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Review

Expression and signaling pathways of Nerve Growth Factor (NGF) and pro-NGF in breast cancer: a systematic review

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Abstract: Breast cancer represents the most frequent cancer and the leading cause of cancer death among women. Thus, the prevention and early diagnosis of breast cancer appears to be of primary urgency as well as the development of new treatments able to improve its prognosis. Nerve Growth Factor (NGF) is a neurotrophic factor that plays a key role in the regulation of neuronal functions thought the binding to the Tropomyosin receptor kinase A (TrkA) and the Nerve Growth Factor receptor or Pan-Neurotrophin Receptor 75 (NGFR/p75NTR). Also, its precursor (pro-NGF) can extert biological activity by forming a trimeric complex with NGFR/p75NTR and sortilin or by binding to TrkA receptors with low affinity. Both *in vitro* and *in vivo* studies showed that NGF is synthesized and released by breast cancer cells and has mitogen, antiapoptotic and angiogenic effects on these cells through the activation of different signaling cascades that involve TrkA and NGFR/p75NTR receptors. Conversely, pro-NGF signaling has been related to breast cancer invasion and metastasis. Other studies suggested that NGF and its receptors could represent a good diagnostic and prognostic tool, as well as promising therapeutic targets for breast cancer. Here, we comprehensively summarize and systematically review the current experimental evidence on this topic.

Keywords: *breast cancer, nerve growth factor (NGF), TrkA, p75NTR, NGFR, pro-NGF, angiogenesis, invasion, metastasis, diagnosis, prognosis, treatment.*

1. Introduction

Breast cancer is a neoplasm of epithelial origin that generally develops in the parts of the breast tissue made up of the glands involved in milk production or in the ducts that connect the glands to the nipple. In women it represents the most frequent cancer [1], with 2.3 million of new cases diagnosed worldwide in 2020 [2], as well as the leading cause of cancer death [3]. The incidence of breast cancer is estimated to increase over the years and to reach 3.2 million in 2050 [4], thus representing a health emergency both from a medical [5] and a psychological point of view [6, 7]. Therefore, prevention and early diagnosis of breast cancer appears to be of primary urgency as well as the development of new treatments able to improve its prognosis.

Nerve Growth Factor (NGF) is a neurotrophic factor that plays a key role in the regulation of neuronal functions thought the binding to the Tropomyosin receptor kinase A (TrkA) and to the Nerve Growth Factor Receptor or Pan-Neurotrophin Receptor 75 (NGFR/p75NTR) [8]. Since the 1990s, several studies have indicated that NGF and its receptors could also play a key role in the pathogenesis of breast cancer and consequently could represent a new therapeutic target [9-13]. Other evidence indicates that both NGF

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and its receptors could be considered as accurate diagnostic and prognostic tools for breast cancer [14-17]. Moreover, the NGF precursor (pro-NGF) signaling pathways were related to breast cancer invasion and metastasis [18,19].

However, as far as we know, this topic has never been systematically reviewed. After providing a general overview of breast cancer and NGF signaling pathways, here we systematically review and comprehensively summarize the current experimental evidence about the involvement of NGF signaling pathways in breast cancer. Based on the findings, significant issues for future studies are then put forward.

2. Breast cancer: an overview

2.1. Classification

There are several classifications of breast cancers. The main ones are based on: i) histologic type, ii) grading; iii) immunophenotype; iv) Tumor Size (T), Nodal Status (N), and Distant Metastasis (M) Staging (TNM classification); and v) molecular subtype [20]. (**Table 1**).

Breast cancer classification

	General classification	*Type*	*Sub-type*
Histological Type	Carcinoma *in situ*	Ductal	Comedo
			Cribiform
			Micropalillary
			Papillary
			Solid
		Lobular	
	Invasive or infiltrating Carcinoma	Tubular	
		Ductal lobular	
		Invasive lobular	
		Infiltrating ductal	
		Mucinous	
		Medullary	
	Grade	*Differentiation*	*Elston-Ellis Score*
Grading	Grade 1 (G1)	Well	3-5
	Grade 2 (G2)	Moderate	6-7
	Grade 3 (G3)	Poor	8-9
	Molecular markers		*Cases*
Immunophenotype	HR positive/HER2 negative		70%
	HER2 positive		15-20%
	Triple-negative		~15%
	Stage	TNM	Category
TNM classification	0	Tis N_0 M_0	Carcinoma *in situ*
	I	T_1, N_0, M_0	Early breast cancer
	II	T_1, N_1, M_0 T_2, NO_1, M_0	Early breast cancer
	III	Any T, N_{2-3}, M_0, T_3 Any N, M_0	Locally advanced
	IV	Any T, any N M_1	Metastatic
	Subtype		*Cases*
Molecular subtype	Luminal A		~40%
	Luminal B		~20%

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Basal-like	15-20%
Claudin-low	12-14%
HER2-enriched	10-15%

Table 1. The main types of classification of breast cancer

2.1.1. *Histological Type*

From a histological point of view, breast cancer can be classified into two broad categories: carcinoma *in situ* and invasive or infiltrating carcinoma [21]. Based on growth patterns and cytological characteristics, breast cancer *in situ* is further sub-classified into ductal and lobular. Ductal carcinoma in situ (DCIS) is much more common than lobular carcinoma in situ (CLIS) and it is further sub-categorized into comedo, cribiform, micropalillary, papillary and solid [22]. Like carcinomas in situ, invasive carcinomas are also a heterogeneous group of cancers categorized into different histological subtypes. The main types of invasive carcinoma are: tubular, ductal lobular, invasive lobular, infiltrating ductal (e.g., intracanalar, apocrine), mucinous (colloid) and medullary [21]. Among these, infiltrating ductal carcinoma (CDI) is the most frequent type, accounting for 70-80% of all forms of breast cancer [23]. CDI is further sub-classified into well differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3) based on nuclear pleomorphism levels, tubule formation, and mitotic index [24].

2.1.2. *Grading*

The histological grade - also called grading - defines how a neoplasm is well, moderately, or poorly differentiated and therefore constitutes a fundamental parameter to evaluate in each newly diagnosed breast cancer [25, 26]. The extent of differentiation is assessed by the pathologists through the observation of several morphological characteristics such as: degree of formation of tubular structures, nuclear pleomorphism and proliferation as indicated by mitotic index [20, 27]. The most widely grading system used worldwide is the Nottingham system (or Scarff-Bloom-Richardson method modified by Elston-Ellis) that evaluates all three parameters just mentioned. A score ranging from 1 to 3 is assigned to each of these parameters; the sum of the three scores determines a global score, based on which the histological grade is defined: i) grade 1 (G1), well differentiated tumor (score from 3 to 5); ii) grade 2 (G2) moderately differentiated tumor (score 6 or 7); iii) grade 3 (G3) poorly differentiated tumor (score 8 or 9) [25].

2.1.3. *Immunophenotype*

Depending on the presence/absence of molecular markers for estrogen receptors (ER), progesterone receptors (PR) (i.e., together known as hormone receptors, HR) and human epidermal growth factor 2 (HER2), breast cancer can also be classified into: i) HR positive/HER2 negative (about 70% of cases); ii) HER2 positive (about 15-20% of cases) and; iii) triple-negative (tumors lacking all three standard molecular markers, about 15% of the remaining cases) [28].

2.1.4. *TNM classification*

The TNM classification was proposed by the American Joint Committee on Cancer and is essentially based on three variables: tumor size (T), lymph node involvement (N) and presence of metastases (M) [21, 29]. The last version also incorporates into the staging system several biological factors such as ER and PR receptors, HER2, histological grade and multigene prognostic assays. Combining these factors, the breast cancer can be

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classified into one of five stages indicated with the Roman numerals I, II, III, and IV (plus 0) [20, 29].

2.1.5. *Molecular subtype*

From a molecular point of view, a distinction can be made among several sub-categories of breast cancer identified through global gene expression profiling studies: luminal A, luminal B, basal, claudin-low and HER2-enriched [20, 21].

The term luminal derives from the similarity in gene expression between these tumors and the normal luminal epithelium of the breast. However, luminal A tumors are also characterized by high expression of ER-related genes, low expression of HER2 genes and low expression of proliferation-related genes (e.g., Ki67<20%). Conversely, luminal B tumors show a low expression of ER-related genes, variable expression of HER2-related genes, and high expression of proliferation-related genes (e.g., Ki6>20%) [30, 31]. Thus, the proportion of the proliferation genes/cells is generally used to carry out the differential diagnosis between luminal A and B tumors [32].

The basal subtype derived its names from the gene expression profile which share some similarities with the basal epithelial cells. However, it is also characterized by a low expression of the luminal and HER2 gene cluster. Most of these forms are triple-negative [33]. However, while most triple-negative breast cancers are basal, not all basal-like forms are triple-negative and there is a significant discordance (up to 30%) between these two classification methods that must always be considered [34, 35]. As well as the basal breast cancer, also the claudin-low are mostly triple-negative despite only a minority of triple-negative breast cancers are claudin-low [36]. In addition, this sub-type is characterized by the low expression of genes involved in cell-cell adhesion such as claudin 3, claudin 4, claudin 7, occludin and E-cadherin [1]. Finally, the HER2-enriched subtype is characterized by a high expression of the HER2 gene cluster and proliferation genes and by a low expression of the genes of the luminal and basal subtype [37].

2.2. *Risk factors*

Literature suggests that several risk factors are associated with breast cancer. These are commonly classified in non-modifiable and modifiable factors. Major non-modifiable factors include female gender, older age, ethnicity (i.e., white non-Hispanic women are the most affected), a family or personal history of breast cancer, presence of genetic mutations (mostly in *BRCA1* and *BRCA2* genes), previous radiation therapy history, reproductive factors (e.g., pregnancy characteristics, late age of menopause) (for a review see: [1, 38]). The main modifiable factors include lifestyle (e.g., obesity, diabetes, alcohol consumption and smoking), poorer vitamin supplementation, hormonal contraceptive or post-menopausal methods, air pollution and night work (for a review see: [1, 38]).

2.3. *Diagnosis*

The standard diagnostic process of breast cancer includes anamnesis, clinical examinations, and medical imaging (e.g., mammography, ultrasonography, magnetic resonance imaging) [39]. Among the medical imaging techniques, mammography is commonly used as a screening tool in many countries, although several researchers are increasingly stressing its limitations such as the radiation exposition, the low sensitivity in women with dense breast tissue [40], the high false-positive and false-negative rates [41], and thus the disadvantageous cost-effectiveness ratio [42]. In addition, it has been shown that molecular biotechnology examinations – aimed to the detection of specific biomarkers such as nucleic acid, proteins, cells and tissues - can diagnose breast cancer earlier than imaging techniques and therefore are increasingly becoming auxiliary methods to diagnose breast cancer and one of the most research topics in this field (for a review see: [39, 41]).

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2.4. Treatments and prognosis

Given the heterogeneity of breast cancer, treatment strategies are chosen based on the type and the extend of the cancer. These include surgery, chemotherapy, radiotherapy, hormonal therapy, and biological therapy. The surgical procedures consist of mastectomy (the complete removal of the breast) or breast-conserving surgery (partial mastectomy, lumpectomy, wide local excision, or quadrantectomy) [1]. Chemotherapy can be both neoadjuvant or coadjutant and is individualized according to the type of breast cancer. Generally, it includes the simultaneous administration of 2 or 3 drugs—carboplatin, cyclophosphamide, 5-fluorouracil/capecitabine, taxanes, and anthracyclines [28]. Although its usage is considered effective, often chemotherapy is accompanied by several side effects, such as hair loss, nausea/vomiting, diarrhea, fatigue, increased risk of infections, bone marrow suppression, irregular menstrual cycle, and infertility [1]. Radiotherapy is a local treatment typically provided after surgery or chemotherapy to ensure that all cancer cells are destroyed and thus to minimize the risk of recurrence [43]. The specific radiotherapy to be applied is chosen based on the type of previous surgery and on the clinical features of breast cancer. The main ones include intraoperative radiation therapy, 3D-conformal radiotherapy, intensity-modulated radiotherapy and brachytherapy [1]. In the same manner of chemotherapy, also radiotherapy is accompanied by side effects such as irritation and darkening of the skin, fatigue, and lymphoedema although is significantly associated with an increased overall survival rate and a lowered risk of recurrence [44]. The main goal of hormonal therapy is to block ER or to decrease the estrogens levels. Thus, it is commonly used in patients with Luminal A or B breast cancer, either as a neoadjuvant or adjuvant (in cases of recurrence or metastasis) treatment [45]. The main drugs include selective ER modulators (i.e., tamoxifen, toremifene) and selective ER degraders (i.e, fulvestrant) to block ER, whereas aromatase inhibitors (i.e., letrozole, anastrazole, exemestane) are applied to decrease the estrogens levels [46, 47]. Finally, the biological therapy can be provided at every stage of breast cancer therapy and the type of drugs are chosen and administered - alone or in combination with other drugs - based on the type of breast cancer and the presence of metastasis: i) trastuzumab, pertuzumab, trastuzumab deruxtecan, lapatinib, and neratinib in HER2-positive; ii) abemaciclib and everolimus in ER positive/HER2 negative; iii) atezolizumab in triple negative and; iv) denosumab in case of metastasis to the bones [48-53].

The advancement on knowledge of breast cancer and the use of personalized medicine, have greatly improved the prognosis and survival rates compared to previous decades [54]. However, as already mentioned, breast cancer continues to be the leading cause of cancer mortality in women, with 685,000 deaths only in 2020 [2], prompting researchers to conduct research on new treatments, especially for the advanced stages, presence of metastasis or cancer types with a poorer prognosis due to chemoresistance, such as triple-negative [55, 56].

Classical prognostic factors of breast cancer include age, tumor stage, type and lymphovascular status [57]. In addition to these features, the American Joint Committee on Cancer (AJCC) also recommended the evaluation of some biological factors (i.e., ER, PR, HER2, grade, and multigene assays) to define the prognosis [58]. Also, Ki67 which is expressed only in cells during the proliferative phases, is widely used as a useful laboratory test to predict the prognosis of breast cancer patients [59]. Other novel and promising prognostic molecular markers are represented by p53, p14ARF, cyclin D1, cyclin E, *TBX2/3*, *BRCA1/2*, and *VEGF* (for a review see: [60].

3. NGF signaling pathways

Neurotrophins are a family of small proteins that play a key role in the growth, survival, and differentiation of developing and mature central and peripheral neurons [61-64]. They also are involved in the apoptotic Programmed Cell Death (PCD) [65]. Four

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types of neurotrophins have been identified in mammals: NGF, Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin 3 (NT-3) and Neurotrophin 4 (NT-4) [66]. NGF was isolated in the 1950s a discovery for which the Nobel Prize in Medicine in 1986 was jointly awarded to Rita Levi-Montalcini [67] and Stanley Cohen [68].

The human *NGF* gene is located on the short arm of chromosome 1 (1p22) and encodes for pro-NGF, a protein which has own biological activities [69]. In turn, pro-NGF can be cleaved by proteases (e.g., plasmin, furin, matrix metalloproteinase MMP7 and MMP3) to produce the mature NGF form [70].

Besides neurons, research has also focused on NGF role in non-neural functions, highlighting its presence and activity in the reproductive, endocrine, cardiovascular, and immune systems [10, 71]. NGF is also synthesized, stored and released by vascular endothelial cells, platelets, fibroblasts [72-75] and cancer cells [10]. In particular, NGF exerts its function by binding two receptors: TrkA and NGFR/p75NTR.

The TrkA receptor was isolated in 1986 from a human colon carcinoma [76] and is encoded by the *NTRK1* gene [77, 78], located on chromosome 1 (1q21-22) [69, 79] in the same manner of the *NGF* gene. Several data showed that the NGF binding to TrkA receptor mediates proliferation, differentiation and survival of both neurons and cancer cells via activation of PI3K/Akt, Ras/MAPK and PLCγ pathways [10].

The second receptor related to NGF is the tumor necrosis factor NGFR/p75NTR, first cloned in 1986 [80] and encoded from the *NGFR* gene located on chromosome 17 (17q21.33) [81]. The NGF binding to NGFR/p75NTR leads to the activation of NF-kB or JNK that mediate opposite effects on survival and apoptosis of both neurons and cancer cells, respectively [10]. The biological effects of NGFR/p75NTR are determined by the level of expression of TrkA receptors. Whether the TrkA receptors are not expressed or under-expressed, NGFR/p75NTR induces apoptotic signals. On the other hand, when NGFR/p75NTR and TrkA receptors are co-expressed, NGFR/p75NTR increases the affinity of the TrkA receptor for NGF and thus mediate the activation of survival pathways [82-85].

As above mentioned, also pro-NGF exhibits neurotrophic activity. In particular, pro-NGF can exert proapoptotic effects by forming a trimeric complex with its high affinity NGFR/p75NTR and sortilin receptors [86, 87]. Interestingly, pro-NGF can also bind with low affinity to TrkA receptors and therefore inducing signaling of cells survival [88].

4. Methods

Study search

A systematic search was conducted in EMBASE, PUBMED and COCHRANE databases. A manual search in the bibliographies of selected articles was also conducted. We used the following Boolean search string considering free text and Medical Subject Heading [MeSH] terms: (“breast cancer”) AND (“nerve growth factor” OR “NGF”) OR (“nerve growth factor precursor”) OR (“nerve growth factor receptor” OR “NGFR”) OR (“tropomyosin receptor kinase A” OR “TrkA”) OR (“sortilin”) synonyms. All returned results were systematically identified, screened then extracted for relevant information following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [89]. (http://www.prisma-statement.org).

Exclusion and inclusion criteria

All studies with the aim to understand the role of pro-NGF, NGF and its receptors in breast cancer were included in the systematic review. Not original research articles (e.g., review, opinion article or conference abstract), articles with unclear design of the study and studies that did not include the role of NGF in breast cancer were excluded from further analysis. Titles, abstracts, and articles were evaluated by two separate reviewers (A.Ma. and F.B.). Titles and abstract were reviewed for subject relevance. The investigators read full-text versions of eligible articles on their own. Disagreements were

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addressed by consensus between the two reviewers. A third investigator (R.M.) was consulted if the two reviewers reached different decisions or when in doubt. Additional research was obtained via appropriate publication reference lists and consulting a specialist in the field.

5. Results

5.1. Included studies

The systematic search in the three databases generated 6,075 entries. As 2548 of them were duplicated across the sources and 155 records were removed for other reasons, a total of 2890 entries were initially screened (**Figure 1**). After applying the inclusion and exclusion criteria, we screened the titles and then the abstracts of all records. We reviewed the full text of the remaining 302 articles with the subsequent removal of 265 not original research (e.g., review, opinion article or conference abstract), 3 articles with unclear study design or setting of the study), and 5 articles did not include specific data on the role of NGF in breast cancer.

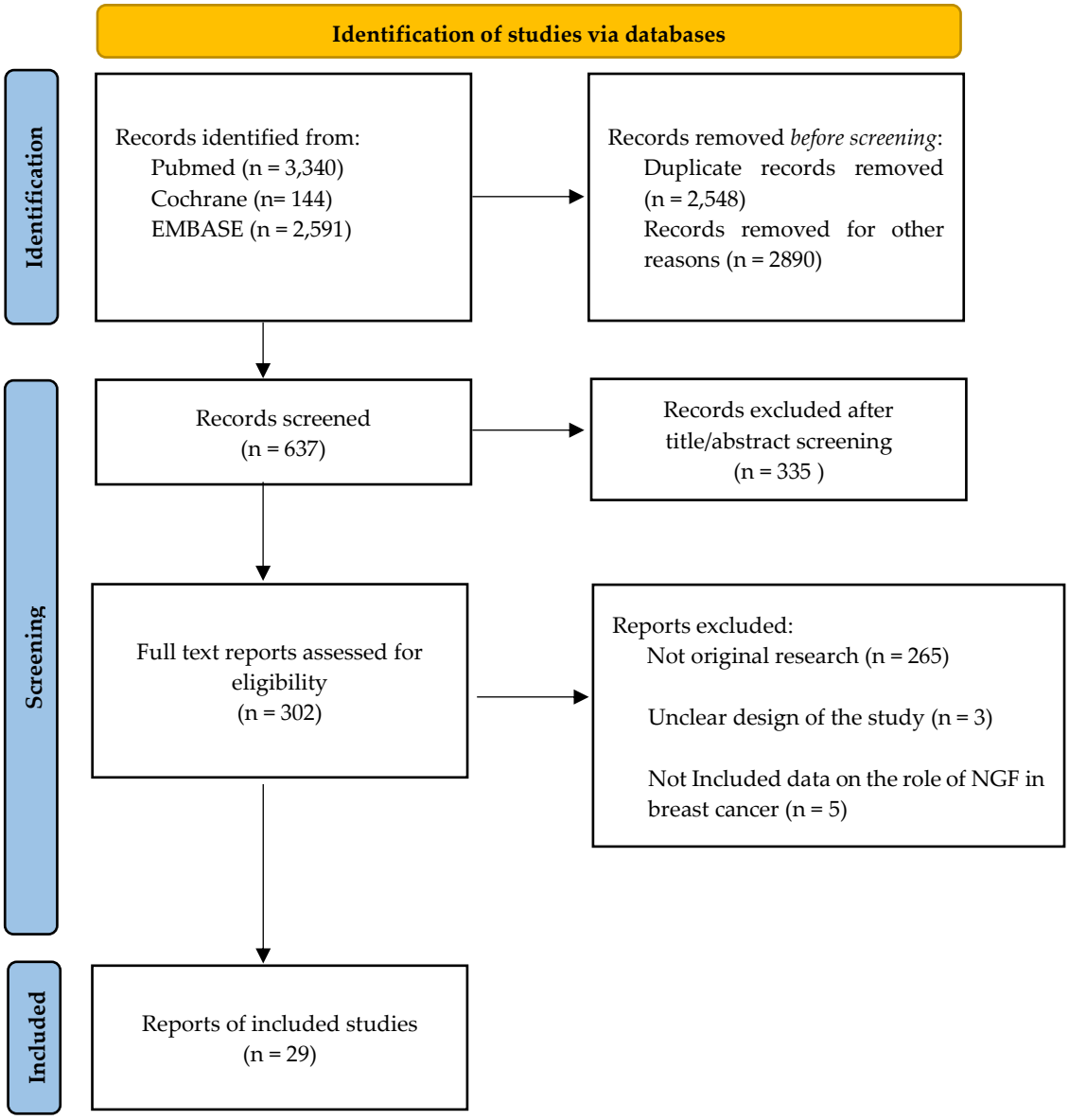

Figure 1. The flowchart of the studies selection

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5.2. NGF and its receptors expression in breast cancer

On the contrary of normal breast epithelial cells, both pro-NGF and NGF are synthesized and released by breast cancer cells [18, 90, 91]. In particular, Dollè et al. [90] found an overexpression of NGF either at the transcriptional and protein level as well as the presence of NGF within classical secretion vesicles in several breast cancer cell lines and breast tumor biopsies of invasive ductal carcinoma. The authors also showed that NGF is biologically active and acts as an autocrine factor for breast cancer development. In 2008, Adriaenssens et al. [91] confirmed the high expression of NGF, particularly concentered in the epithelial cancer cells, in several histological types of breast cancer (i.e., invasive ductal, invasive lobular, colloid, apocrine, epidermoid metaplastic, tubular and intracanal carcinomas) through the immunostaining with anti-NGF of human breast cancer tissue sections obtained from a series of biopsies.

More recently, Kumar et al. [92] reported an overexpression of NGF in benign phyllodes, a rare fibro-epithelial breast tumor, demonstrating that this neurotrophin could play a role not only in the pathogenesis of malignant tumors. Beyond NGF, also NGFR/p75NTR and TrkA receptors are expressed in breast cancers cells either at the transcriptional and protein level [93-96].

5.3. Mitogenesis of breast cancer cells

The first evidence that NGF stimulates the proliferation of breast cancer cells emerged in 1998 from an *in vitro* study carried out by Descamps et al. [97]. Through the use of MCF-7 and MDAMB-231 cell lines for the study of invasive ductal and triple-negative breast cancers (TNBC), respectively, the authors showed that NGF not only induced cells in the G0 phase to reenter the cell cycle, but also reduces the duration of the cell cycle. The NGF-stimulated proliferation seems to happen in a concentration-dependent manner, at least in MCF-7 cells [98]. Moreover, Sakamoto et al. [99] reported a correlation between NGF and cell proliferation assessed by Ki67 index, in 71 specimens of invasive ductal carcinoma.

These effects are probably due to the activation of TrkA/MAPK cascade [100] as well as the NGF-induced phosphorylation of $P185^{HER2}$ [101], a kinase receptor of the HER family *per se* overexpressed in breast cancer and that stimulate the cell growth also through the MAPK pathway [102-104]. Com et al. [105] also reported a list of TrkA signaling partners in breast cancer cells such as IQGAP1m VCP and actin, by using proteomics on MCF-7 cell line. The authors speculated that IQGAP1 could also represent a scaffold protein in the TrkA/MAPK mitogenic pathway and that VCP and actin proteins, could be involved in the TrkA/PI3K/Akt pathway although other studies are needed to confirm this hypothesis. In addition, it has been shown that breast cancer cells overexpressing TrkA show increased tumorgenicity [78] and that also in TNBC cells NGF activated the TrkA receptors leading to the formation of the TrkA/β1-integrin/FAK/Src complex involved in mitogenesis [106]. Moreover, Bashir et al. [96] found an upregulation of NGFR/p75NTR in breast tumor tissue, breast cancer cell line MCF7 and isolated cancer stem cells (MCF7-CSCs) that induces the activation of NF-κB pathway to mediate cell proliferation after the binding to NGF.

5.4. Anti-apoptosis and survival of breast cancer cells

As mentioned previously, the binding of NGF to NGFR/p75NTR can activate the NF-kB signaling pathway for the regulation of cell survival [10]. The same pathway could be involved in the anti-apoptosis effect of NGF on breast cancer cells, as demonstrated by Descamps et al. [100] and Bashir et al. [96]. These data are in line with the results of Naderi et al. [107] and Chakravarthy et al. [17] on a subset of ER-positive breast cancer with

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overexpression of *BEX2* gene and in TNBC cells line, respectively. Conversely, Sakamoto et al. [99] found a correlation between the apoptotic index and the expression of NGFR/p75NTR in 71 specimens of human invasive ductal breast carcinoma.

According to Com et al. [105], also TrkA receptor may play a role in the NGF-induced anti-apoptotic cascade by involving the DNA repair protein Ku70. In particular, the authors showed that TrkA and Ku70 co-localize and interact upon NGF stimulation. This could lead to the TrkA-mediated tyrosine phosphorylation of Ku-70 and, thus, to MCF-7 breast cancer cells survival. Furthermore, Ku70 depletion induces a strong potentiation of apoptosis in TrkA-overexpressing cells. Thus, Ku70 could represent a promising therapeutic target to induce the selective apoptosis of breast cancer cells overexpressing TrkA, although further *in vitro* and *in vivo* studies are required to confirm this hypothesis. Last, but not least, Com et al. [105] showed that nucleophosmin, a protein involved in cell growth and proliferation, could be involved in the cytoprotective activities of NGF upon TrkA activation in breast cancer cells.

5.5. Angiogenesis of breast cancer

Tumor angiogenesis is the process by which new blood vessels are generated, starting from existing ones, to supply nutrients to the cancer [108]. The Vascular Endothelial Growth Factor (VEGF) is considered one of the most efficient proangiogenic factors in breast cancer [109, 110]. Interestingly, Romon et al. [111] demonstrated that NGF increased the secretion of VEGF in both endothelial and breast cancer cells. In addition, the authors collected both *in vitro* and *in vivo* data supporting the view that NGF stimulate *per se* the breast cancer angiogenesis through several TrkA downstream signaling pathways including PI3K and ERK, leading to the activation of MMP2 and nitric oxide synthase. Moreover, Lagadec et al. [95] also showed that the increased tumor angiogenesis of breast cancer cells was related to TrkA overexpression.

5.6. Breast cancer invasion and metastasis

Invasion and metastasis are extremely complex processes involving different classes of proteins, such as enzymes (extracellular proteases), glycoproteins (integrins), and immunoglobulins [112]. These processes cause biological changes in the functioning of the cells that begin from tumor invasion into blood and lymph vessels adjacent to the primary tumor site to metastatic colonization at other sites of the body [113].

NGF and its receptors TrkA and NGFR/p75NTR, due in part to their proliferative and anti-apoptotic activities, also appear involved in the invasion and metastasis of breast cancer cells. In 2001, Aragona et al. [93] observed a rapid metastatic spreading in NGFR/p75NTR-negative breast cancer patients suggesting, for the first time, the involvement of this NGF receptor in breast cancer natural history. In 2004, Davidson et al. [114] characterized the expression of NGF, NGFR/p75NTR and phospho-TrkA (p-TrkA activated receptor) during the progression of breast carcinoma from primary tumor to pleural effusion in sections from 42 malignant pleural effusions from breast cancer patients and 65 corresponding solid tumors (34 primary, 31 metastatic). From the results of this study emerged that NGF expression in effusions significantly predicted a shorter time to progression (TTP). In addition, the authors reported a downregulation and an upregulation of NGFR/p75NTR and TrkA, respectively, compared to primary breast tumors. The levels of TrkA were also upregulated in locoregional recurrences compared to early lymph node metastases [114]. NGF strongly increased invasion, cord formation and the monolayer permeability of endothelial cells [111] and metastasis of xenografted breast cancer cells in immunodeficient mice [95]. In particular, Lagadec et al. [95] reported bigger metastatic foci in the lungs, liver and brain of mice that received TrkA overexpressing cells. These effects are probably due to the overexpression of TrkA receptor and the consequent activation of the downstream PI3K/Akt and Ras/MAPK signaling pathways [78, 95, 111], as well as to the NGF-induced increased secretion of

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VEGF [111]. Trouvilliez et al. [115] demonstrated that the NGF stimulation also causes the binding of the v3 isoform of CD44 to the TrkA receptors leading to breast cancer cell tumor development and metastasis *in vivo*. Regarding triple-negative breast cancers, Di Donato et al. [106] found that the NGF-induced activation of TrkA receptor results in the formation of the TrkA/β1-integrin/FAK/Src complex, leading to cell migration and invasion and increased spheroid size in MDA-MB-231 and MDA-MB-453 cells line. In addition, a recent study in a population of TNBC cells reported an overexpression of NGFR/p75NTR and an interaction between NGFR/p75NTR and TrkC receptors that affects tumor growth and metastasis through the Trk MEK-ERK1-ZEB1 and PI3K-AKT signaling pathways [15]. Moreover, NGFR/p75NTR appears aslo to be involved in the proliferation and metastatization of invasive ductal carcinoma through the NF-kB pathway, as demonstrated on MCF-7 cell [96].

Beyond NGF, two studies [18, 19] demonstrated the involvement of pro-NGF signaling pathways in breast cancer invasion and metastasis. Demont et al. [18] reported the existence of an autocrine loop stimulated by the overexpression of pro-NGF and mediated by TrkA plus sortilin, with the activation of Akt and Src, that leads to the stimulation of breast cancer cell lymph node invasion in breast cancer cells. Interestingly, the authors also reported that the pro-NGF appears to have a greater invasive effect than mature NGF [18]. Moreover, Lévêque et al. [19] identified EphA2, a membrane receptor tyrosine kinase, as a key element of the pro-NGF signaling in breast cancer cells. In particular, the authors showed that the binding of proNGF to sortilin leads to the formation of a sortilin/TrkA/EphA2 complex that induces cell invasion.

5.7. NGF and its receptors as diagnostic markers for breast cancer

As mentioned above, several evidence indicated that NGF is synthesized and released by breast cancer cells but not from normal breast epithelial cells [18, 90, 91]. In particular, it has been reported a transcript, protein and immunological expression of NGF in the majority of human breast tumors [91], making it a broader diagnostic potential than ER or HER-2 [17]. Noteworthy, several studies have also shown the overexpression of TrkA and NGFR/p75NTR receptors in most of the human breast cancers as compared to the expression of these receptors in normal cells [94, 114]. Moreover, Islam et al. [14] demonstrated that Russell's Viper Venom (RVV)-NGFa (an NGF isoform), labelled with Fluorescein Isothiocyanate (FITC), establishes strong binding to TrkA and NGFR/p75NTR receptors in breast cancer cells but not in non-cancerous cells. This is a promising result for the future development of a tool using a fluorescent molecule-tagged RVV-NGFa binding technique to differentiate cancerous from non-cancerous cells and thus to diagnose breast cancer [14].

In addition to represent a potential new biomarker for the diagnosis of breast cancer *per se*, the analysis of NGF and its receptors could also be useful for making the differential diagnosis between various types of breast cancer. Tsang et al. [116] reported that NGFR/p75NTR could represent a potential marker for specific molecular subtypes of breast cancer through the comparison of its immunohistochemical expression in 602 specimens of luminal A (ER and/or PR+, HER2−, Ki67<14), luminal B (ER and/or PR+, HER2+ and/or Ki67≥14), HER2-overexpressed (HER2-OE) (ER−, PR−, HER2+), basal-like (ER−, PR−, HER2−,CK5/6+ and/or EGFR+) and unclassified subtypes (ER−, PR−, HER2−, CK5/6−, EGFR−). From this study emerged that the NGFR/p75NTR expression was positively correlated with basal markers, including Ki67, Cytokeratin (CK5/6), CK14, p63, c-kit and EGFR, but negatively with HR. Regarding the molecular subtypes, NGF was positively associated with luminal B and basal-like breast cancer, with a comparable or better specificity than other basal markers or ER, PR, HER2 and Ki67, respectively. NGF was also negatively associated with luminal A. In addition, the results of Wu et al. [15] provide the basis for the future better characterization and use of NGFR/p75NTR as a diagnostic marker for determining the metastatic potential of TNBC cells. Finally, the

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study of Kumar et al. [92] opens the prospect of using NGF as a biomarker to distinguish also benign tumors from each other, as breast phyllodes tumors overexpress NGF up to five times more than fibroadenomas tumors.

5.8. NGF and its receptors as prognostic markers for breast cancer

The prognostic value of NGF, TrkA and NGFR/p75NTR expression was evaluated in several studies [15-17, 19, 93, 94, 99, 114, 117].

Davidson et al. [114] posed NGF as first molecular marker able to predict the time interval to progression. Namely, the authors found a mean of time to progression of 6.3 and of 4 years for NGF-negative and -positive effusions, respectively. Noh et al. [16] analyzed the immunohistochemical expression of NGF in breast cancer tissues obtained from 145 women affected by invasive ductal carcinomas (n= 137) and invasive lobular carcinomas (n= 8). The level of NGF resulted significantly associated with heme oxygenase-1 (HO1) expression, histologic grade, HER2 expression, and latent distant metastasis. In addition, it predicted shorter overall survival and relapse-free survival. More specifically, the patients with tumors expressing NGF had the shortest survival whereas the NGF-/HO1-phenotype was associated with favorable prognosis. A recent study also provided the first evidence of the unfavorable prognostic value of the high expression of NGF in serum-derived exomes in a cohort of 129 patients mainly affected by invasive ductal carcinoma (96.9%) undergoing to neoadjuvant chemotherapy [117].

About NGF receptors, Descamps et al. [94] reported that the overall TrkA mRNA expression predicts a more favorable prognosis in a highly variable cohort of 363 primary breast carcinoma. However, as mentioned above, Davidson et al. [114] showed that the levels of p-TrkA were associated with tumor progression to effusion in metastatic breast carcinoma, thus correlating the dysregulation of p-TrkA with poor prognostic outcome in a more uniform cohort of 39 patients. Furthermore, the immunohistochemical expression of NGFR/p75NTR receptor was reported as a positive and negative prognostic factor for non-TNBC and TNBC, respectively. By analyzing the tissues of 46 patients affected by different histological types of breast cancer (i.e., infiltrating ductal not otherwise specified, infiltrating ductal comedo, lobular invasive, mucinous, medullary), the expression of NGFR/p75NTR was associated with a longer disease-free survival, in addition to ER positivity, small tumor dimension, low histologic grade (G1–G2), old age and menopause [93]. In the same way, by analyzing 71 specimens of invasive ductal carcinoma, Sakamoto et al. [99] found that immunohistochemical NGF-positive and NGFR/p75NTR-negative show a lower disease-free survival rates whereas the opposite pattern was associated with more favorable outcome. Wu et al. [15] found that NGFR/p75NTR was expressed at a high level in TNBC patients compared to non-TNBC patients and negatively correlated with the overall survival of TNBC patients. Indeed, it has been reported that the NGF-mediated upregulation of NGFR/p75NTR can contribute to chemoresistance of TNBC cells [17]. As a proof of this evidence, in a sub-type of ER-positive breast cancer with an overexpression of the *BEX2* gene and treated with tamoxifen a more favorable prognosis was reported since *BEX2* modulates the activation of NF-kB due to NGFR/p75NTR and enhances the antiproliferative effect of tamoxifen [107]. Lévêque et al. [19] demonstrated that high TrkA/EphA2 levels, a pro-NGF-induced complex, were associated with poor prognosis in breast cancer patients. Finally, Jung et al. [117] also reported an alternation of DNA copy number amplifications and mRNA upregulation of NGF that was correlated with a worse survival in the same cohort of patients manly affected by invasive ductal carcinoma and who underwent to neoadjuvant chemotherapy.

5.9. NGF signaling pathways as a therapeutic target for breast cancer

Preliminary *in vitro* and *in vivo* data indicated that NGF and its receptors could represent promising target for the treatment of breast cancer. In particular, Adriaenssens

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et al. [91] reported that both antibodies against NGF and or small interfering RNA (siRNA) against NGF induced a decrease in cell proliferation with a concomitant increase in apoptosis of breast cancer cells and an inhibition of tumor angiogenesis and metastasis. Dollè et al. [90] also reported that the use of antibodies against NGF lead to a reduction of the constitutive growth of breast cancer cell lines (i.e, MCF-7, MDA-MB-231, T47-D and BT20) in a dose-response manner. As mentioned above, Romon et al. [111] reported that NGF increased the secretion of VEGF in both endothelial and breast cancer cells. Interestingly, the Inhibition of VEGF with a neutralizing antibody reduced about half of NGF-induced endothelial cell invasion and angiogenesis *in vivo*. In addition, the treatment of TNBC cells with NGF-neutralizing antibody or NGF inhibitors (Ro 08-2750 and Y1086) reduced the NGF-induced increased levels of NGFR/p75NTR involved in anti-apoptosis [17].

Through the use of several cell lines, it has been shown that NGF-mediate proliferation of breast cancer cells could be reduced or inhibited by the TrkA phosphorylation inhibitor K252a [97, 98], the selective inhibitor of the MAPK cascade PD98059 [97], the antiestrogen drug tamoxifen [98], the TrkA inhibitor larotrectinib [78] and endocannabinoids [118]. In addition, some evidence indicated that K252a also inhibited growth [90,; 91], abolished invasion [111] and reduces metastasis [91, 95] of breast cancer. However, as mentioned above, Tagliabue et al. [101] found that TrkA cooperates with HER2 to activate breast cancer cell proliferation under NGF stimulation. Indeed, the TrkA phosphorylation inhibitor K252a did not affect the NGF-mediated activation of HER2, suggesting targeting also these receptors for the inhibition of breast cancer cell proliferation. Moreover, the use of other TrkA inhibitors, such as LY294002 and PD98059, lead to a complete abolition of NGF-induced invasion in MDA-MB-231 cell line [111] whereas GW441756, another TrkA inhibitor that blocks the formation of the TrkA/β1-integrin/FAK/Src complex, could reverse proliferation, migration and invasion in MDAMB-231 and MDA-MB-453 cells [106]. Trouvilliez et al. [115] tested the effects of the administration of the CD44v3 mimetic peptide 4 on the formation of the TrkA/CD44v3 complex finding that it could impair clonogenicity and invasion of breast cancer cells *in vitro* and tumor growth and metastasis in a mouse xenograft model [115]. Zhang et al. [119] demonstrated that the downregulation of TrkA receptors through siRNA in MCF-7 cell and tumor xenograft mice model inhibit the proliferation of cancer cells and arrested cell cycle at G0/G1 phase via inactivation of NF-κBp65. Moreover, TrkA siRNA increased the efficacy of the chemotherapeutic agent paclitaxel and decreased the incidence of lung metastasis in tumor xenografted mice. Moreover, Demont et al. [18] reported that the pharmacological inhibition of TrkA with K252a and siRNA and of sortilin with siRNA resulted in the abolition of proNGF-induced invasion and migration in different breast cancer cell lines [18]. Lévêque et al. [19] also found that the simultaneous inhibition of TrkA via lestaurtinib and siRNA and of EphA2 via siRNA reduced the breast tumor aggressiveness and, in particular, colony formation *in vitro*, primary tumor growth and metastatic dissemination towards the brain *in vivo* suggesting that the inhibition of these pathways may improve the therapeutic benefit in patients overexpressing TrkA, EphA2 and proNGF.

Beyond NGF and TrkA, other researchers targeted NGFR/p75NRT signaling pathways for the treatment of breast cancer. Descamps et al. [100] tested the use of the NF-kB pharmacological inhibitor SN50 in the MCF-7 cell line, finding a reduction of NGF antiapoptotic activity. Also, the transfection of MCF-7 cells with IkBm, another inhibitor of NF-kB reduced the anti-apoptotic effect of NGF. Bashir et al. [96] reported that the treatment of MCF-7 and MCF7-CSCs cells with thymoquinone downregulated mRNA expression of NGFR/p75NTR and its downstream target NF-κB1. Thymoquinone also altered the expression of target gene of NF-κB pathway, such as Sox2 and Nanog, involved in proliferation and survival of cancer cells and cancer stem cells. Chakravarthy et al. [17] demonstrated that the knock-down of NGFR/p75NTR using short hairpin RNA (shRNA) or small molecule inhibition of NGF-NGFR/p75NTR interaction (i.e., Ro 08-2750) sensitized TNBC cells to the apoptosis induced by the cytotoxic/genotoxic drugs used as adjuvant therapies in breast cancer treatment. As previously reported, Wu et al. [15]

showed that NGFR/p75NTR exerted its premetastatic effects by binding with TrkC mainly through a ligand-independent manner in TNBC cells. Interestingly, the use of shNGFR/p75NTR and shTrkC to silence the gene expression of the two receptors, reduced invasive capacity *in vivo* and sphere growth *in vitro*, respectively, and increased the sensitivity of TNBC cells to the anti-Trk drug entrectinib. As mentioned above, Naderi et al. [107] identified a novel subtype of ER-positive breast cancer characterized by overexpression of the *BEX2* gene that regulates the NGF-mediated inhibition (through NF-kB activation) of C2-induced apoptosis. Interestingly, *BEX2* modulates apoptosis of breast cancer cells in response to estradiol and tamoxifen. Furthermore, BEX2 overexpression enhances the antiproliferative effect of tamoxifen at pharmacologic dose suggesting that NGF/BEX2/NF-kB pathway is involved in modulating response to tamoxifen in primary tumors.

6. Conclusions

In conclusion, the evidence reported and discussed in this systematic review demonstrated the pivotal role of pro-NGF, NGF and their receptors expression in breast cancer development, proliferation, growth, angiogenesis, migration, invasion and metastasis. These characteristics make pro-NGF, NGF, TrkA and NGFR/p75NTR good candidates as diagnostic and prognostic tools and therapeutic targets for different types of breast cancer as indicated by another lines of research.

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