sheets. In the other hand, the N-domain is formed by four α-helixes (αA, αB, αC and αD) bound by loops [112]. It is likely that αB, αC and αD form a coiled coil, a structure involved in oligomerization of proteins (**Fig.** 4A).

The N-terminal domain drives the assembly of PTX3 as a tetramer due to its unique structural features, enabling Cys-Cys interactions. The N-terminal Cys^{47} and Cys^{49} residues drive the formation of dimers, which are then linked by a Cys^{103} - Cys^{103} disulphide bridge, forming a tetramer. In the other hand, PTX3 monomers can also associate into dimers independently of covalent bounds, thus forming a "dimer of dimers". The morphological differences between the covalently bound tetramer and the "dimer of dimers" may be explained by the different structural arrangement adopted by the coiled coil forming α-helixes in the N-terminal. The coiled coil like structures may acquire an extended conformation when the protomers associate through interchain disulphide bridges or a more compact organisation when the protomers self-associate, forming an anti-parallel helix bundle. Finally, both tetramers are linked to each other through a disulphide bridge between Cys^{317} and Cys^{318}, in the C-terminus, forming the complete PTX3 octamer. Therefore, the native form of PTX3 is composed by two morphologically different tetramers connected by a stalk [111, 113]. The structural differences between the two tetramers confer PTX3 with a pseudo 4-fold symmetry, in contrast to the typical pentameric symmetry found in other pentraxins (**Fig.** 4B).

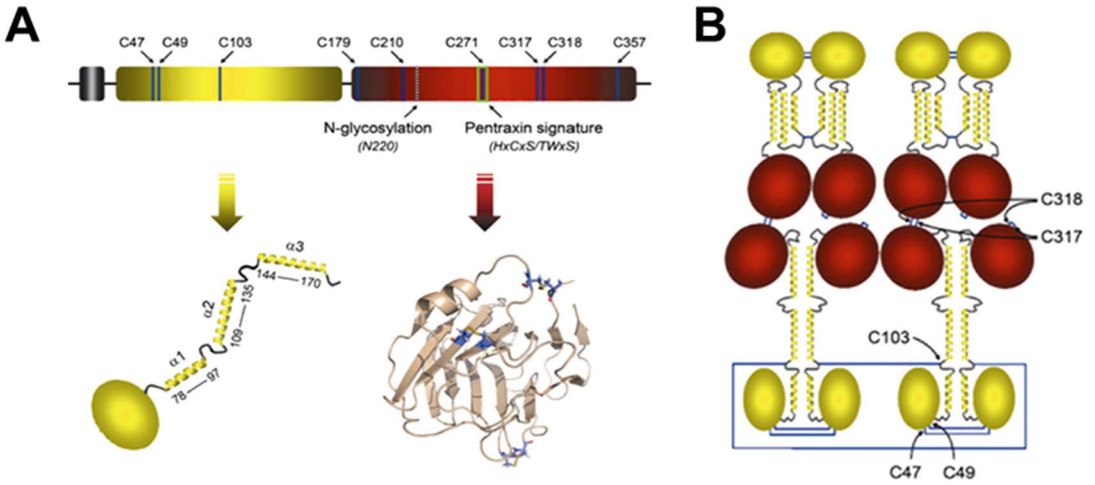

Figure 4. Monomeric and multimeric structure of PTX3. **A)** Secondary structures of the N- (yellow) and C-terminal (red) domains. **B)** Octameric organisation of PTX3. Two morphologically different dimers, non-covalently (up) and covalently linked (down), associated through disulphide bridges in the C terminal domains. Cysteine residues involved in covalent bounds are highlighted [113].

A single N-glycosylation site is present in PTX3, located in the Asn^{220} residue in the C terminus. This site is occupied by complex oligosaccharides, mostly by fucosylated and syalylated biantennary sugars, although a fraction of tri- and tretra-antennary glycans has been described. The syalylated sugars interact with polar amino acids in the C-terminal domain, concealing protein binding sites. When the sialic acid is removed, these sites become accessible resulting in the restored binding ability for some ligands. Thus, these glycans can modulate the biological activity of PTX3. Interestingly, the composition of the N-linked oligosaccharides changes depending on the cellular origin of the protein, indicating that the pattern of sialylation and fucosylation is key for the context specific roles of PTX3 [114].

1.3.3. Functions and ligands of PTX3.

Consistent with its innate PRM activity, PTX3 recognises and binds pathogen associated molecular patterns (PAMPs). *Aspergillus fumigatus* was the first microbial ligand described for PTX3. The conidia of these fungi are more effectively cleared by macrophages in the presence of PTX3, due to its opsonic activity [115]. In fact, PTX3 can bind selected viruses, bacteria and fungi, such as human cytomegalovirus, *Pseudomonas aeruginosa* and *Paracoccidoides brasiliensis,* respectively. In contrast, PTX3 is unable to bind other pathogens, such as the opportunistic fungi *Candida albicans*. Moreover, PTX3 recognises conserved microbial moieties like the outer membrane protein A from *Klebsiella pneumoniae* [106, 107].

In the host defence against pathogens, PTX3 acts either as an opsonin, enhancing clearance of microbes, or as an immune modulator, interacting with key molecules of the immune system to improve the immune response. PTX3 opsonic activity is mediated by various mechanisms that have not been fully characterised yet. The best understood mechanism of PTX3 driven opsonisation is mediated by Fcγ receptors (FcγR), expressed on the surface of macrophages and polymorphonuclear cells (PMN). The interaction with FcγR is also observed in short pentraxins. PTX3 binds FcγRIII and FcγRIIA with high and low affinity, respectively. Phagocytosis of *A. fumigatus* conidia by PMN cells for example, is mediated by PTX3 - FcγR interaction. Upon binding of PTX3-opsonised conidia to FcγRIIA, an inside-out activation of complement receptor 3 (CR3) occurs, inducing phagocytosis. Interestingly, the PTX3 involved in this process is endogenous, derived from the PTX3-enriched granules in neutrophiles [116]. Thus, PTX3 is able to recognise and bind microbial ligands, leading to the activation of immune cells and clearance of pathogens.

The interaction of the complement system with PTX3 is essential for the fine tuning of inflammation and pathogen clearance. The complement protein C1q binds the pentraxin domain of PTX3. This is in line with the C1q binding ability of CRP. In contrast to CRP, PTX3 binds C1q in a calcium independent manner. It has been reported that when C1q is plastic-bound, mimicking the coating of a microbial surface, PTX3 binds to it, initiating the recruitment of other members of the complement system (C3 and C4), resulting in the activation of the classical complement pathway. However, when PTX3 binds C1q in fluid phase, it inhibits the onset of the complement cascade by blocking key binding sites in C1q [117]. The glycosylation state of PTX3 regulates the interaction with the complement system. Indeed, the binding of PTX3 to C1q is enhanced when sialic acid is removed from the N-linked oligosaccharide of PTX3, while the interactions is inhibited when it is enriched in sialic residues. This mechanism contributes to the fine tuning of the complement activation [114].

PTX3 also binds ficolin-1, ficolin-2 and mannose binding lectin (MBL). The interaction of PTX3 with both MBL and ficolin-2 results in enhanced deposition of complement proteins in the surface of *A. fumigatus* and *C. albicans*, respectively. In particular, the formation of PTX3-MBL complexes on *C. albicans* promotes its phagocytosis. In turn, the binding of immobilised PTX3 and ficolin-1 results in the activation of the lectin dependent complement pathway [118]. On the other hand, PTX3 regulates complement activation through its binding with the inhibitory protein Factor H. Surface bound PTX3 recruits Factor H, which enhances iC3b deposition modulating the onset of the alternative complement pathway, avoiding an exaggerated activation of the complement system. In this way, PTX3 plays a dual role in the innate immune system. First, it promotes the immune response against pathogens through direct binding and activation of the complement, resulting in increased phagocytosis and microbe clearance. On the other hand, PTX3 modulates the excessive activation of the complement system,

either by interacting with Factor H or by alterations in the sugar moiety thus inhibiting C1q deposition [119, 120].

Another mechanism by which PTX3 regulates inflammation is through the inhibition of neutrophil recruitment to the inflammation site. Although neutrophiles are essential for a correct response to pathogenic infections, an exaggerated recruitment and activation often results in tissue injury. Neutrophiles are recruited into the inflammation site from the blood stream through the interaction between P-selectin on endothelial cells, and P-selectin glycoprotein 1 (PSGL-1) on the neutrophile surface. The binding between P-selectin and PSGL-1 induces in the rolling of neutrophiles along the endothelium, followed by extravasation to the inflammation site. PTX3 binds P-selectin through its N-linked oligosaccharide, thus inhibiting P-selectin binding to PSGL-1. Therefore, PTX3 attenuates excessive neutrophile recruitment to the inflammation site, preventing an exacerbated tissue-damaging response [121].

Consistent with its opsonic activity, PTX3 binds and recognises apoptotic cells, limiting the development of autoimmune diseases. In inflammatory contexts, PTX3 binds apoptotic cells limiting their immediate clearance. In this way, it prevents the cross-presentation of apoptotic self-antigens to CD8+ T cells, while inhibiting the uptake of self-antigens by specialised antigen presenting cells. In contrast, following late neutrophile apoptosis after inflammation, the granule stored PTX3 is secreted as an opsonin in the surface of neutrophiles, enhancing their clearance by macrophages. This two-sided function of PTX3, both inhibiting and eliciting apoptotic cell phagocytosis is context dependent [122, 123].

It has been reported that PTX3 is involved in tissue remodelling and repair. In a model of skin wound, TLR and IL-1 receptor activation resulted in PTX3 expression by macrophages and mesenchymal cells, promoting their migration to the wound site. In PTX3 deficient mice, an increased fibrin accumulation was observed in injured skin, liver and lungs. Fibrin is a key component of the ECM during tissue repair, as it is transiently deposited in the site of injury. Later on, fibrin degradation allows tissue repair and scar formation. PTX3 contributes to the degradation of fibrin through the interaction of its N-terminal domain with both fibrin and plasminogen in acidic conditions. The injury site presents an acidic pH as a result of cell metabolism adaptation in response to tissue hypoperfusion and hypoxia. The acidic environment is necessary for the formation of the tripartite interaction among fibrin, PTX3 and plasminogen, thus acting as a selective switch, only activated in sites with trauma and tissue damage. Once the ternary complex has been formed, a prompt plasmin-induced fibrin degradation takes place, resulting in macrophage and mesenchymal cell migration to the wound site, favouring tissue repair [124, 125].

A relevant role for PTX3 has been reported in female fertility. In fact, PTX3 deficient female mice are severely subfertile due to the defective organisation of the oocyte associated ECM. This matrix is produced by the *cumulus oophorus* and

granulosa cells in the follicle, and it is essential for a successful fertilisation *in vivo*. Hyaluronic acid (HA) is the main component of the matrix and it is responsible for its viscoelastic properties. However, HA requires the presence of inter-α-trypsin inhibitor (IαI), TNF-α induced protein 6 (TSG-6) and PTX3 for a proper assembly. In the process of matrix formation, TSG-6 mediates the covalent linking between IαI and HA chains. For this to occur, PTX3 stabilises TSG-6 through its N-terminal domain, allowing its interaction with IαI. Thus, PTX3 is necessary to provide structural integrity to the cumulus matrix and its deficiency results in a severely impaired fertility [111, 126].

Finally, several members of the Fibroblast Growth Factor (FGF) family have been described as PTX3 ligands. Canonical FGFs exert different biological functions through the stimulation and subsequent dimerization of tyrosine kinase FGF receptors (FGFR). These receptors activate multiple signalling pathways among which PI3K/Akt and Ras/MAPK, modulating tissue repair and angiogenesis among other processes. FGF2 is the prototypic member of the FGF family, and an important inducer of angiogenesis through the stimulation of proliferation in endothelial cells. PTX3 binds to FGF2 with high affinity through its N-terminal domain, thus preventing the stimulation of cell surface FGFR, averting its effects on cells. Therefore, PTX3 regulates neovascularisation containing the proangiogenic effect of FGF2. In the same way, PTX3 binds other members of the FGF family such as, FGF8b, FGF6, FGF10 and FGF17, although with lower affinity than FGF2 [112, 127].

1.3.4. PTX3 in cancer.

Inflammation is a ubiquitous process in tumour development. Most, if not all cancers, present immune cell infiltrates and activated inflammation-related pathways. In the last decades, it has become evident that inflammation is a necessary event in tumour progression, and it has been described as a hallmark of cancer. Considering the role of PTX3 as a regulator of the immune response, its involvement in cancer related inflammation and progression is not surprising. As a consequence of its biological versatility, PTX3 has been described to be involved in different steps of tumour progression, such as tumour onset, angiogenesis, proliferation, invasion and metastasis. The participation of PTX3 in tumour development has been observed in several cancers. However, these observations are contradictory, due to the fact that PTX3 may exert both pro- or anti-tumoral functions in a cancer-specific and microenvironment-dependent way [128].

Several malignancies, including pancreatic, gastric, cervical, prostatic and breast cancers, show a local overexpression of PTX3 when compared to matched normal tissue. In these cancers, PTX3 has been described to play a protumoral role enhancing migration, proliferation and invasion, among many other events. Furthermore, PTX3 levels are correlated to tumour progression and bad prognosis in several malignancies [129].

In head and neck squamous cell carcinoma, PTX3 is overexpressed and has been reported to induce tumour cell migration, invasion and EGF upregulation. Additionally, it has been shown that EGF drives the expression of PTX3, forming a positive feedback signalling loop, while promoting the expression of vimentin and matrix metalloproteinase 9 (MMP-9). The upregulation of these proteins results in enhanced cell motility and invasion. Thus, PTX3 overexpression contributes to the metastatic behaviour of head and neck squamous cell carcinoma [130].

PTX3 expression is upregulated in gastric cancer. High levels of PTX3 in this cancer promote tumour cell migration and macrophage recruitment to the tumour site, thus contributing to the formation of an inflammatory milieu. Additionally, PTX3 participates in the osteolytic activity of tumour cells in the bone metastatic niche, enhancing the direct interaction between tumour and osteoblasts. Therefore, PTX3 might be employed as a marker for bone metastasis in gastric cancer patients [131].

In cervical cancer, PTX3 expression is associated with tumour grade and differentiation. In fact, PTX3 is upregulated in the tumour when compared to surrounding normal tissue. Its silencing in cervical cancer cell lines results in decreased cell viability, proliferation and colony formation ability. *In vivo*, PTX3 knockdown decreases lung metastasis through the downregulation of MMP-2, MMP-9 and urokinase plasminogen activator [132].

Similar to cervical cancer, PTX3 is also correlated with tumour grade and severity in gliomas. Locatelli and colleagues have proposed the employment of PTX3 as a biomarker for tumour development and cancer related inflammation in these tumours. PTX3 plays an important role in glioma progression and metastasis, and SPOCD1 has been recently identified as a driver of proliferation and invasion in gliomas able to enhance PTX3 expression. In turn, PTX3 is able to attenuate the antitumoral effect of SPOCD1 silencing, suggesting that SPOCD1 effect on glioma is partly mediated by PTX3 [133].

Prostate cancers are also characterized by high levels of PTX3, together with other proinflammatory molecules, suggesting that cancer related inflammation is elevated in these tumours. Accordingly, local and serum levels of PTX3 are increased in prostate cancers when compared to premalignant lesions and normal prostate tissue. It is possible that the role of PTX3 in prostate cancer is linked to the tumour escape from complement-mediated immune surveillance. The modulation of the complement system may result in defective opsonisation of tumour cells, enhancing immune scape. Thus, the protumoral role of PTX3 in prostate cancer might be mediated by complement interactions [134].

PTX3 local and serum levels are elevated in lung cancer patients and its expression seems to be positively correlated with lung tumour grade. Moreover, increased PTX3 expression is associated with reduced overall and disease-free survival in lung cancer patients [135]. Ahmmed and colleagues reported that

deglycosylation of PTX3, either through inhibition of N-linked glycosylation or glycosylation site mutation, resulted in a significant decrease of migration and survival of lung cancer cells. This study highlights the importance PTX3 glycosylation in its protumoral functions [136].

In hepatocellular carcinomas, PTX3 expression is elevated in tumour when compared to normal liver tissue. In fact, PTX3 overexpression is correlated with aggressive and malignancy-related features like tumour size and intrahepatic metastases. Patients with hepatocellular carcinomas expressing high levels of PTX3 showed a dramatically decreased overall survival. This effect on tumour progression seems to be mediated by the induction of EMT by PTX3 [137].

In contrast to the protumoral functions listed above, PTX3 also exerts antitumoral functions and can be considered an oncosupressor in several cancers. This tumour suppressor activity is mainly driven through the modulation of the complement system and the inhibition of FGF-dependent biological effects (mainly angiogenesis and proliferation).

It has been reported that PTX3 knockout mice present an increased susceptibility to mesenchymal and epithelial carcinogenesis, supporting the oncosuppressive role of PTX3. Bonavita and colleagues showed that in a chemically induced sarcoma model, PTX3 deficient mice showed more aggressive tumours, characterised by increased cancer related inflammation, cytokine production, angiogenesis, macrophage recruitment and M2 differentiation. In addition, these tumours suffered from increased genetic instability, as represented by TP53 mutation rate. The authors demonstrated that this inflammatory response was initiated as a consequence of an exaggerated and uncontrolled activation of the complement cascade, represented by an increased deposition of C3. The hyperactivation of the complement system is due to the lack of PTX3 mediated modulation. PTX3 hampers the complement activation through the interaction with Factor H. Indeed, Factor H inhibition resulted in a phenotype similar to that in PTX3 deficient mice. Thus, PTX3 acts as an intrinsic oncosupressor limiting the overactivation of the complement system, preventing the formation of a tumour promoting inflammatory environment [138].

In addition, PTX3 exhibits antitumoral functions acting as an antagonist of the FGF/FGFR system, which plays a key role in different tumours. For instance, the ability of PTX3 to bind and sequester FGF2 (and other FGF family members) has been reported to be a relevant anti-angiogenic/anti-tumour feature in bladder and prostate cancer, as well as in fibrosarcoma [127, 139]. Also, FGF inhibition by PTX3 has been shown to play relevant role in the invasiveness and EMT of melanoma cells [140].

An additional, evidence of the tumour supressing activity of PTX3 in cancer is given by the fact that PTX3 is epigenetically silenced in several tumours. In colorectal cancer for example, two PTX3 enhancers are deeply methylated. Interestingly, these methylations occur in different stages of tumour progression.

Enhancer 1 is silenced in the early stages, suggesting that PTX3 activity is incompatible with tumours onset, whereas enhancer 2 is methylated in stages II or III, emphasising oncosuppressive role of PTX3 in tumour progression [141].

In conclusion, PTX3 plays a dual and controversial role in cancer. It seems clear that PTX3 can prevent FGF induced proliferation and neovascularisation in tumours, but it has no inhibitory effect in other proangiogenic factors such as vascular growth factor (VEGF-A). In the same way, the interaction between PTX3 and the immune system might both contribute to or inhibit the cancer related inflammation, a key event in tumour progression. Altogether, the role of PTX3 in cancer is not yet fully elucidated and further characterisation is needed to determine whether PTX3 itself or processes in which PTX3 is involved can be targeted therapeutically.

1.3.5. PTX3 in breast cancer.

Several studies have reported the overexpression of PTX3 in breast cancer and its positive correlation with tumour grade and severity, thus suggesting a possible pro-tumoral role.

Upregulation of PTX3 expression in breast cancer has been linked to hypoxia, a very frequent event in cancer progression. Breast cancer cells activate the HIF pathway under hypoxic conditions, resulting in the nuclear translocation of the HIF-1α transcription factor and overexpression of inflammatory factors such as cyclooxygenase 2 and PTX3. In breast cancer, the NF-κB mediated upregulation of proinflammatory genes, including PTX3, under hypoxic conditions strictly contributes to the formation of a protumoral inflammatory microenvironment [142].

Additionally, Choi and colleagues have reported that PTX3 is associated to bone metastasis in advanced breast cancer. Indeed, PTX3 has been found upregulated in breast cancer bone metastases in comparison to metastases in other sites, such as liver, lung and brain, suggesting that PTX3 plays a protumoral role in the bone niche [131]. PTX3 expression was elevated in breast cancer cells upon stimulation with TNF-α. The upregulation of PTX3 was mediated by NF-κB, resulting in increased cancer cell migration, osteoclast differentiation and macrophage recruitment. Indeed, PTX3 appeared to increase RANKL levels in osteoblasts, resulting in the stimulation of osteoclastogenesis by PTX3-induced RANKL, thus potentiating breast cancer metastasis progression in the bone [143].

Epithelial to mesenchymal transition is one of the most important events in tumour progression. Tumour cells that undergo EMT show increased motility and invasive ability. NF-κB is involved in EMT while also upregulating PTX3 expression. In fact, PTX3 expression is elevated in poorly differentiated breast carcinoma cells, characterised by mesenchymal-like traits [144]. These results coincide with the observation made by Chen and colleagues, where PTX3 was significantly more expressed in aggressive breast cancer cell lines when compared to less aggressive

cancer cells. Additionally, PTX3 was the most upregulated gene among aggressive breast cancer cells which outlines the importance of PTX3 in advanced tumour progression [145].

PTX3 is also overexpressed in basal-like breast cancer. The PI3K/Akt signalling axis is often upregulated in basal-like breast cancer due activating mutations in PI3K or other members of this pathway. The over-activation of this pathway in breast cancer cells results in PTX3 overexpression, mediated by both PI3K and NF-κB. Interestingly, PTX3 acts as a necessary intrinsic effector of PI3K/Akt driven stemness in these cells. Accordingly, abrogation of PTX3 expression almost totally reverts this stem-like phenotype. The mechanism by which PTX3 enhances stemness are not fully understood, although a possible autocrine signalling has been proposed [146].

A recent study has confirmed the role of PTX3 in the induction of stem-like traits in breast cancer cells. The authors describe that the scaffold protein SH3RF3 is highly expressed in breast cancer stem cells, enhancing the activation of the JNK/c-Jun signalling pathway. In this model, SH3RF3 drives the assembly of a JNK1 activating complex resulting in the activation of c-Jun, which in turn induces PTX3 expression. Indeed, PTX3 expression is necessary and sufficient for the acquisition of tumorigenicity and stemness characteristics in breast cancer cells. The mechanisms through which PTX3 induces stemness in tumour cells are not fully characterised, but there are evidenced of the involvement of Hedgehog and YAP signalling [147].

Finally, the role of breast cancer microenvironment derived PTX3 has also been described in scientific literature. Chi and colleagues reported that the transcription factor CEBPD is upregulated by tumour associated macrophages (TAMs) and cancer associated fibroblasts (CAFs) in breast cancers when treated with 5-fluoroacyl and cisplatin. This transcription factor in turn induces PTX3 expression, which enhances stemness, migration and invasion in tumour cells. Thus, the authors conclude that PTX3 expressed by microenvironment cells after treatment may contribute to resistance to therapy in breast cancer [148].

2. AIM OF THE WORK.

Breast cancer is among the most frequent malignancies worldwide, being the second cause of cancer-related death in women. In the last decades, important progress has been made in breast cancer treatment and prevention. The diffusion of targeted therapies for example, has yielded significant results in breast cancer treatment. A considerable effort has been put in the blocking of ER and HER2 signalling, since these receptors are collectively present in 85 to 90% of breast cancers and are essential for tumour progression. The hampering of these pathways with drugs such as tamoxifen and trastuzumab led to a better prognosis and overall survival in ER and HER2 positive breast cancer patients.

However, 10-15% of breast cancers are triple negative thus, they express neither ER nor HER2, rendering these targeted therapies useless for these patients. Additionally, TNBCs are characterised by an aggressive clinical behaviour, and patients bearing these tumours are diagnosed with poor prognosis. Thus, the research for new targetable pathways and molecules in TNBC is of vital interest.

PTX3 is a member of the pentraxin superfamily. It is produced locally at the site of inflammation and it plays a role in several physiological processes, such as host defence, innate immune system modulation, tissue repair and tumour biology. In cancer development, PTX3 acts both as an oncosupressor and as an oncogene, in a context dependent matter. As a consequence, the role of PTX3 in cancer remains controversial.

In breast cancer, PTX3 supports tumour progression mediating EMT, invasion and stemness. It has been reported that PTX3 is overexpressed in basal-like breast cancer. Most of these tumours are histologically triple negative and clinically aggressive.

The aim of this thesis project was to investigate and better characterise the role of PTX3 in triple negative breast cancer exploiting *in vitro* and *in vivo* models. Unravelling the role of PTX3 in triple negative tumours will open new therapeutical options in the treatment of this cancer.

3. MATHERIAL AND METHODS.

3.1. Reagents and cell culture.

Human MDA-MB-231 and MDA-MB-468 TNBC cells are from American Type Culture Collection (ATCC) and cultured in DMEM *plus* (GIBCO) 10% heat inactivated FBS (GIBCO).

Breast cancer cells were infected with a pLentiPGK-Puro (Addgene Plasmid #19070) lentiviral vector harbouring or not the full length human PTX3 cDNA (GenBank accession n° X63613). For silencing, cells were infected with lentiviral vector containing short-hairpin RNA (shRNA) targeting human PTX3 (TRCN0000436981 or TRCN0000430959) or a non-targeting/control sequence (SHC002V, Merck). Transduced cells were selected with 1µg/ml puromycin. Cells were maintained at low passage, returning to original frozen stocks every 3 to 4 months, and tested regularly for Mycoplasma negativity.

3.2. *In vitro* assays.

Cell proliferation. Cells were seeded (5×10^3) in 48-well culture plates in complete medium, detached at different time points and counted using the MACSQuant Analyzer (Miltenyi Biotec).

Clonogenic Assay. Five hundred cells were seeded in 6-well culture plates and incubated in complete growth medium until visible colonies were formed. Then, the supernatant was removed, and cells stained with 0.1% crystal violet/20% methanol; plates were photographed (colonies counted using Image J software) and solubilized with 1% SDS solution (to measure absorbance at 595 nm).

Soft Agar Assay. Cells (5×10^4) were suspended in 3ml of complete growth medium containing 0.3% agar and poured on to 2ml pre-solidified 0.6% agar in a 6-well plate. After 3 weeks of incubation, colonies were observed under a phase contrast microscope, photographed, and their area was measured using the ImageJ Software and the SA_NJ algorithm.

Wound-Healing assay. Confluent cells were scraped with a 200 µl tip to obtain a 2-mm-thick denuded area. After 24 hours, wounded monolayers were photographed, and the width of the wounds was measured in 3-independent sites per group.

Analysis of variance (ANOVA) test was carried out to evaluate the statistical differences for all assays.

3.3. Western Blot analysis.

Breast cancer cells were harvested from 80-90% confluent monolayers and homogenised with RIPA buffer (NaCl 5 M, Triton 100x 1%, sodium deoxycholate 0.5%, Tris HCl pH 7.5 25mM) in the presence of protease and phosphatase inhibitors. Subsequently, the protein samples were diluted in water and reducing loading buffer before being boiled. The samples were resolved by gel electrophoresis (10% polyacrylamide) in running buffer and transferred to activated PVDF membranes. After blocking with BSA, the membranes were incubated with the following primary antibodies: anti-PTX3 (Humanitas), anti-phospho-Akt (Ser473), anti-phospho-JNK (Thr183/Tyr185) and anti-phospho-Jun (Ser73) from Cell Signalling; anti -NF-κB p65, anti-phospho- NF-κB p65 (Ser311), and anti-GAPDH from Santa Cruz; anti-Actin β from Sigma.

After the incubation with primary antibodies, the membranes were reincubated with HRP-conjugated secondary antibodies. Protein levels were determined by Clarity Western Peroxide Reagent and Clarity Western Luminol/Enhancer Reagent (Clarity™ Western ECL Substrate, BioRad) and the chemiluminescence levels were detected using ChemiDoc™ Touch Imaging System (BioRad). The densitometric analysis was carried out using ImageJ.

3.4. Genome-wide expression profiling (GEP).

GEP was performed on cells MDA-MB-231 shNT/shPTX3 and MDA-MB-468 mock/PTX3. A cut-off of p-value < 0.01 (FDR corrected) and Log2 fold change ± 2 was applied to select differentially expressed genes. Total RNA was extracted using TRIzol Reagent according to manufacturer's instructions (Invitrogen). RNA integrity and the purity of the treated cells were assessed using a Bioanalyzer (Agilent Technologies). Hybridization to an Illumina Microarray (Illumina) was performed. Robust spline normalization and L2T were performed in R software, using the Lumi package from Bioconductor open source software (http://www.bioconductor.org/). Normalized data were imported into Partek Genomic Suite 6.6 software (Partek). After quality controls, Analysis of variance (ANOVA) test was performed to assess the effects of PTX3 modulation on gene expression, comparing MDA-MB-231 shNT *vs* MDA-MB-231 shPTX3 and MDA-MB-468 mock *vs* MDA-MB-468 PTX3. In order to show how samples group together based on similarity of features, we generated hierarchical clustering of gene expression changes. To identify significantly enriched or depleted groups of genes involved in the same biological pathways, Gene Set Enrichment Analysis (GSEA) was performed (http://software.broadinstitute.org/gsea/index.jsp). ANOVA test was performed to determine the impact of PTX3 levels in gene expression.

3.5. Tumour sphere formation assay and ALDH analysis.

Five thousand cells were resuspended in DMEM/F-12 medium (GIBCO) containing 10 ng/ml basic Fibroblast Growth Factor (bFGF), 10 ng/ml Epidermal Growth Factor (EGF) and 2% of B27 supplement (Sigma) and plated into each well of 24-well Ultra-Low Attachment Plates (Corning). After 7 days of incubation, tumour spheres were counted and assayed for ALDH activity using the Aldefluor kit (Stem Cell technologies) according to manufacturer's instructions. ALDH-positive cell analysis was performed by FACS (MACSQuant cytofluorimeter). Samples treated with the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) were used as controls to set the gates defining the ALDH-negative and the ALDH-positive regions (not shown). ANOVA test was performed to assess the differences in tumour growth.

3.6. *In vivo* studies.

Animal experiments were approved by the local animal ethics committee (OPBA, Organismo Preposto al Benessere degli Animali, Università degli Studi di Brescia, Italy) and were performed in accordance with national guidelines and regulations. Seven-week-old NOD/Scid female mice were injected orthotopically into the mammary fat pad with 4×10^6 MDA-MB-231 (shNT or shPTX3) and 8×10^6 MDA-MB-468 (mock or PTX3). Tumours were measured with callipers and the volume was calculated according to the formula $V = (D \times d2)/2$, where D and d are the major and minor perpendicular tumour diameters, respectively. Tumour volume data were analysed with a 2-way analysis of variance, and individual group comparisons were evaluated by the Bonferroni correction. At the end of the experimental procedure, tumours were surgically removed, weighed and paraffin embedded for immunohistochemical analysis.

3.7. Immunohistochemistry.

Formalin-fixed, paraffin-embedded samples were sectioned at a thickness of 3µm, dewaxed in xylene, hydrated and stained with hematoxylin and eosin (H&E) for histological analysis or alternatively processed for immunohistochemistry. The following primary antibodies were used: rabbit polyclonal anti-PTX3, anti-CD31 (Dako), rabbit anti-phospho-Histone H3 (Ser10) (Millipore). Sections were then incubated with HRP labelled polymer anti-rabbit or anti-rat and subsequently in Vectastain Elite ABC kit (Vector Laboratories). Positive signal was revealed by 3,3'-diaminobenzidine staining (Roche) and counterstained with Carazzi's haematoxylin to identify nuclei, dehydrated and mounted in DPX (Sigma) before analysis by light microscopy. Images were acquired with the automatic high-resolution scanner Aperio System (Leica Biosystems, Wetzlar, Germany, EU). ANOVA test was performed to evaluate the differences in tumour growth and pHH3, CD31 and CD44 expression levels.

4. RESULTS.

4.1. Preliminary data.

Thomas and colleagues demonstrated that the expression of PTX3 in upregulated in basal-like breast cancer. We sought to confirm these results employing Gene expression-based Outcome for Breast cancer Online (GOBO), a publicly available online tool that provides gene expression levels in a dataset of 1881 breast cancer samples and 51 breast cancer cell lines. Database mining revealed that PTX3 levels are elevated in basal-like breast cancer when compared to other subtypes (**Fig.** 5A). In the same way, PTX3 expression is positively correlated with tumour grade (**Fig**. 5B). These results corroborate previous observations where PTX3 overexpression was associated with basal-like tumours [146]. To further confirm this, a Western Blot analysis was performed on samples from triple negative, triple positive and HER2 positive tumours and, as expected, PTX3 was mainly expressed by TN tumours (**Fig.** 5C).

To confirm that breast cancer cell lines reflect breast tumours, we evaluated PTX3 expression in a panel of 51 breast cancer cell lines in GOBO (**Fig.** 6A). Every cell line was then clustered depending on its intrinsic type. PTX3 mRNA expression was significantly higher in basal B cell lines followed by basal A and finally luminal cell lines (**Fig.** 6B). Interestingly, the basal-B subtype in breast cancer cell lines reflect a TNBC phenotype [149]. In agreement with this, when the cell lines were clustered in clinical subtypes, the triple negative cluster displays significantly higher levels of PTX3 mRNA than HER2 positive and hormone receptor positive cell lines (**Fig.** 6C). A Western Blot analysis performed in triple negative, ER/PR+ and HER2+ breast cancer cell lines, confirmed that PTX3 expression is increased in TNBC cells (**Fig.** 6D).

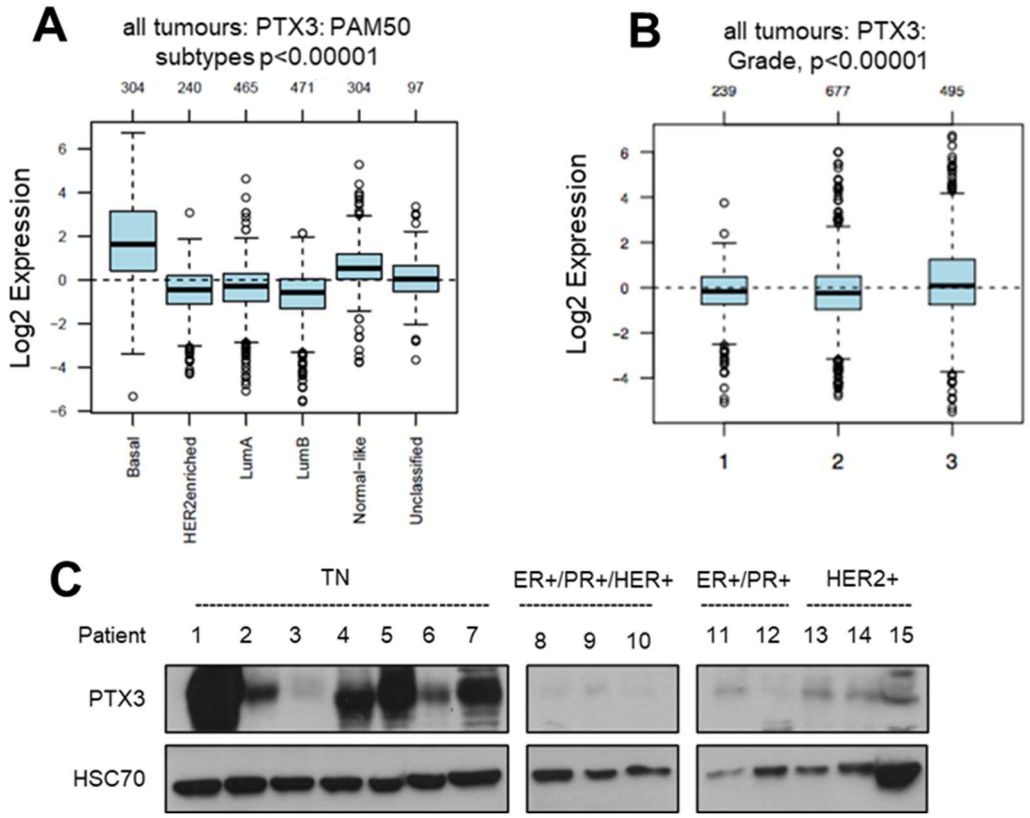

Figure 5. PTX3 expression is increased in basal-like breast cancers and it is correlated with higher tumour grade. A) PTX3 expression in breast tumours sorted in PAM50 subtypes. **B)** PTX3 expression correlates with tumour grade in breast cancer. **C)** PTX3 expression in 15 breast cancer patients classified by ER, PR and HER2 expression.

4.2. PTX3 contributes to the aggressive behaviour in TNBC cells.

Based on preliminary *in silico* and Western Blot data, we selected a prototypical human TNBC cell line, MDA-MB-231 which shows elevated expression of PTX3. To investigate the effect of PTX3, we silenced its expression with a short hairpin RNA (shRNA). Thus, we produced two MDA-MB-231 clones, one where PTX3 expression is silenced (shPTX3), and a sh Non-Targeting (shNT) as control.

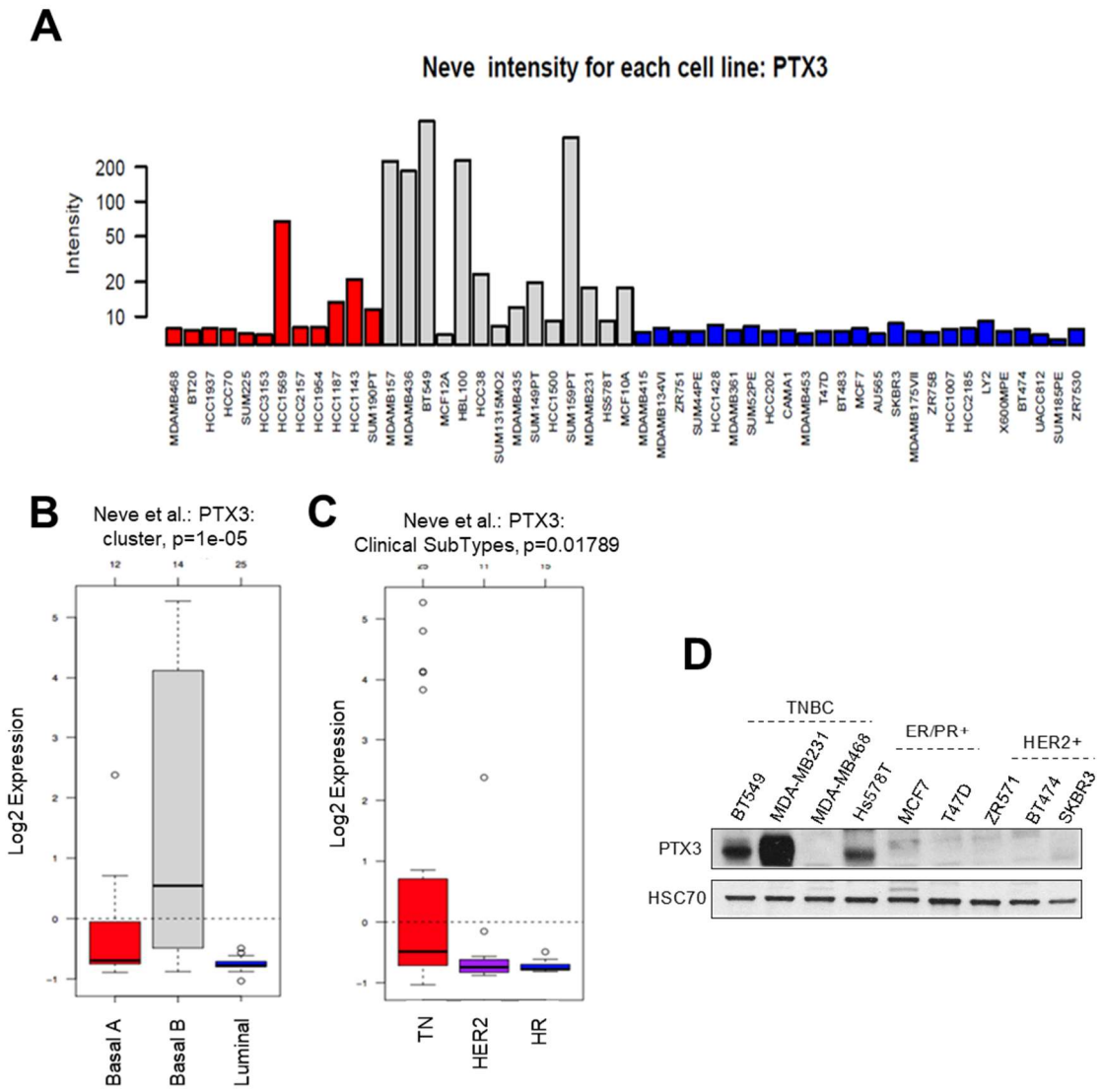

Figure 6. **PTX3 is most expressed in triple negative and basal B breast cancer cell lines**. **A)** PTX3 expression in 51 breast cancer cell lines clustered in basal A (red), basal B (grey) and luminal (blue) subtypes. **B)** Analysis of PTX3 expression in the plotted 51 cell lines, clustered in basal A, basal B and luminal and **C)** in clinical subtypes TN, HER2 positive (HER2) and Hormone Receptor positive (HR). **D)** Western blot analysis of PTX3 expression in triple negative, ER/PR positive and HER2 positive breast cancer cell lines.

The transduction of the short harpin successfully downregulated PTX3 production when compared with MDA-MB-231 wild type (wt) and shNT (**Fig.** 7A). Next, we characterised the effect of PTX3 downregulation *in vitro*, evaluating key tumoral processes. We analysed the expression of genes involved in proliferation and cell cycle by GSEA in MDA-MB-231 shNT and shPTX3. We found that targets of E2F, a transcription factor involved in proliferation, and genes involved in G2-M transition were significantly downregulated after PTX3 downregulation in MDA-MB-231 shPTX3 cells (**Fig**. 7B). Accordingly, MDA-MB-231 shPTX3 cells proliferate

significantly less than wt and shNT counterparts, confirming the results obtained in the GSEA (**Fig.** 7C), and showed decreased clonogenic capacity (**Fig.** 7D). Also, when tested for anchorage independent growth in a soft agar growth assay, MDA-MB-231 shPTX3 cells formed fewer colonies when compared to wt and shNT cells (**Fig**. 7E). Finally, PTX3 silencing significantly reduced the MDA-MB-231 motility and migratory potential as demonstrated by wound healing assay (**Fig.** 7F).

4.3. PTX3 modulation affects stem-like features in MDA-MB-231.

Cancer stem cells are key players in cancer progression and resistance to treatment. In fact, most cases of therapy failure are associated with cancer stem cell populations [150]. In addition, it has been reported that PTX3 is involved in the induction of stem cell-like traits in basal-like breast cancer cells [146]. Here, we show that PTX3 silencing significantly decreases the tumour sphere formation ability in MDA-MB-231 cells (**Fig**. 8A). Accordingly, we found that the expression of genes associated with breast cancer progenitors are downregulated in MDA-MB-231 shPTX3 in comparison with shNT cells (**Fig.** 8B). In addition, the evaluation ALDH activity (a functional marker of stem cells) in MDA-MB-231 shNT and shPTX3 cells revealed that the proportion of ALDH positive cells in MDA-MB-231 shPTX3 was significantly lower than in shNT cells (**Fig.** 8C). Altogether these data suggest a relevant role of PTX3 in the promotion of stem cell like traits in TNBC.

In silico analysis employing the Ingenuity Pathway Analysis (IPA) of differentially expressed genes revealed a significant modulation in NF-κB and Akt centred pathways in relation to PTX3 expression (**Fig.** 9A). In agreement with this, Western Blot analysis revealed that the phosphorylation of both Akt and the NF-κB p65 subunit were reduced in MDA-MB-231 shPTX3 (**Fig.** 9B). These observations were confirmed by GSEA, showing that PTX3 silencing results in downregulation of genes involved in NF-κB signalling (**Fig.** 9C).

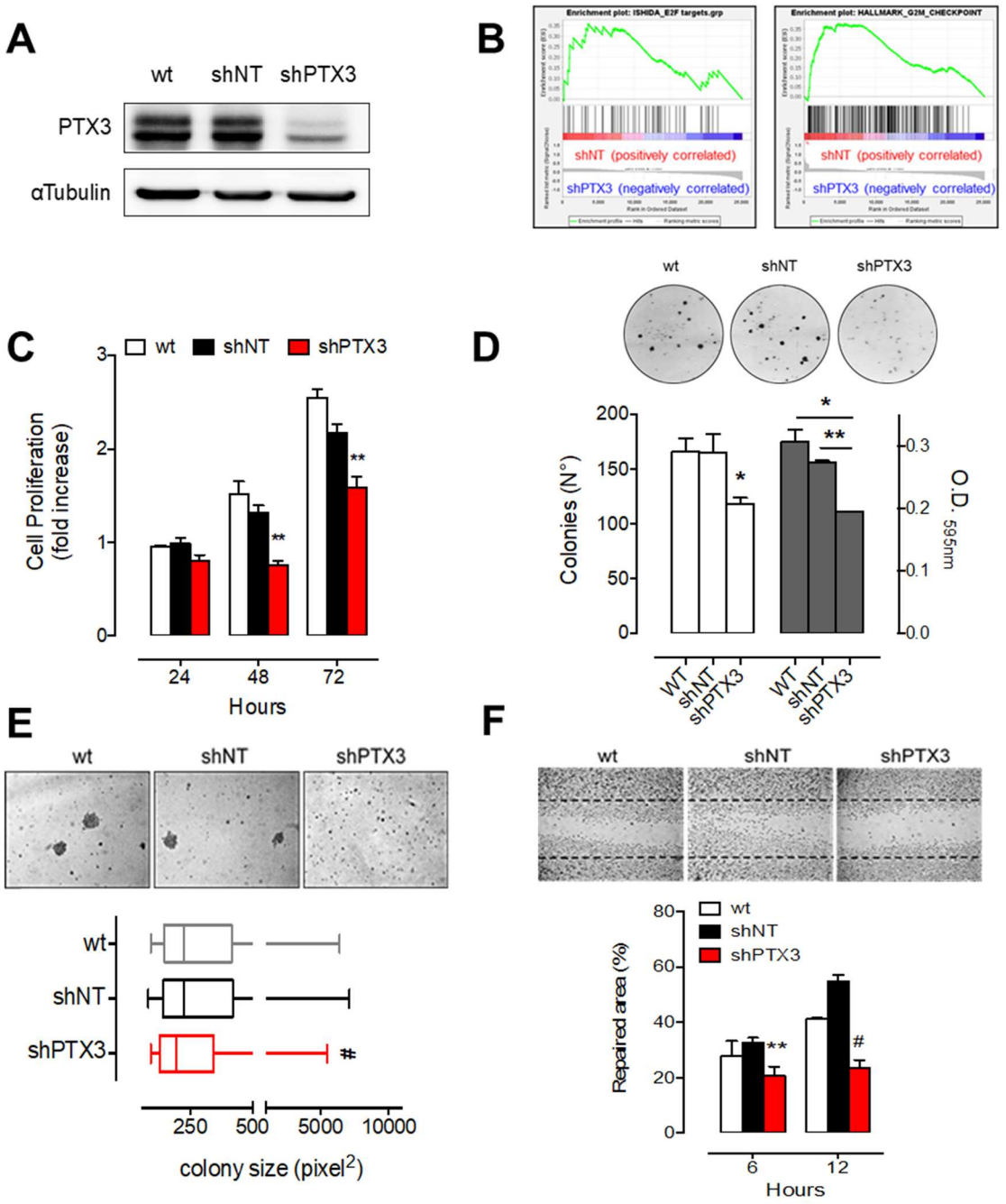

Figure 7. PTX3 silencing in MDA-MB-231 cells decreases proliferation, clonogenic ability, anchorage independent growth and motility. A) Western blot analysis of PTX3 expression in MDA-MB-231 wt, shNT and shPTX3 cells**. B)** GSEA of E2F target genes and G2/M phase transition related genes in MDA-MB-231 shNT and shPTX3 cells. **C)** Measurement of MDA-MB-231 wt, shNT and shPTX3 proliferation in complete medium; *p<0.01 **D)** Effect of PTX3 silencing in the clonogenic ability of MDA-MB-231 cells. Results shown in number of colonies and optical density of crystal violet; *p<0.05, **p<0.01. **E)** Anchorage independent growth in MDA-MB-231 wt, shNT and shPTX3; #p<0.001. **F)** Cell motility and migratory potential in MDA-MB-231 wt, shNT and shPTX3 cells evaluated by scratch wound assay; **p<0.01, #p<0.001.

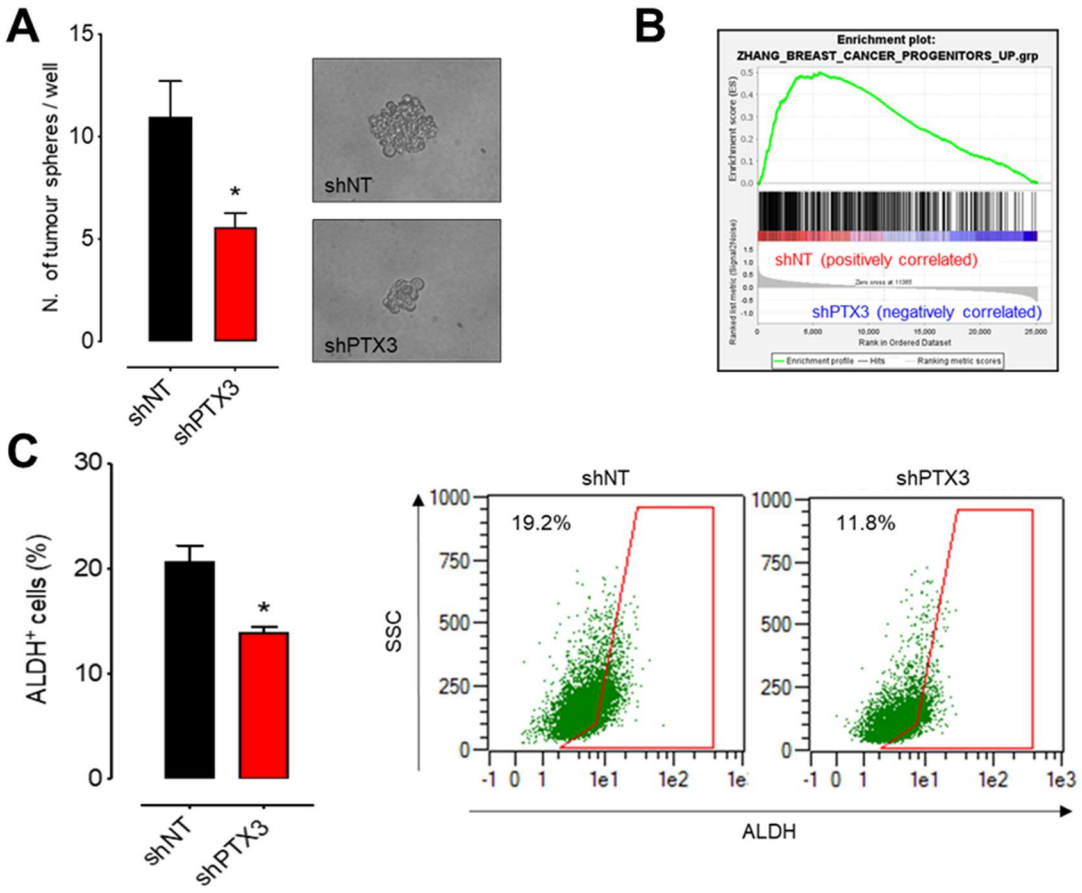

Figure 8. PTX3 silencing reduces stem cell-like characteristics in MDA-MB-231 cells. A) Tumour sphere formation is reduced in MDA-MB-468 shPTX3 when compared to shNT cells; *p<0.01. **B)** GSEA of breast cancer progenitor related genes are downregulated in MDA-MB-231 shPTX3 when compared with shNT. **C)** Assessment of the ALDH positive subpopulation in MDA-MB-231 shNT and shPTX3 cells by FACS. PTX3 downregulation results in reduced ALDH positive cell fraction; *p<0.01.

Zhang and colleagues showed that PTX3 can be regulated by JNK/c-Jun signalling in TNBC [147]. Therefore, we assessed the activation and nuclear translocation of JNK and c-Jun by Western Blot in our cell model. As shown in **Figure** 9D, MDA-MB-231 shPTX3 cells exhibited a decreased nuclear translocation of both JNK and c-Jun. As a confirmation, a GSEA of genes in involved in JNK signalling revealed a significant downregulation of these genes in shPTX3 cells (**Fig.** 9E).

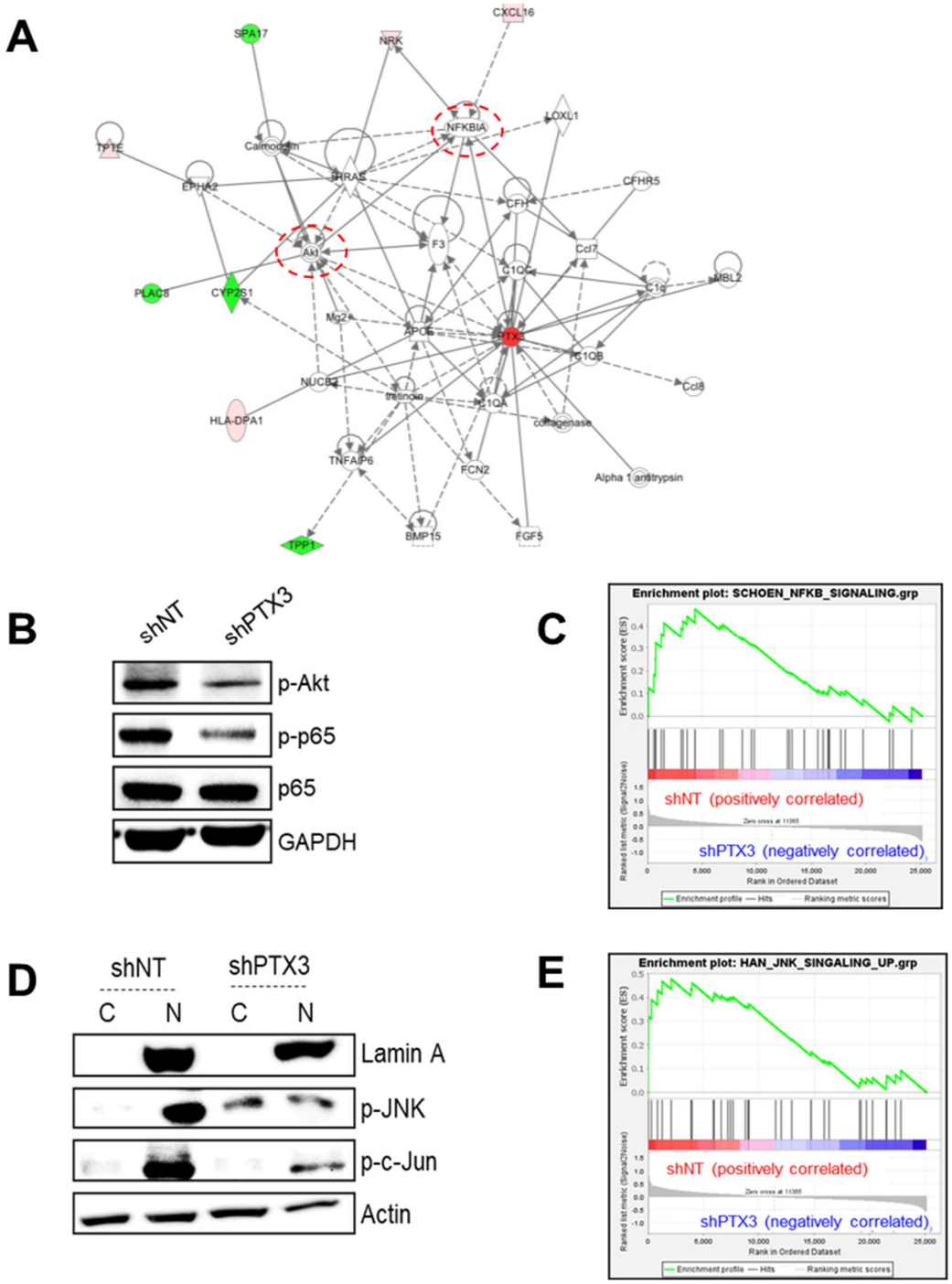

Figure 9. PTX3 silencing modulates Akt/NFκB and JNK/c-Jun pathways. A) Ingenuity pathway analysis of differentially expressed genes shows that Akt and NF-κB pathways are modulated in relation with PTX3. **B)** Western blot analysis of Akt and NF-κB subunit p65 activation in MDA-MB-231 shNT and shPTX3. **C)** Confirmation by GSEA, NF-κB signalling genes are downregulated in shPTX3 cells. **D)** Western blot analysis of JNK/c-Jun pathway activation in MDA-MB-231 shNT and shPTX3 cells. Actin and Lamin A blotted as loading controls of the cytoplasmic (C) and nuclear (N) fractions. Nuclear levels of both phospho-JNK and phospho-c-Jun are decreased in shPTX3 cells. **E)** This result is further confirmed by a GSEA that shows the downregulation of JNK signalling in shPTX3 cells.

4.4. PTX3 overexpression enhances aggressiveness and stemness in MDA-MB-468 cells.

Considering the results obtained by PTX3 silencing in MDA-MB-231 cells, we sought to determine whether PTX3 overexpression in low-PTX3 expressing cells could "recapitulate" this phenotype. To this purpose, we selected MDA-MB-468 cells, a TNBC cell line with low expression of PTX3, to stably overexpress this protein. The biological features of MDA-MB-468 PTX3 were then compared with wt and control/mock counterparts (**Fig.** 10A).

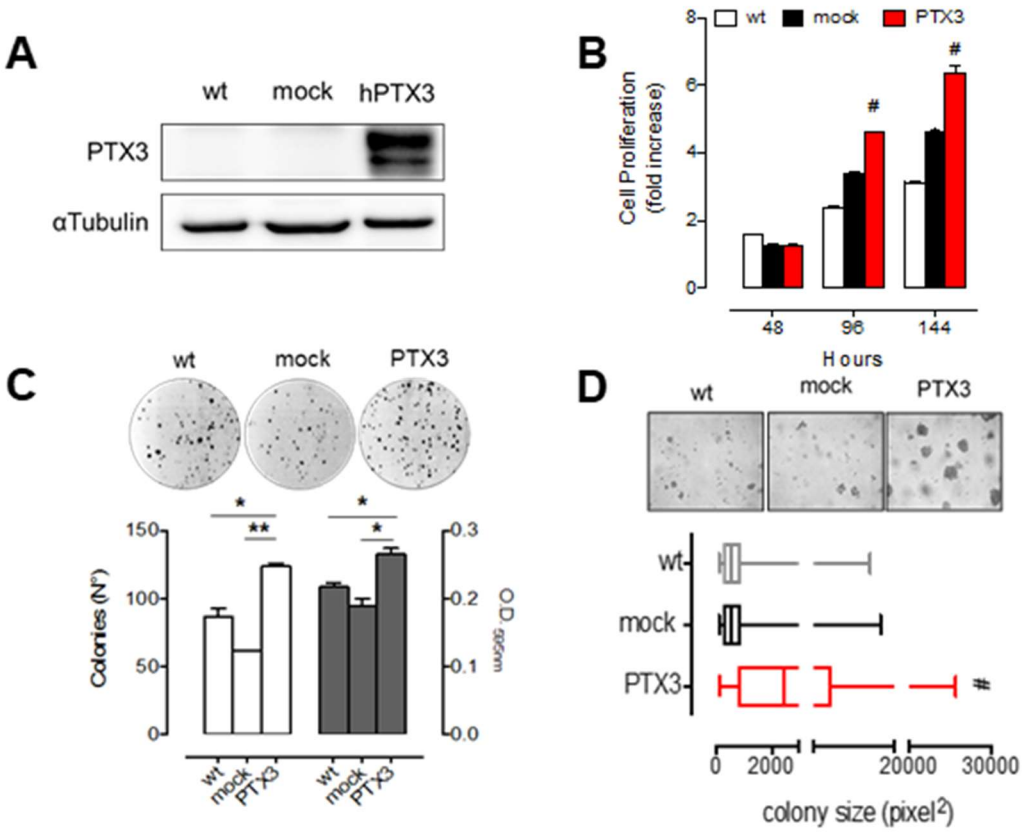

Figure 10. PTX3 overexpression in MDA-MB-468 cells enhances proliferation, clonogenic ability and anchorage independent growth. A) Western blot analysis of PTX3 expression in MDA-MB-468 WT, mock and PTX3 overexpressing cells**. B)** Measurement of MDA-MB-468 WT, mock and PTX3 proliferation in complete medium; #p<0.001. **C)** Effect of PTX3 overexpression in the clonogenic ability of MDA-MB-468 cells. Results shown in number of colonies and optical density of crystal violet; *p<0.05, **p<0.01. **D)** Assessment of anchorage independent growth in MDA-MB-468 WT, mock and PTX3 cells by soft agar colony formation assay; #p<0.001.

PTX3 overexpressing MDA-MB-468 cells showed significantly increased proliferation (**Fig.** 10B), clonogenic ability (**Fig.** 10C) and anchorage independent growth (**Fig.** 10D) when compared to wt and mock cells. In the same way, we sought to determine whether PTX3 overexpression had any effect on stemness. In agreement with previous results, MDA-MB-468 PTX3 formed more tumour spheres than mock cells (**Fig.** 11A). These results were further supported by a GSEA showing that genes expressed in cancer progenitors are upregulated in PTX3 overexpressing MDA-MB-468 cells (**Fig.** 11B). Accordingly, we detected more ALDH positive cells in MDA-MB-468 PTX3 than in mock cells (**Fig.** 11C).

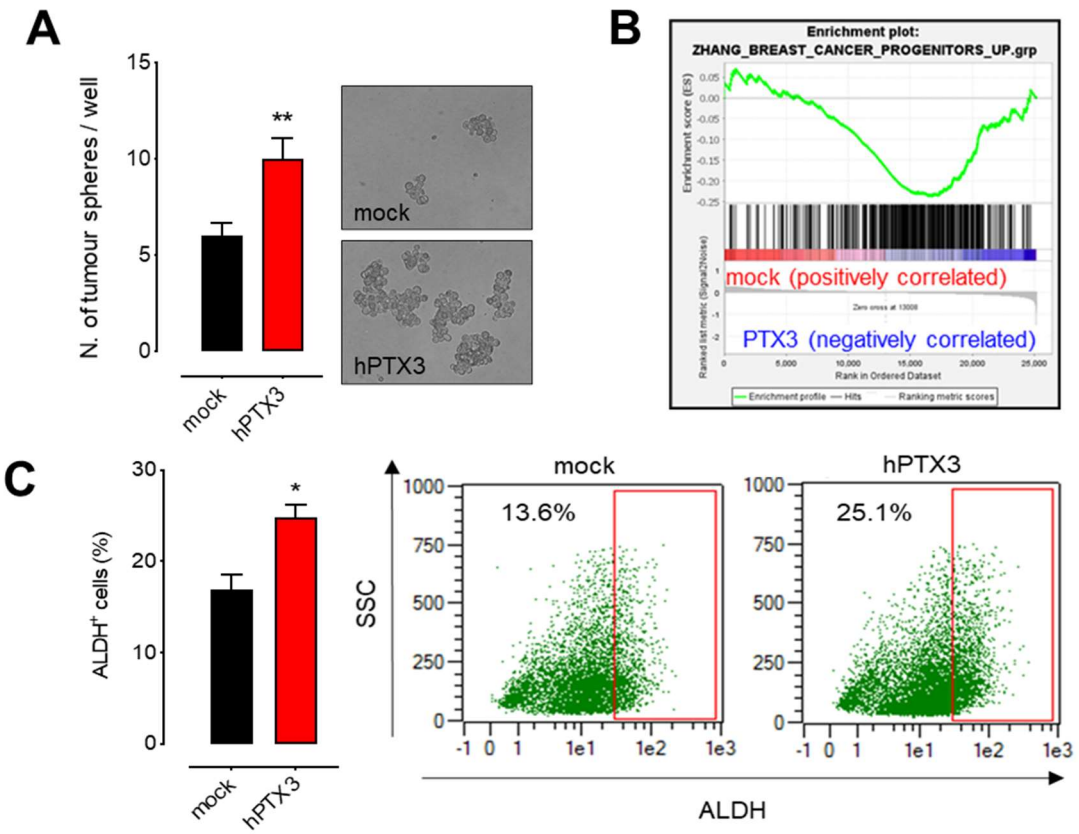

Figure 11. PTX3 overexpression enhances stem-like characteristics and tumorigenicity in MDA-MB-468 cells. A) Tumour sphere formation is increased in MDA-MB-468 PTX3 when compared to mock cells; **p<0.01. **B)** GSEA of breast cancer progenitor related genes are upregulated in MDA-MB-468 PTX3 when compared with mock cells. **C)** Assessment of the ALDH positive subpopulation in MDA-MB-468 mock and PTX3 cells by FACS; *p<0.05.

4.5. PTX3 modulation determines TNBC cell growth *in vivo*.

Considering the *in vitro* and *in silico* results, we evaluated the effect of PTX3 modulation in tumour growth *in vivo*. To this purpose, human MDA-MB-231 and MDA-MB-468 with PTX3 modulation were grafted orthotopically into the mammary fat pad of immune compromised NOD/Scid female mice, and tumour growth was then followed.

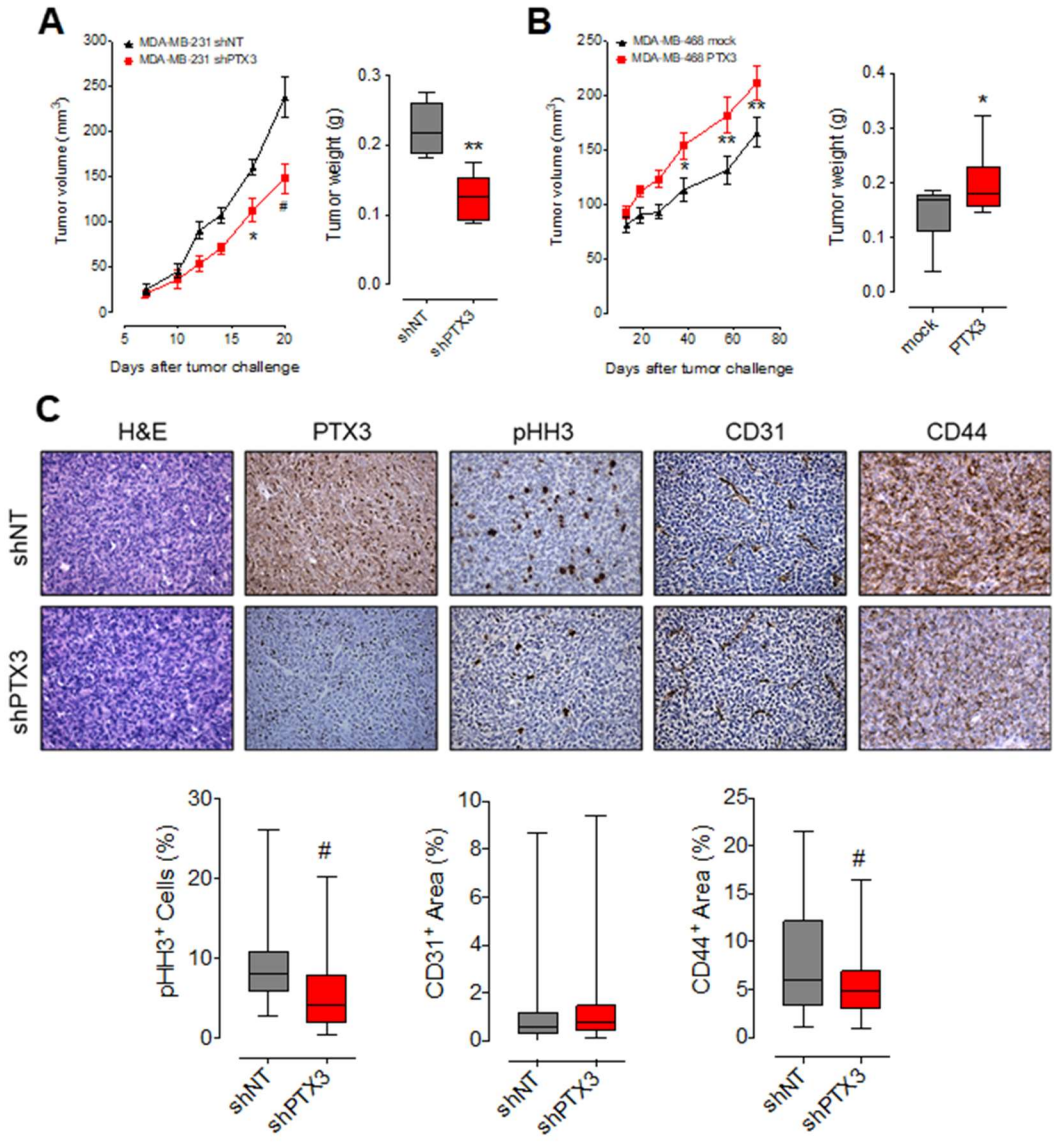

Figure 12. PTX3 expression enhances *in vivo* tumour growth and is associated with increased proliferation and stemness in MDA-MB-231 derived tumours. A) Tumour growth of orthotopically grafted MDA-MB-231 shNT and shPTX3 cells in immunocompromised mice; *p<0.05, **p<0.01. **B)** Tumour growth of orthotopically grafted MDA-MB-468 mock and PTX3 cells in immunocompromised mice. **C)** IHC analysis performed in MDA-MB-231 shNT and shPTX3 derived tumours. The tumours were stained for hematoxylin and eosin and PTX3. Additionally, we evaluated the positive area for proliferation (pHH3), angiogenesis (CD31) and stem cell markers (CD44); #p<0.001.

As shown in **Figure** 12A-B, MDA-MB-231 shPTX3 cells formed significantly smaller tumours than shNT cells, and accordingly, MDA-MB-468 PTX3 tumours grew faster than MDA-MB-468 mock tumours. In accordance with the *in vitro* observations, immunohistochemical analysis performed on representative MDA-MB-231-derived tumours revealed that both cell proliferation (pHH3) and stemness (CD44) related markers, were significantly reduced in MDA-MB-231 shPTX3 tumours. Interestingly, no differences were found in the levels of endothelial marker CD31 among shNT and shPTX3 tumours (**Fig.** 12C).

5. DISCUSSION AND CONCLUSION.

The designing of new therapies against breast cancer during the last decades has significantly improved the overall survival for many patients. Targeted therapies against ER, PR and HER2 have been efficiently used in the treatment of breast tumours bearing these markers, accounting for more than 75% of the total. TNBC makes up 10 to 15% of all breast cancers, and in these patients the abovementioned therapies are not useful due to the lack of these targetable receptors. In the same way, TNBCs are known to be more aggressive and patients bearing these tumours have bad prognosis, with a 70% death risk during the first five years after diagnosis [151]. Therefore, the research for new targetable pathways is of essential interest for these tumours.

Several studies have pointed out key molecular mechanisms that could be therapeutically targeted. Unfortunately, these new strategies have not been successful so far. New fields in TNBC treatment are represented by ADP-ribose polymerase (PARP) inhibitors (PARPis) that exploit the defects in double-stranded DNA (dsDNA) repair mechanisms that are frequently present in TNBC as a result of mutations in BRCA1/2 and other genes of DNA repair [152, 153]. Considering the relevant role of the immune system on the response to standard chemotherapy and prognosis of TNBC, several immune modulatory drugs (targeting PD-1/PD-L1 or CTLA-4) have entered the clinical trials testing with limited activity as monotherapy, but promising results in combination with chemotherapy [154-157]. In addition, PI3K-Akt-mTOR (PAM) pathway is frequently dysregulated in cancer, promoting cell proliferation and tumorigenesis, *PIK3CA* gene being mutated in almost 10% of TNBC [158, 159]. Despite this key role, the clinical application of PAM targeting drugs, mainly mTOR and panPI3K inhibitors, has led to disappointing results due to high toxicities and the activation of resistance feedback loops [160, 161]. Thus, more selective inhibitors, or alternative targeting of Akt have been tested, with more beneficial results, as reported for the α-selective PI3K inhibitor alpelisib combined with nab-paclitaxel [162], and for the Akt inhibitors ipatasertib or capivasertib combined with chemotherapy [163, 164].

PTX3 is a pattern recognition molecule involved in several biological processes. It is upregulated in inflammatory contexts where it exerts pleiotropic functions through its multiple interactions with the ECM or complement system. Consistent with the presence of an inflammatory environment during tumour progression, PTX3 has been linked to cancer. However, it seems clear that PTX3 exerts a dual role in cancer development, as it might act as an oncosupressor or as an oncogene, depending on the context. To date, all reports point to a pro-tumoral role for PTX3 in breast cancer.

It has been shown that PTX3 produced by tumour-associated M2 macrophages and fibroblasts in TNBC models may exert a tumour promoting function [148]. In this work we show that in TNBC patients, PTX3 expression correlates with tumour aggressiveness, and that the main source of PTX3 in the tumour context is represented by cancer cells themselves. This aspect sheds light on the fact that PTX3 produced in the tumour microenvironment may play relevant roles, but tumour-produced PTX3 is a key player in conditioning cancer cell behaviour. Indeed, high levels of PTX3 confer aggressive biological features to TNBC cells, such as increased proliferation and stemness.

Stemness in particular, is a key feature in cancer progression. Due to their low proliferative rate, cancer stem cells (CSCs) show resistance to anticancer therapy, resulting in tumour recurrence and worse disease-free survival. Thus, it is of outmost importance to unravel the mechanisms involved in the establishment and maintenance of the CSC population in TNBC.

In this study we demonstrate that elevated levels of PTX3 result in an increased subpopulation of stem cell-like cancer cells, as shown by the augmented ALDH activity and tumour sphere formation ability [165, 166]. Although these features are considered sufficient to point towards a stem cell-like phenotype, other protein markers have been described for the recognition of CSCs. In fact, Ricardo et al report that the best suited markers to identify TNBC stem cells are CD44+/CD24-/ALDH+ [167]. These three proteins could improve the detection of TNBC stem cells permitting a deeper study of the role of PTX3 in stemness. Additionally, these markers would permit us to confirm our results on the role of PTX3 in the amplification of TNBC stem cells.

Our data demonstrate that the increased proliferation and stemness are strictly linked to the activation of Akt and JNK/c-Jun pathways as a consequence of PTX3 overexpressed in TNBC cells. Notably, these observations are in accordance with reports by Thomas *et al.* showing that PTX3 is a PI3K-regulated biomarker and a potential critical mediator of PI3K signalling able to prompt PI3K-induced stem cell-like traits [146]. In addition, it has been shown that, in breast cancer cells, the scaffold protein SH3RF3 promotes stem-like properties through activation of JNK/c-Jun pathway and expression or PTX3 [147]. In our experimental models, gene expression profiling confirmed that Akt and JNK/c-Jun represent relevant “hubs” for the determination of stem-like features in TNBC cells.

Additionally, NF-κB emerged as another molecular mediator significantly called into question and modulated together with PTX3. Interestingly, this is in line with observations reporting that, upon activation of HIF-1α and NF-κB, PTX3 and other pro-inflammatory genes, are significantly upregulated in human mammary invasive carcinoma [142]. NF-κB is frequently upregulated in breast cancer [168].This was also reported by Scimeca and colleagues that described the

correlation between elevated PTX3 levels and NF-κB expression in high grade infiltrating ductal carcinomas [144]. Furthermore, hypoxia-induced NF-κB activation results in the upregulation of PTX3 in breast cancer cells [142]. This suggests that hypoxic tumour cells may develop a coordinated pro-inflammatory gene response where PTX3 is functionally involved, leading to a more malignant and invasive phenotype [142].

In the reports by Thomas *et al* and Zhang *et al*, PTX3 acts as a downstream mediator of PI3K and JNK/c-Jun signalling pathways, respectively[146, 147]. Our results show that the silencing of PTX3 in tumour cells results in the inhibition of the abovementioned pathways, suggesting that PTX3 may act as an autocrine/paracrine tumour promoting factor in TNBC cells. Moreover, the fact that PTX3 is associated both upstream and downstream with PI3K, JNK/c-Jun and NF-κB signalling pathways might imply a positive feedback loop, although further experimentation is needed to confirm this.

Overall, our results show that PTX3 is a key player in the progression of TNBC, enhancing tumour growth both *in vitro* and *in vivo,* and fostering stemness. Therefore, even though the exact mechanisms of action of PTX3 in TNBC requires further clarification, we show that the PTX3-mediated functional rewiring in TNBC goes through the acquisition of proliferative and differentiation capacities controlled by other key signalling players, such as Akt, JNK/c-Jun and NF-κB. In a therapeutic perspective, these observations reinforce the meaning of Akt targeting strategies that have been tested and are still in phase II/III clinical evaluation. On the other hand, these data open the possibility to further explore and target other mediators (PTX3 itself, JNK/c-Jun and NF-κB) for the treatment and the prognostic evaluation of TNBC.

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