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Analyzing the impact of metronomic scheduling and dosing of 5-Fluorouracil and Vinorelbine in triple-negative breast cancer and endothelial cells

Surname: SCAGLIOTTI Name: ARIANNA

Registration number: 765715

Tutor: Prof. Marialuisa Lavitrano

Co-tutor: Dr. Maria Grazia Cerrito

Coordinator: Prof. Andrea Biondi

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Chapter 1

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Introduction

Breast cancer

Breast cancer is the most common type of cancer and the fourth leading cause of cancer-related death worldwide. The American Cancer Society estimates, every year, the number of new cancer cases and mortality in the United States. For female breast cancer it has predicted about 276,480 new cases in 2020 [1].

The risk factors starting the neoplastic transformation can be subdivided into two major groups identified as intrinsic and extrinsic factors. The first group includes age, sex, race, and genetic background, which promote the familial event of the cancer disease or benign tumors of the mammary gland [2]. The second group includes extrinsic factors due to lifestyle, diet, or prescriptions such as oral hormonal contraceptives or hormone replacement medicine, and their impact on the neoplastic process can be changed until a certain point [2].

The risk of developing female breast cancer increases with age, with a median of around 62 years, except for the triple-negative subtype, which occurs on average in 50-years-old-women [3]. The main mutations associated with a higher risk of breast cancer are localized in the tumor-suppressing genes BRCA1 and BRCA2 (Breast Cancer genes 1 and 2), that help to repair damaged DNA (DNA Repair Associated), increasing the percentage to develop breast cancer from 10 to 70% in 80 years older women [4]. Also, mutations in PALB2 (Partner and Localizer of BRCA2), TP53 (Tumor Protein 53), PTEN (Phosphatase and Tensin Homolog), STK11 (Serine/Threonine

Kinase 11), and CDH1 (Cadherin 1) have been indicated as breast cancer risk factors [5]. Others intrinsic risk factors are related to personal characteristics, such as race (Caucasian women tend to develop more likely breast cancer than African-American), breast and bone mineral density [6,7], endogenous postmenopausal hormone levels [8], menstrual cycles [9].

Among the extrinsic breast cancer risk factors, excess body weight [10], physical inactivity [11], excessive alcohol consumption [12] are relevant. The risk of developing breast cancer associated by these individual habits may be due to several factors, including the increase in circulating estrogen levels induced by diet or alcohol, or by exposure to mutagenic substances contained in alcohol [12,13]. Also tobacco use may increase the risk of breast cancer development [8], probably because of the presence in the breast fluid of carcinogenic substances contained in tobacco smoke, where they can promote tumor transformation of mammary epithelial cells [14].

Breast cancer is a complex disease due to high heterogeneity. This heterogeneity concerns both clinical characteristics (tumor size, lymph node involvement, histological grade), and the expression or non-expression of molecular biomarkers. For these reasons, breast cancer can be divided into many subtypes on the bases of different parameters.

Histological classification

Since the first breast tumors classification of the World Health Organization (WHO) in 1968, the breast cancer diagnosis is by

histology. Although the insights into the molecular and genetic settings, histology is still considered the basis of the breast tumors classification [15].

According to the histological classification, breast cancer is divided into in situ carcinoma, if limited to the epithelium of the ducts (in situ ductal carcinoma, DCIS) or the lobules that supply the ducts with milk (in situ lobular carcinoma, LCIS), and invasive (infiltrating) carcinoma, if the tumor has invaded the stroma [16]. Invasive breast carcinoma, with an incidence of about 81%, constitutes the majority of breast cancer cases [17]. Based on the size, shape, and arrangements of cancer cells, breast cancers can be divided into ductal carcinoma in situ, lobular carcinoma in situ, invasive ductal carcinoma, invasive lobular carcinoma, invasive tubular carcinoma, invasive medullary carcinoma, and invasive mucinous carcinoma (figure 1) [16,18].

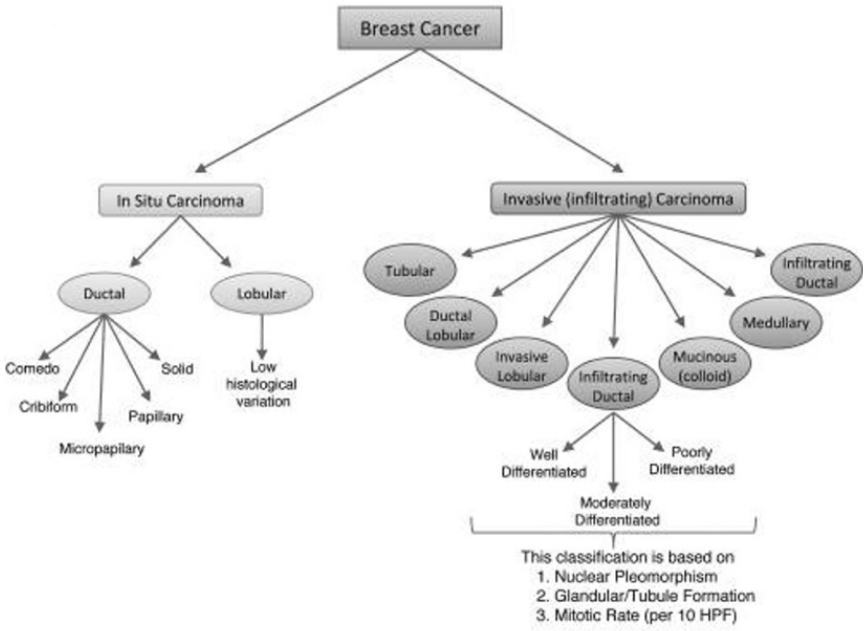

Figure 1. Histological classification of breast cancer [18]***.***

Ductal carcinoma in situ (DCIS). DCIS is characterized by cellular and nuclear atypia and the potential malignant capacity of the ducts' epithelial cells [16]. DCIS includes a group of tumors characterized by a heterogeneous architecture: the comedo, solid, cribriform, papillary, and micropapillary [16,18]. Based on the degree of nuclear atypia, intraluminal necrosis, mitotic activity, and calcification, DCIS can be classified in low-grade, intermediate-grade, and high-grade. DCIS is considered a precursor lesion of invasive breast carcinoma. The risk of developing invasive breast carcinoma is proportional to the grade of DCIS [16].

Lobular carcinoma in situ (LCIS). LCIS is characterized by a uniform population of round, small-to-medium-sized cells with normochromic nuclei. Pleomorphism, mitosis, and necrosis are rarely present in this subtype of breast cancer [16,19,20]. LCIS is predominantly multicentric (70% of cases), but it can also be bilateral (30-40%) [16]. LCIS has a high risk of becoming invasive lobular carcinoma [20].

Invasive ductal carcinoma (IDC). IDC represents the most common invasive subtype of breast cancer, with about 70-80% of incidence [18,21]. IDC has heterogeneous histology and can be sub-classified based on the levels of nuclear pleomorphism, glandular/tubule formation, and mitotic index in: well-differentiated, moderately differentiated, or poorly differentiated [16,18]. Approximately 70–80% of IDCs are hormone receptor (HR) positive, and the 15% express high levels of Human Epidermal growth factor Receptor 2 (HER2) [22]. IDC also has a

heterogeneous molecular profile; indeed, it falls into different molecular subtypes: luminal A, luminal B, HER2 positive, and triple-negative [22]. The prognosis of IDC is related to the tumor grade, clinical stage at diagnosis, and to its molecular profile [22].

Invasive lobular carcinoma (ILC). ILC incurs in about the 5%–15% of invasive breast carcinoma and usually affects older age group women [22]. ILCs are frequently multifocal and have a variable mitotic activity. ILCs are composed of small, uniform, epithelial cells with intracytoplasmic lumina and show a reduction of cell adhesion molecules; therefore, they are generally non-cohesive [22]. Molecular characteristics suggest that ILCs are frequently categorized as luminal A, even if a small group of tumors falls into luminal B, HER2- or basal-like [23].

Invasive tubular carcinoma (ITC). ITC accounts for approximately 2% of invasive breast cancer, and over 90% of ITC is associated with an excellent prognosis within 10-year survival rates [16,22]. ITC is histologically characterized by a proliferation of open tubules arranged in a desmoplastic stroma. The tubules are coated from a single layer of epithelial cells with mild nuclear pleomorphism and low mitotic activity [22].

Invasive medullary carcinoma. Invasive medullary carcinoma affects women about 50 years of age and is usually triple-negative [16]. It represents the main type of breast tumors in patients with BRCA1 germline mutations, even if only 13% of patients with medullary carcinoma have BRCA1 germline mutations [16,22]. Medullary carcinomas are generally well-

circumscribed and composed by cells with high-grade nuclei. There is a significant lymphocytic infiltrate [22].

Invasive mucinous carcinoma. Invasive mucinous carcinoma is a rare subtype of invasive breast cancer (2%) and usually is found in patients over 55 years old [22]. Histologically, this type of tumor has high extracellular mucin content in which nests or islands of neoplastic cells are floating. Tumors may be classified in hypocellular or type A when a significant mucin content characterized them compared to the cellular one, and hypercellular or type B, characterized by the predominance of the cellular component over the mucinous one [24]. These tumors are usually diagnosed early and are associated with a good prognosis with a 10-year survival rate of 80–100% [22].

Molecular classification

Despite the high molecular heterogeneity, it has been possible to classify breast cancer in molecular subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and other proliferation markers. In the clinical practice, the molecular classification provides useful information regarding prognosis, risk of relapse, and therapy response. According to the molecular profile, breast cancer can be classified in luminal A, luminal B, basal-like, HER2-enriched, normal-like, and claudin-low (figure 2).

Luminal A (HR+/HER2-). The luminal A breast cancer is the most frequent subtype of breast cancer (~40%), and it is

associated with a better prognosis than other subtypes because of the high response to hormone therapy [18,25,26].

Luminal B (HR+/HER2+). Together with luminal A, this subtype of breast cancer has a gene expression profile that looks like the breast's normal luminal epithelial cells. Luminal B breast cancers have a higher grade and the worst prognosis than Luminal A, due to the increased expression of proliferation markers HER2 and Ki67 [25,26].

Basal-like (HR-/HER2-). This breast cancer subtype is also called triple-negative breast cancer because of the lack of ER, PR, and HER2 expression. Basal-like is a high heterogeneous subtype representing about 15% of breast cancer and occurs in young women. The basal-like subtype is characterized by a poor prognosis and an increased risk of relapses within five years after diagnosis [18,26,27].

HER2-enriched (HR-/HER2+). This breast cancer subtype does not express hormone receptors but is characterized by HER2 overexpression. Like the basal-like, HER2-enriched breast tumors have a poor prognosis and a high risk of distant recurrences. However, these tumors are responsive to anthracycline and taxane-based chemotherapy [18,26,28].

Normal-like. The normal-like is a rare subtype of breast tumor (only 5-10% of cases) and is also poorly characterized. This subtype of cancer expresses genes related to adipose tissue and often does not express HR and HER2; therefore, most of these tumors are triple-negative, like the basal-like subtype. Normal-

like cancers have an intermediate prognosis between the luminal and the basal-like subtype and generally do not respond to chemotherapy [18,29].

Claudin low. This breast cancer subtype is characterized by the low or absent expression of luminal differentiation markers, the high expression of EMT-associated genes, and immune-response genes. Due to the low expression of proliferation genes, the claudin-low subtype is associated with cancer stem cells' phenotype acquisition. Most of the claudin-low breast cancers are triple-negative, have a poor prognosis, and are unresponsive to chemotherapy [18,29,30].

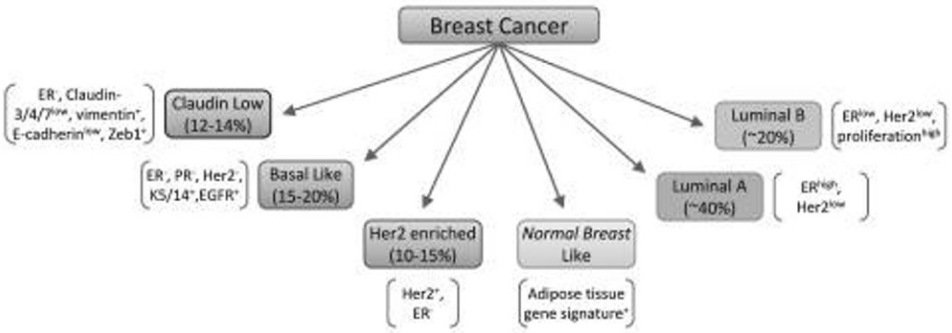

Figure 2. Molecular classification of breast cancer [18]***.***

TNM classification

The cancer stage classification is crucial in defining appropriate treatment based on previous patients' knowledge and results with the related stage. The most used staging system in clinical is the Tumor, Node, and Metastasis (TNM) staging system. The American Joint Committee on Cancer (AJCC) defines five different breast cancer staging (0, I, II, III, and IV) using the TNM

classification, which is based on the tumor size (T), the status of regional lymph nodes (N) and distant metastases (M) information (figure 3).

			ER+, PR+, HER2+	ER+, PR+, HER2-	ER+/PR-, HER2+	ER-/PR+, HER2+	ER-, PR-, HER2+	ER+, PR-, HER2-	ER-, PR+, HER2-	ER-, PR-, HER2-	Anatomic stage
TisN0	M0	G1-3	0	0	0	0	0	0	0	0	0
T1N0 T0N1mi T1N1mi		G1	IA	IA	IA	IA	IA	IA	IA	IA	IA
		G2	IA	IA	IA	IA	IA	IA	IA	IB	IA
		G3	IA	IA	IA	IA	IA	IA	IA	IB	IA
T0N1 T1N1 T2N0		G1	IA	IA	IB	IB	IIA	IB	IB	IIA	IIA
		G2	IA	IA	IB	IB	IIA	IIA	IIA	IIA	IIA
		G3	IA	IB	IIA	IIA	IIA	IIA	IIA	IIA	IIA
T2N1 T3N0		G1	IA	IA	IIB	IIB	IIB	IIB	IIB	IIB	IIB
		G2	IB	IB	IIB	IIB	IIB	IIB	IIB	IIB	IIB
		G3	IB	IIA	IIB	IIB	IIB	IIB	IIB	IIIA	IIB
T0N2 T1N2 T2N2 T3N1 T3N2		G1	IB	IB	IIIA	IIIA	IIIA	IIIA	IIIA	IIIA	IIIA
		G2	IB	IB	IIIA	IIIA	IIIA	IIIA	IIIA	IIIB	IIIA
		G3	IIA	IIB	IIIA	IIIA	IIIA	IIIA	IIIA	IIIC	IIIA
T4N0 T4N1 T4N2 AnyN3		G1	IIIA	IIIA	IIIB	IIIB	IIIB	IIIB	IIIB	IIIB	IIIB
		G2	IIIA	IIIA	IIIB	IIIB	IIIB	IIIB	IIIB	IIIC	IIIB
		G3	IIIB	IIIB	IIIB	IIIB	IIIB	IIIC	IIIC	IIIC	IIIB
Any	M1	Any	IV	IV	IV	IV	IV	IV	IV	IV	IV

Figure 3. Pathologic prognostic stages defined committee in the 8th edition by AJCC. *ER, PR, and HER2 status are incorporated with TNM (Tumor-Node-Metastasis) classification to define the stage of breast cancer. T= Tumor; N= Node; M= Metastasis; G= Grade; Tis= Tumor in situ* [31].

The T grade is assigned according to the invasive tumor's size or the most massive invasive tumor if multiple tumors are present. The N grade is defined by the extension of regional lymph node involvement and currently represents the most potent prognosis indicator. The M parameter may have grade 1 or 0 based on the presence or absence of distant metastasis. Tumors with M1 are automatically assigned to the IV stage, independently from T and N grade [22,31]. The tumor stage also considers the tumor grade (G), which reflects the tumor differentiation and is determined by the microscopic evaluation of three parameters: mitotic activity, tubule/gland formation, and nuclear pleomorphism. Tumor, based on the score assigned to

each parameter, can be well-differentiated (G1), moderately differentiated (G2), or poorly differentiated (G3) [22,31]. In the 8th edition of AJCC, expression levels of ER, PR, and HER2 were included at the TNM grade to better define the prognostic staging and improve the probability of a positive response to chemotherapy [25,31,32].

Diagnosis

Breast cancer can be diagnosed by monitoring symptoms or a palpable mass of patients or with imaging techniques. Mammography is the standard technique for the detection of breast cancer [33]. This technique has high sensitivity and specificity, is inexpensive and well-tolerated, even if it is frequently associated with the feeling of anxiety and pain and exposes patients to radiation. False-positives are also frequent with mammography [34]. Ultrasounds are another fundamental imaging technique for breast cancer detection that do not use ionizing radiation and have high sensitivity [34]. Magnetic resonance imaging (MRI), Positron Emission Tomography (PET), and Single-photon emission computed tomography (SPECT) are other imaging techniques that could be used for diagnosing breast cancer [34].

Therapeutic strategies

Surgery. Surgery aims to eradicate cancer and, to date, remains the first choice for local and regional breast cancer treatment [35]. Surgical treatment involves mastectomy, consisting of the surgical removal of the entire breast, or breast-conserving

surgery (BCS), also known as partial mastectomy or lumpectomy. It consists of removing only cancerous tissue and the normal tissue on the tumor margin [35,36]. To be sure that cancer has not spread, one or a few regional lymph nodes are removed from the armpit during BCS and mastectomy with a surgical operation called axillary node dissection [36]. In most cases, mastectomy and BCS are followed by radiotherapy or chemotherapy.

Radiotherapy. Radiation is a locoregional therapy often used after surgery to eliminate the remaining tumor cells. It has been demonstrated that after BCS, radiation therapy reduces the risk of local recurrence by about 50% at 10 years and 20% of death at 15 years after treatment [37]. Radiotherapy may be administered as external or internal radiation therapy (brachytherapy) or as a combination of both [38].

Chemotherapy. Chemotherapy is a systemic therapy that can be used as neoadjuvant therapy if administered before surgery to reduce tumor size, or adjuvant therapy if administered after surgery to destroy the undetectable cells [39]. There is no single optimal chemotherapy regimen for breast cancer due to the high heterogeneity of this tumor. Therefore, the choice of chemotherapy regimen depends on the tumor's characteristics, as HR and HER2 status, stage, grade, and lymphovascular involvement [35]. The toxic effects of chemotherapy are high; of particularly concern are nausea and vomiting, myelosuppression, fatigue, hair loss, mucositis, and neuropathy. Due to the severe toxicity sometimes related to chemotherapy,

the patient's age and life expectancy should be considered when chemotherapy regimens are proposed [35].

Endocrine therapy. The binding of estrogens, including estrone, estradiol, and estriol, to estrogen receptors, leads to the ER's dimerization and the activation of the downstream signaling pathways that promote estrogen-regulated genes transcription. About 80% of breast cancers express hormone receptors (HR) and can be treated with estrogen inhibitors [40]. Among ER-blocking drugs, there is Tamoxifen, which competitively inhibits estrogen binding to the estrogen receptor and Aromatase inhibitors (Anastrozole, Exemestane, and Letrozole), which inhibit the conversion of androgens to estrogen, decreasing circulating estrogen levels [41,42]. Tamoxifen has shown strong efficacy in pre- and postmenopausal women, while Aromatase inhibitors are effective only in postmenopausal women [41] due to physiological reasons: in premenopausal women, estrogens are mainly produced by ovaries, while in postmenopausal women, aromatase converts androgens released by the adrenal glands into estrogen [42].

Targeted therapy. HER2 is a tyrosine kinase receptor generally expressed at a low level on the surface of epithelial cells. High levels of HER2 constitutive stimulate HER2 phosphorylation, which leads to activation of downstream signaling pathways that promote cell proliferation, apoptosis inhibition, angiogenesis, and metastasis development [43]. Trastuzumab is a monoclonal antibody that efficiently blocks HER2 receptors. Treatment with Trastuzumab and chemotherapy has shown an improvement in

patients' survival with early-stage HER2-positive breast cancer. Due to its safety and efficacy profile, Trastuzumab is proposed, both in mono- or combined therapy, as a first-line option to treat early and advanced HER2-positive BC [43].

Cyclin-Dependent kinases (CDKs) are serine/threonine kinase enzymes whose activity is regulated by interaction with cyclins and CDK inhibitors. CDKs control cell cycle progression by responding to external stimuli (i.e., nutrients) and internal signals (i.e., DNA damage). Consequently, the inhibition of specific CDKs represents an attractive therapeutic target in cancer. For instance, in breast cancer, CDK4/6 inhibitors such as Palbociclib, Ribociclib, and Abemaciclib prevent cancer cell proliferation-inducing cell cycle arrest in the G1 phase [44]. These oral selective, reversible inhibitors have been approved by the Food and Drug Administration (FDA) to treat HR+ metastatic breast cancer combined with specific endocrine therapies [45–48]. However, the primary and acquired resistance limits CDK4/6 inhibitors therapy. Several preclinical data showed the pre-existence or inducted resistance mechanism in the breast cancer cell lines: loss of retinoblastoma (RB), hyperphosphorylation of PDK1, or hyperactivation of cyclin A/CDK2 or cyclin E/CDK2, has been correlated with the development of resistance state to CDK4/6 [44].

BRCA1 and BRCA2 mutations incur in about 10% of all breast cancers and about 30% of hereditary breast cancers [49]. BRCA1 and BRCA2 are essential proteins of the homologous recombination repair (HRR) system that resolve DNA double-

strand breaks. Loss of BRCA1 and BRCA2 activity leads to genome instability and a high risk of tumor development [50]. BRCA1 mutations are associated with TNBC tumors, while BRCA2 mutated expression is found overall in ER+ or PR+ breast cancers [50]. BRCA1 and BRCA2 deficient tumors strongly depend on PARP (poly ADP-ribose polymerase) activity to resolve DNA breaks because of the impairment of the homologous recombination mechanisms in this group of patients. The accumulation of double-strand breaks and genomic instability induced by PARP inhibition in BRCA-deficient cells leads to cell cycle arrest and apoptosis [51]. Olaparib, Rucaparib, and Niraparib are PARP inhibitors approved by the FDA to treat ovarian cancer [50]. Rucaparib and Veliparib are currently in clinical development for breast cancer that is BRCA-mutated. At the same time, the FDA recently approved Olaparib for use in BRCA mutated HER2-negative metastatic breast cancer previously treated with chemotherapy [50].

Anti-angiogenic therapy.

Angiogenesis plays a crucial role in the development of breast cancer and metastasis progression [50]. High levels of angiogenesis growth factors correlate with reduced survival of breast cancer patients. Preventing the outset of angiogenesis and its progression could provide effective treatments for breast cancer patients [52]. Anti-VEGF (Vascular Endothelial Growth Factor) and anti-VEGFR (VEGF Receptor) therapeutic antibodies, such as Bevacizumab and Ramucirumab, have been developed in order to inhibit tumor angiogenesis. Bevacizumab

is a humanized monoclonal antibody directed against VEGF-A. After a first clinical study in which Bevacizumab has shown a significant increase of PFS (11.8 *vs.* 5.9), in 2008, FDA approved Bevacizumab for the treatment of HER-2 negative breast cancers [53]. Subsequent studies demonstrated the high toxic effect of this drug, which encouraged the FDA to withdraw Bevacizumab's approval for metastatic HER2-negative breast cancer [53,54]. Bevacizumab is still approved by EMA (Medicine European Agency) to treat this breast cancer subtype in combination with Capecitabine or Paclitaxel [53].

Another anti-angiogenic strategy is the use of tyrosine kinase inhibitors (TKI), through which Sunitinib, Sorafenib, and Axitinib, that bind and inactivate receptors involved in pro-angiogenic signaling pathways, such as VEGFR2 or PDGF (platelet-derived growth factor) [53,55]. Although these agents have both an anti-angiogenic and antitumorigenic activity, the effect of anti-angiogenic monotherapy appears to be limited, and their association with other chemotherapy drugs strongly increases toxicity [53].

Immunotherapy. Immunotherapy stimulates the immune system response against tumors and is an emerging therapeutic area for breast cancer. Immune checkpoints are a regulatory machine that controls T-cells activation preventing an excessive immune response. In tumors, immune checkpoints' enhancement stops the tumor's immune response, favoring tumor evading [56]. Immune checkpoint inhibitors, such as PD1 (Programmed Cell Death 1) and PDL-1 (Programmed Cell

Death-Ligand 1) inhibitors, are used with chemotherapy to treat some breast cancers, in particular the triple-negative subtype [35]. Another potential type of immunotherapy is represented by breast cancer vaccines, which help treat and prevent breast cancer. Currently, clinical trials of phase I using DNA vaccines for HER2 are ongoing [35].

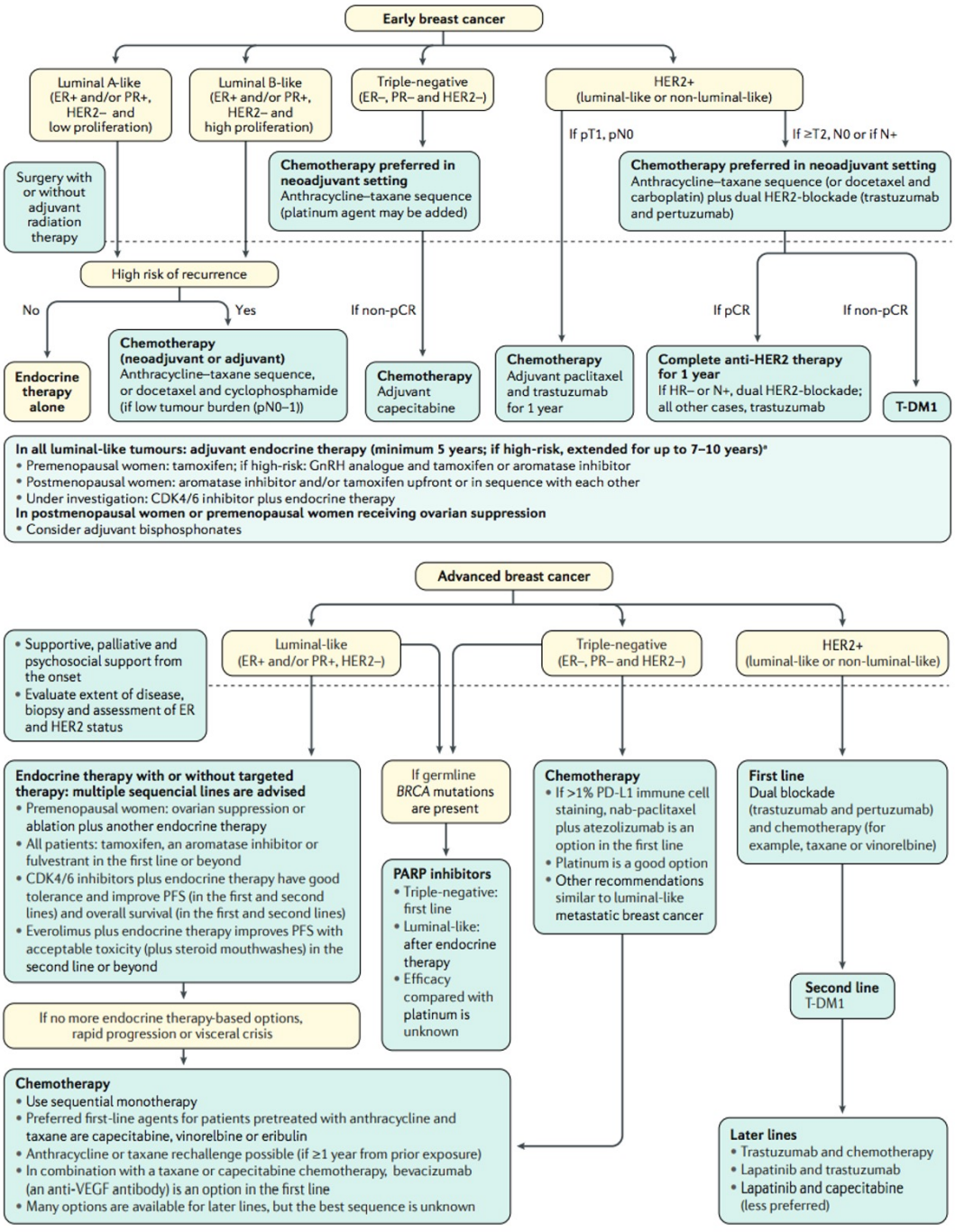

Figure 4. Algorithm for the treatment of early and advanced breast cancer*. Different therapeutic strategies are proposed based on the disease's progression and the tumor's molecular characteristics* [5].

Triple-negative breast cancer

Triple-negative breast cancer (TNBC), defined by the lack of ER, PR, and HER2 amplification, affects young females under the age of 50, with an incidence of about 10-20% [57]. The risks of developing TNBC are principally related to lifestyle and genetics. Young age and ethnicity are the most critical risk factors of TNBC. Several studies have demonstrated the prevalence of the triple-negative subtype, compared to other breast cancer subtypes in African-American and African women under 50 related to white women [58–61]. Late pregnancies increase the risk of developing TNBC, while breastfeeding has a protective effect, probably due to non-hormonal mechanisms [62]. Differently from other breast cancer subtypes, early age of menarche is not associated with an increased risk of TNBC [62]. Obesity increases the risk of developing TNBC because of the high levels of circulating insulin [63]. Insulin resistance is also associated with a greater risk of TNBC by increasing free estrogen and androgen levels that promote breast epithelium proliferation. Women with insulin resistance produce elevated insulin levels that activate Akt/mTOR, and the activation of Akt/mTOR is associated with a more aggressive TNBC [63]. Family history is another TNBC risk factor: mutations in BRCA genes are responsible for developing hereditary ovarian and breast cancers, of which approximately 75% presents a triple-negative phenotype [64,65]. Among the TNBCs, 10-42% are BRCA-mutated [62].

The poor tumor differentiation, the absence of symptoms and the unavailability of specific and sensitive screening tests contribute to the tardive diagnosis of TNBC [66,67]. When diagnosed, patients usually present unfavorable histopathologic features with high grade, large tumor size, and lymph node positivity [68]. Triple-negative breast cancer is an aggressive tumor with an increased tendency to metastasize, especially in the lungs, brain, and bone. Distant recurrences often occur within about three years after being diagnosed, and the mortality within 5-years after diagnosis is significantly higher in TNBC compared to other breast cancer subtypes [69].

From a histopathological point of view, TNBC is characterized by a high nuclear grade, increased mitotic activity, a high nuclear-cytoplasmic ratio, and an elevated tumor proliferation rate [70]. The majority of triple-negative breast cancers are unifocal invasive ductal carcinomas (90%), and a minority is classified as medullary, secretory, apocrine, metaplastic, and invasive lobular carcinomas [57]. Except for the absence of hormone receptors and HER2, the molecular profile of TNBC is high heterogeneous: the 75% of TNBCs cluster in the basal-like subtype, while there is a 25% that belongs to the normal-like and claudin-low breast cancer subtypes [71–73]. Thanks to the development of gene expression technologies, it has been possible to better characterize cancer's molecular profile to determine breast cancer patients' clinical outcomes. Different stratifications of TNBC have been made based on the gene expression profile and the epigenetic modifications (figure 5).

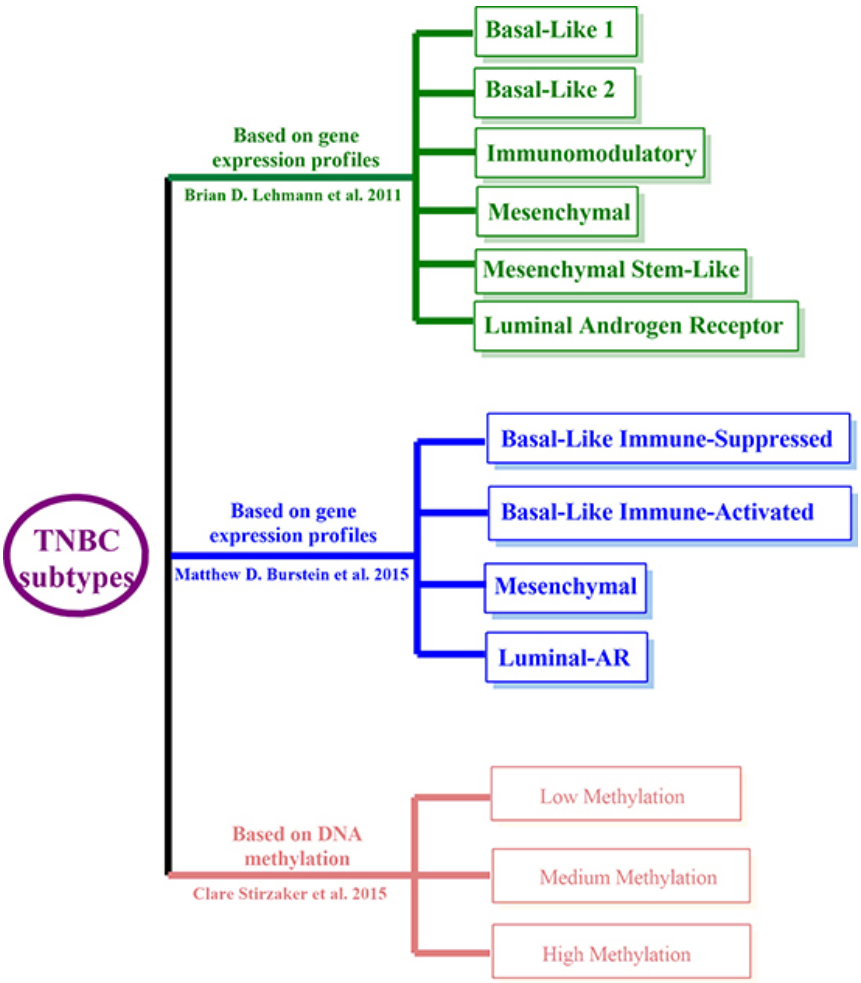

Figure 5. TNBC classifications. *Gene expression profile-based classification drawn up by Brian is reported in green, and Matthew's one is reported in blue. Clare's DNA methylation-based subtyping is written in red* [74].

Molecular classification of TNBC

Based on the gene expression profile, Lehman et al. subdivided triple-negative breast cancers into basal-like, immunomodulatory, mesenchymal, and luminal androgen receptor subtypes (figure 5) [75]. More recently, Burstein et al. reexamined the TNBC classification, identifying four TNBC subtypes: basal-like immune-suppressed, basal-like immune-activated, mesenchymal, and luminal androgen receptor [76].

Basal-Like Subtypes (BL). BL represents the most frequent subtype of TNBC (~80%). Lehman et al. distinguish the BL-1 subgroup, which is characterized by high expression of cell cycle checkpoint and DNA damage response genes (ATR/BRCA), and the BL-2 subgroup, which is described by high expression of genes involved in metabolic (glycolysis and gluconeogenesis) and in growth factor pathways (EGF, NGF, MET, Wnt/β-catenin and IGF1R). Instead, Burstein et al. identified two basal-like subtypes based on the downregulation (basal-like immune-suppressed, BLIS) or upregulation (basal-like immune-activated, BLIA) of genes involved in pathways that regulate immune system cells [74,77,78].

Immunomodulatory Subtype (IL). IL is similar to the BLIA type defined by Burstein, and it is characterized by the strong activation of cytokine, antigen processing presentation, and immune signaling pathways. Moreover, IL-TNBC also strongly expresses STAT pathway-related genes [74,77,78]. Based on the different grades of tumor infiltrating lymphocytes (TIL), TNBC can also be distinguished in immune “hot” (high-TIL) and “cold”

(low-TIL) tumors, which correlate with a different response to immunotherapy [79].

Mesenchymal Subtypes. Mesenchymal (M) and Mesenchymal stem-like (MSL) are characterized by the expression of genes involved in the epithelial to mesenchymal transition (EMT) and the maintenance of cancer stem cells (CSCs). These subtypes strongly depend on the expression of genes related to cell motility, extracellular receptor interaction, and cell differentiation pathways, such as Notch and WNT/β-catenin signaling pathways. Mesenchymal subtypes are often resistant to cytotoxic drugs due to the overexpression of ABC transporter and antiapoptotic proteins [74,77,78].

Luminal Androgen Receptor Subtype (LAR). LAR is characterized by the overexpression of androgen receptor (AR) and its pathway [74]. LAR is also enriched in hormonally regulated pathways, including steroid synthesis, porphyrin metabolism, and androgen/estrogen metabolism [75]. LAR subtype has a favorable prognosis since it often correlates with a low clinical-stage, low histologic grade, and low mitotic score [80]. TNBCs dependent on AR overexpression are inherently resistant to chemotherapy but have shown good response to AR-inhibitors in clinical trials [81].

Epigenetic classification of TNBC

Genetic alterations and epigenetic modifications in tumor cells introduce DNA sequences modifications, changing their gene expression profile. Through the most characterized epigenetic

modifications, gene promotors methylation is accepted as a marker of epigenetic silencing. According to the different methylation grades, Stirzaker et al. divided TNBC into three subtypes (figure 5): low, medium, and high methylation [82]. The low-methylation subtype has a better survival within 5-years post-diagnosis than the other two, while the medium methylation cluster is associated with the worst survival [83].

Post-translational modification of histone proteins, chromatin modifications, nucleosome positioning, chromosomal looping, and noncoding RNAs (ncRNAs) are other epigenetic modifications associated with tumorigenesis differentially expressed between non-TNBC and TNBC as well as within the triple-negative subtype [83]. Therefore, other TNBC subtypes have been identified based on these epigenetic modifications [83].

Therapeutic strategies for TNBC

Targeted therapy. Despite the good efficacy in breast cancers, hormone or Trastuzumab-based therapies cannot be used in TNBCs due to the lack of ER, PR, and HER2. The discovery of new molecular targets in TNBC has allowed testing of some targeted agents in this breast cancer [57,84].

Among the 10-50% of TNBCs expressed high levels of the Androgen Receptor (AR) [85]. The binding of androgenic hormones, including dihydrotestosterone or testosterone to AR leads its translocation from the cytoplasm to the nucleus, regulating the transcription of genes related to cell proliferation

and apoptosis [85]. Enzalutamide is an AR inhibitor approved for prostate cancer that binds the ligand-binding domain of AR and inhibits its translocation to the nucleus, the recruitment of cofactors, and the AR binding to DNA [86]. A recent phase II clinical trial has highlighted the promising efficacy of Enzalutamide in AR-positive TNBC, with a median PFS of 14.7 weeks and only grade 3 or higher adverse events [87]. The AR inhibitor Bicalutamide, is also in a clinical trial for the LAR subtype. Bicalutamide has shown a progression-free survival of 12 weeks with no grade 4/5 treatment-related adverse events [88].

About 15% of TNBCs express BRCA1 mutations [89]. As previously reported, several PARP inhibitors, thorough which Velaparib and Rucaparib, are currently in clinical trials for BRCA-deficient TNBCs [50,90]. Although BRCA-mutated breast tumors shown a good sensitivity, TNBCs mutated in BRCA1, and BRCA2 did not respond as expected [91,92]. New predictive biomarkers should be explored to improve the outcome of treatment with PARP inhibitors in TNBC.

EGFR (Epidermal Growth Factor Receptor) is a tyrosine kinase receptor involved in cell proliferation, migration, and survival. Even though 70% of TNBCs overexpress EGFR1, the monoclonal antibody anti-EFGR Cetuximab has shown a modest or clinical non-significant activity in TNBC patients [84]. On the contrary, the EGFR-targeted TKIs, including Erlotinib and Lapatinib, have good antitumor activity in TNBC, although in a restricted subgroup [93]. The major limitation of the EGFR

inhibitors is represented by the constitutive activation of the EGFR downstream pathway, often sustained by KRAS (Kirsten Rat Sarcoma Viral Oncogene Homolog) mutation/amplification or CRYB AB expression [94,95].

PI3K/AKT/mTOR pathway is one of the most important signaling pathways that control cell survival and proliferation. PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha) mutations are frequent in MSL and AR+ TNBCs, with an incidence of 23% and 40%, respectively [96]. Several PI3K (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase) inhibitors, through which Buparlisib (BKM120), Pictilisib, Alpelisib, and Serabelisib, are currently in early-phase clinical trials but have shown promising activity in this breast cancer subtype [97].

Several AKT (AKT Serine/Threonine Kinase) inhibitors have been investigated in patients with triple-negative breast cancer. The Phase I/III trial of the AKT inhibitor MK2206 in TNBC has been suspended due to the manifestation of hyperglycemia, pruritus, fever, rash, and mucositis despite the reduction of dose twice [98]. In a randomized phase II trial (LOTUS), Ipatasertib, in combination with Paclitaxel, has shown a significant increase in the progression-free survival (PFS) in TNBC patients compared to the placebo (6.2 *vs*. 4.9 months) [99].

Mutations in mTOR (Mammalian Target Of Rapamycin) incur in about 72% of TNBCs and limit the treatment with PI3K and AKT inhibitors [97]. Two classes of mTOR inhibitor agents have been developed: rapamycin analogs, also Rapalogs, and TORC1/2

kinase inhibitors. Several ongoing clinical trials have compared the mTOR kinase inhibitor Everolimus, given in monotherapy versus the combined use of it with other chemotherapy agents, such as Paclitaxel or Cisplatin [100,101]. The addition of Everolimus to neoadjuvant chemotherapy with Paclitaxel is well tolerated and showed an improvement of the RR (Relative Risk) at 12 weeks [100]. Moreover, the treatment with Everolimus seems to sensitize breast cancers to DNA damaging agents, including Cisplatin [101].

The use of PI3K/AKT/mTOR inhibitors in TNBC is limited by multiple factors like mutations in TP53, MYC, and RB or alteration in the WNT/β-catenin signaling pathway [102].

Src is a non-receptor tyrosine kinase protein involved in cell proliferation, survival, and invasion. TNBC, especially the mesenchymal and the mesenchymal stem-like subtypes, expresses higher Src levels than other breast cancer tumors [103]. However, in the phase II clinical trial, the Src inhibitor Dasatinib has shown limited efficacy as a single agent in patients with TNBC [104]. Only a subpopulation of TNBC patients has been found to respond to this inhibitor, for this breast cancer subtype's high heterogeneity [104].

Immunotherapy. Immune checkpoint inhibitors have shown a good response in those TNBCs that express high levels of tumor-infiltrating lymphocytes, such as the immunomodulatory and the basal-like subtypes. PD-1 and PD-L1 inhibitors, such as Pembrolizumab, Nivolumab, Durvalumab, and Atezolizumab, are currently in clinical trial for metastatic' TNBCs as

monotherapy or in combination with other chemotherapy agents, such as Capecitabine [77,79,105]. Despite the encouraging results, their use is restricted to tumors that express intratumoral PD-1 or PDL-1, about 20% of TNBCs [106].

Anti-angiogenic therapy. The anti-angiogenic therapy, based on VEGF inhibition, has improved the clinical outcomes in many types of cancer. Although VEGF's high intratumoral expression in many patients with TNBC [52], anti-angiogenic therapy has shown only small clinical benefits in these patients, increasing toxicity. Treatment of TNBC patients with the anti-VEGF antibody Bevacizumab has improved the ORR (42% *vs.* 23%) and the PFS (8.1 *vs.* 5.4 months; hazard ratio 0.63; $p < 0.0001$) but did not have an impact on the OS (18.9 *vs.* 17.5 months; hazard ratio 0.96; ns) [107]. The BEATRICE trial, a large adjuvant phase III trial with 2,591 TNBC patients, showed no statistically significant effect in DFS or OS in adding Bevacizumab to chemotherapy in the adjuvant setting for patients with TNBC [108]. The VEGFR tyrosine kinase inhibitor Sorafenib improves the outcomes of TNBC patients when associated with Capecitabine, increasing the median PFS of two months (2.5 *vs.* 4.3 months; HR = 0.596 (95% CI 0.3–1.1) [109]. However, Sorafenib's addition to chemotherapy is associated with higher rates of grade 3/4 toxicities [109].

Chemotherapy. Despite the targeted agents having a promising efficacy with controlled toxicity in some TNBCs, a high molecular heterogeneity strongly limits their efficacy in this tumor' subtype. Therefore, election therapy for triple-negative breast cancer

remains classical chemotherapy with anthracyclines, platinum agents, antimetabolites, taxanes, or vinca alkaloids (figure 6) [110].

Anthracyclines are DNA intercalating agents that promote cell death by destabilizing DNA and inhibiting DNA topoisomerases I and II [111]. Anthracyclines, such as Doxorubicin and Epirubicin, have shown promising efficacy in TNBC treatment [112]. However, p53-mutated TNBCs have been demonstrated to be resistant to anthracyclines chemotherapy [110].

Platinum agents make intra-strand and inter-strand double-stranded DNA crosslinks, avoiding the formation of the replication fork and generating double-strand breaks and replication lesions. Because of the inability to repair DNA damages, BRCA-mutated TNBCs show good sensitivity to alkylating agents such as Cisplatin and Carboplatin [113].

Antimetabolites, such as Methotrexate, Capecitabine, and 5-Fluorouracil, interfere with DNA synthesis by incorporating chemically altered nucleotides or blocking the availability of deoxynucleotides [114].

The antimetabolite, 5-Fluorouracil (5-FU), is a pyrimidine analog that blocks the uridine conversion into thymidine by making an irreversible aggregate with thymidylate synthase [101]. Consequently, there is an insufficiency of thymidine for DNA synthesis which leads to cell death [115].

5-FU can also be converted into fluorouracil-triphosphate (FUTP), incorporated into RNA in place of uridine, thereby

inhibiting RNA processing, interfering with cell metabolism and viability. Moreover, 5-FU can be incorporated into DNA when converted into fluorodeoxyuridine triphosphate (dFUTP) [115].

Taxanes, such as Docetaxel and Paclitaxel, are a group of tubulin polymerizes that inhibit cell proliferation by preventing disassembly of microtubules and the mitotic spindle. Taxanes have demonstrated a higher efficacy in TNBC compared to receptor-positive breast cancers [112].

Vinca alkaloids have a mechanism of action opposite to taxanes: they inhibit cell proliferation and avoid microtubule and mitotic spindle polymerization [114]. Vinorelbine, Vinblastine, and Vincristine are the principal representatives of this antitumoral class of agents. Vinorelbine, differently from the other vinca alkaloids, blocks cell proliferation through several mechanisms: inhibition of mitosis by interacting with tubulin, interfere with amino acid, cyclic AMP, and glutathione metabolism, and alter calmodulin-dependent Ca^{++}-transport ATPase activity, cellular respiration, and nucleic acid and lipid biosynthesis [116].

Although these agents have shown promising initial efficacy in TNBC patients, this type of systemic chemotherapy has shown severe side effects, inadequate long-term response, and overall do not completely eradicate tumor [39,57].

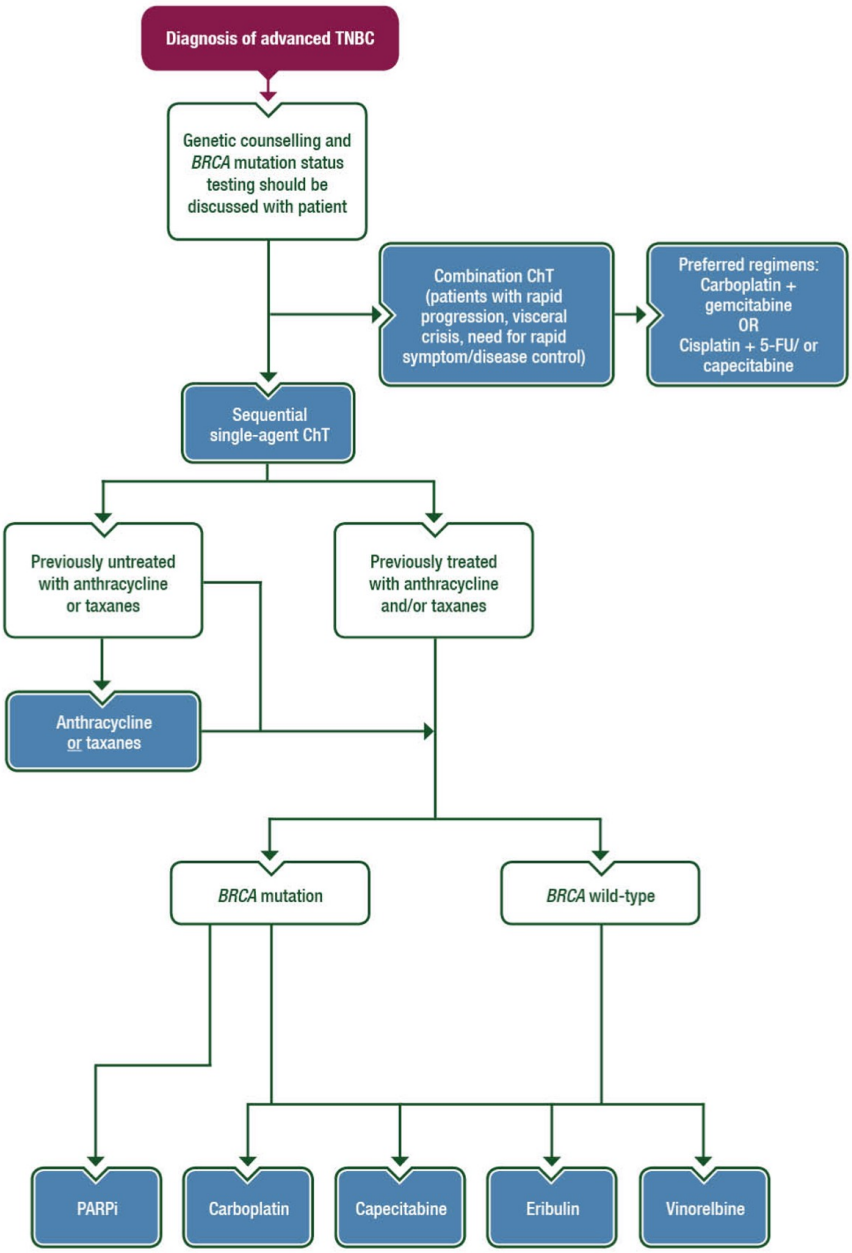

Figure 6. Algorithm for the treatment of advanced TNBC. *Basing on the mutational status of BRCA genes, different chemotherapy (ChT) strategies are suggested to treat TNBC* [117].

Metronomic chemotherapy

Despite advances in cancer treatments, many types of cancer do not benefit from effective targeted therapies, and therefore they are treated with chemotherapy based on the cyclic administration of the maximum tolerated dose (MTD). MTD-chemotherapy has shown promising efficacy in curing or controlling cancer, but the high toxicity limits the use of this treatment. In 2000, Judah Folkman and Robert Kerbel demonstrated that frequent low-dose chemotherapy drugs could reduce tumor growth in preclinical models targeting tumor angiogenesis [118,119].

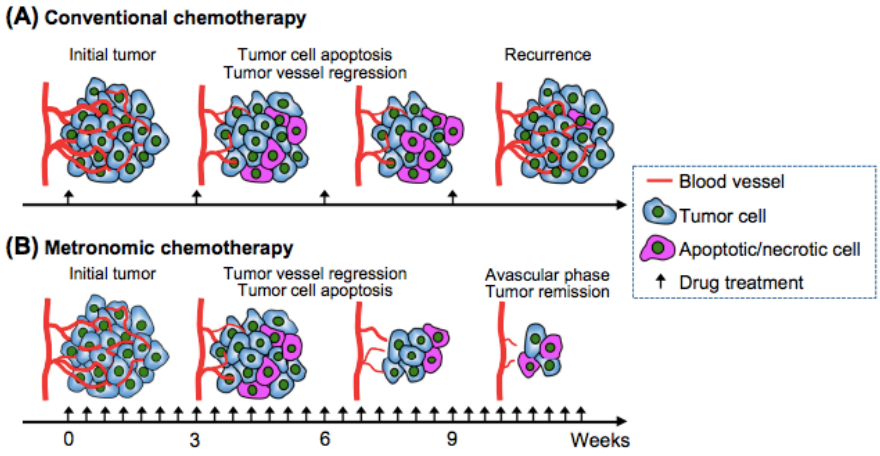

Figure 7. Comparison between conventional and metronomic chemotherapy effects on tumor progression. ***(A)*** *Conventional chemotherapy regimen based on the cyclic administration of the MTD of drugs leads to an initial regression of the tumor mass followed by a regrowth of the tumor and endothelial cells, leading to tumor recurrence.* ***(B)*** *Metronomic chemotherapy regimen based on the continuous administration of a low dose of chemotherapy drugs effectively targets tumor angiogenesis and tumor growth leading to a complete remission* [120].

Based on these findings, Douglas Hanahan coined the term “metronomic” to define the frequent administration of low doses of chemotherapy agents without prolonged drug-free breaks [121].

Due to the drug administration's modality, the ideal agents useful in metronomic chemotherapy should be oral, inexpensive, and well-tolerated. The most studied drugs under metronomic regimen are Cyclophosphamide (CTX), Methotrexate, 5-Fluorouracil, Paclitaxel, Vinblastine, and Vinorelbine [122]. The low-toxic profile of drugs used in metronomic protocols allows to propose both monotherapies and combination therapies with other antitumoral agents under metronomic protocol or targeted, antiangiogenic or immunologic therapies [123]. Moreover, metronomic chemotherapy can be used as maintenance therapy after the MTD regimen, using a chemo-switch protocol [124,125]. Indeed, a significant improvement of the overall survival in patients with high-risk non-metastatic rhabdomyosarcoma treated with conventional chemotherapy followed by a maintenance therapy based on Vinorelbine and low-dose oral Cyclophosphamide has been reported [126]. Also the phase III CAIRO3 trial has shown the benefits of the chemo-switch protocol: the maintenance treatment with metronomic Capecitabine and Bevacizumab in metastatic colorectal cancer patients, previously treated with conventional chemotherapy, doubled the median PFS-1 from 4.1 months of the control group to 8.5 months of the maintenance one (hazard ratio [HR] 0.43, 95% CI 0.36–0.52; P <0.0001) and increased the PFS-2 in

patients on the maintenance treatment from 8.5 months to 11.7 months (HR 0.67, 95% CI 0.56–0.81; P <0.0001) [127,128].

Many clinical trials on metronomic chemotherapy are going on in several types of tumors. All these clinical trials have demonstrated that metronomic chemotherapy is well-tolerated [129]. No or rare high-grade toxicity is found in patients treated with the metronomic schedule. The most common toxic effects were mild nausea and vomiting, mild to moderate anemia, neutropenia, leucopoenia, lymphopenia, and low-grade fatigue [122,130]. Different metronomic regimens are used in patients with breast, ovarian, prostate, lung cancers, non-Hodgkin lymphoma, advanced multiple myeloma, and others [129,131].

Differently from the MTD-based chemotherapy, which targets only cells in active proliferation, metronomic chemotherapy can inhibit both tumor and its microenvironment through multiple mechanisms of action, which includes the inhibition of angiogenesis, the improvement of the immune system response against the tumor, and the inhibition of tumor cell proliferation [130,132]. Metronomic chemotherapy can be, therefore, considered a multi-targeted therapy (figure 8) [132].

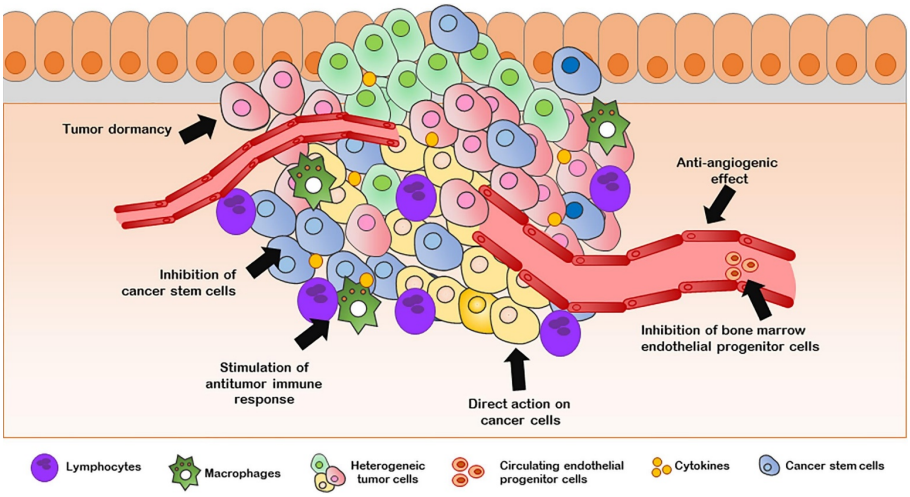

Figure 8. Schematic representation of the multitarget effect of metronomic chemotherapy [133]***.***

Metronomic chemotherapy and angiogenesis

Tumor growth and metastasis spread strongly depend on the vascular network. The development of new vessels within the tumor is called tumor angiogenesis and is a multistep process regulated both by pro- and anti-angiogenic factors. The modulation of the angiogenic factors in favor of the pro-angiogenic ones promotes new vessel formation through the vessel board's degradation and the migration and proliferation of endothelial cells [134]. Folkman and Kerbel first demonstrated the anti-angiogenic property of some antitumor agents, especially CTX and Vinblastine, if administered at low doses [118,119]. Later, other studies demonstrated that metronomic chemotherapy inhibits neo-angiogenesis by directly killing endothelial cells, modulating angiogenesis-related factors, and

inhibiting the circulating endothelial progenitor cells (CEPs) [135].

Endothelial cells. Metronomic administration of some antitumor agents (Taxanes, Epothilone B and 4-HC) inhibits proliferation and induces apoptosis in HUVECs (Human Umbilical Vein Endothelial Cells) and HMVECs (Human Microvascular Endothelial Cells) *in vitro* [136]. After, it has been established that low doses of temozolomide (TMZ) have a significant antitumor effect in *in vivo* models of glioblastoma due to its antiangiogenic activity assessed by microvessel density [137]. Most recent studies have proved that metronomic Topotecan or Melphalan significantly inhibits *in vitro* tube formation in HUVECs and endothelial progenitor cells (EPCs) and *in vivo* tumor volume in preclinical models of glioblastoma [138]. Vinorelbine in the metronomic schedule also inhibits HUVECs migration, tube formation, and sprouting in normal and severe hypoxia conditions. Moreover, continuous low doses of Vinorelbine reduce HUVECs proliferation by shifting the cell population to the G1 phase in normal but not in severe hypoxia conditions [139].

Angiogenesis-related factors. Several studies highlighted the capacity of metronomic chemotherapy to change the balance between pro- and anti-angiogenic stimuli in favor of the anti-angiogenic ones. High levels of the anti-angiogenic factor TSP-1 (Thrombospondin-1) were found in the plasma of PC-3 human prostate cancer-bearing mice after treatment with frequent low doses of Cyclophosphamide [140]. Metronomic Docetaxel also inhibits angiogenesis *in vitro* and *in vivo,* increasing the

expression of TSP-1 and reducing VEGF expression in preclinical models of gastric cancer [141]. Etoposide administered under metronomic protocol alters the angiogenic switch in tumors by inhibiting the secretion of VEGF-A and FGF2 (Fibroblast Growth Factor 2) in several types of tumors and by increasing plasma levels of the angiogenesis inhibitor endostatin in mice [142]. Clinical studies confirmed the modulation of angiogenic factors by the metronomic administration of drugs. VEGF and PDGF-BB (Platelet-Derived Growth Factor BB) levels decreased in cancer patients treated with metronomic Capecitabine or CTX, Methotrexate, and Thalidomide [143,144]. Moreover, TSP-1 serum levels were upregulated in cancer patients treated with metronomic CTX, even if its upregulation did not correlate with clinical benefits [145].

Circulating endothelial precursor cells. Circulating endothelial progenitor cells (CEPs) are precursor cells recruited from the circulation in the angiogenic site, where, via differentiating in endothelial cells, contribute to the generation of new vessels [146]. It has been reported that following acute therapy, CEPs significantly contribute to the regeneration of damaged tumor vasculature, promoting tumor regrowth [135]. In contrast, metronomic chemotherapy destroys the number of circulating bone marrow-derived proangiogenic cells (BMDCs), such as CEPs. A strong reduction of CEPs was also found during metronomic treatment with CTX, suggesting the systemic angiogenesis inhibition induced by metronomic treatment [131].

On the contrary, the number of CEPs in the blood of lymphoma-bearing mice significantly increased after treatment with MTD-regimen CTX. Moreover, a reduction of CEPs in mice blood was observed after metronomic treatment with Vinblastine, Cisplatinum, or Vinorelbine [132,133]. These studies showed the correlation between the minimum CEPs level and the maximum antiangiogenic effect, and therefore, CEP has been proposed as a possible pharmacodynamic biomarker of therapeutic outcome [147]. This correlation has also been observed in patients treated with metronomic Trofosfamide, Cyclophosphamide, and UFT [148–150].

Metronomic chemotherapy and immune system

The immune system is an effective defense in cancer control [151]. The use of the MTD-chemotherapy, which targets actively proliferating cells, strongly affects immune system cells, causing the host immunosuppression [152]. Unlike MTD-chemotherapy, which abolishes immune surveillance via neutropenia and lymphopenia [153,154], metronomic chemotherapy enhances the host immune response against tumors. In particular, it has been demonstrated that low doses of some antitumoral agents, such as Cyclophosphamide and Temozolomide, inhibit regulatory T-cells (T-reg) [155,156], $CD4^+CD25^+Foxp3^+$ lymphocytes that inhibit antigen-specific immune response suppressing $CD8^+$ cytotoxic T lymphocytes, $CD4^+$ T helper cells, and natural killer cells [157].

In addition, metronomic doses of Vinblastine, Paclitaxel, and Etoposide enhance immune system response promoting dendritic cell maturation [158].

Metronomic chemotherapy and cancer cells

For years, based on the log-kill curve, it was believed that a short period of exposure to high doses of cytotoxic drugs was more effective in killing cancer cells than chronic administration of low doses of drugs. Hence, oncologists adopted the concept of MTD-chemotherapy [159]. Around the 90s, the more potent cytotoxic effects of Paclitaxel and Topotecan on cancer cells when administered at low and frequent doses compared to MTD protocol both *in vitro* and *in vivo* has been reported [160,161]. More recently, other studies confirmed the direct effect of continuous low doses of chemotherapy drugs on tumor cells. For example, Orlandi et al. demonstrated the inhibition of non-small-cell lung cancer (NSCLC) cell proliferation by metronomic Vinorelbine through the inhibition of ERK and AKT phosphorylation as well as the reduction of cyclin-D1 gene expression [162].

Studies comparing MTD and continuous low dose protocols have shown that drugs have dose- and schedule- dependent mechanisms of action. For example, among the three mechanisms of action of 5-Fluorouracil using MTD-protocols, the thymidylate synthase inhibition is the only one activated if given at low and frequent doses [163]. The DNA synthesis inhibitor Gemcitabine at low doses stabilizes TRF2 (telomeric repeat-binding factor 2), causing telomere shortening in HeLa cells,

while metronomic Paclitaxel inhibits the nuclear import of the calcium-binding protein S100A4, reducing metastasis propagation in cholangiocarcinoma models [164,165]. The different activity between MTD and metronomic administration of drugs results in different cell death mechanisms.

It has been reported that metronomic chemotherapy promotes cancer cell death through the activation of apoptosis, autophagy, and senescence.

Apoptosis. Apoptosis is an active programmed cell death process involved in different biological events such as tissue homeostasis, cell differentiation, or elimination of damaged cells [166]. Apoptosis can be activated by extrinsic or intrinsic signals, which trigger the caspases cascade activation and subsequent DNA fragmentation, cytoskeletal and nuclear proteins degradation, apoptotic body formation, and finally, the uptake by phagocytic cells [167]. Apoptosis activation can be considered as a protection mechanism against cancer [168].

Different studies have demonstrated the activation of apoptosis after metronomic chemotherapy drug treatment. For example, low doses of Actinomycin D inhibit neuroblastoma cell proliferation-inducing p53-dependent apoptosis *in vitro* as well as tumor regression *in vivo* [169]. The combination treatment with metronomic Topotecan plus the TKI Pazopanib efficiently inhibits TNBC cell proliferation in tumor tissue samples and *in vitro,* inducing an increase of cleaved caspase 3, leading to apoptosis activation [170].

Autophagy. Autophagy is a physiological catabolic process activated by cells under extra- or intracellular stress conditions, such as nutrient deprivation, organelle damage, or abnormal protein accumulation [171]. Indeed, through the degradation of intracellular macromolecules into autophagosomes, the cell can obtain the energy necessary for the minimal cell functioning under nutrient deprivation. However, excessive activation of autophagy has been demonstrated to result in autophagic cell death; therefore, autophagy can act as a tumor suppressor [166,171]. In support of this hypothesis, it has been recently demonstrated that colorectal cancer (CRC) cells die via apoptosis and autophagy after treatment with photodynamic therapy (PDT). This anticancer procedure consists of applying a photosensitizer and its stimulation by light of the appropriate wavelength and intensity [172]. Apoptosis and autophagy are activated after both metronomic and acute protocols, consisting of a short-term fluence rate with higher intensity than the metronomic one. However, metronomic PDT induces a higher cell death rate of CRC cells than the acute treatment due to a long-lasting autophagy activation accompanied by the greater activation of apoptosis [173].

Senescence. Senescence is a state of irreversible growth arrest activated by the shortening of telomeres or the exposure to acute or chronic exogenous or indigenous stressors, such as oxidative stress, DNA damage, or oncogenic signals [174]. Physiologically senescence prevents tumorigenesis by definitely blocking cell proliferation, representing a promising outcome for a

chemotherapy treatment [175]. It has been recently demonstrated that low dose Topotecan treatment of neuroblastoma cells induces DNA-damage, p21 up-regulation, and senescence activation resulting in tumor regression *in vitro* and *in vivo* [176]. Even the ribonuclease reductase inhibitor hydroxyurea (HU) induces senescence in primary neuroblastoma cell lines *in vitro* if administered long-term at a low dose [177]. Moreover, metronomic combination with Everolimus and Etoposide strongly affects non-Hodgkin lymphoma (NHL) cell lines by inducing cell cycle changes and activating senescence together with apoptosis and autophagy [178].

Metronomic chemotherapy and cancer stem cells

Cancer stem cells (CSCs) are pluripotent cells considered tumor-initiating cells because of their involvement in tumor development, growth, and dissemination [179]. CSCs are intrinsic resistant to chemotherapy and radiotherapy and, therefore, responsible for metastasis spread and cancer recurrences [180].

It has been reported that the metronomic administration of chemotherapy drugs can reduce stemness in several types of tumors [159]. Metronomic Cyclophosphamide significantly reduces both primary and secondary glioma spheroids isolated from drug-treated patients [181] and reduces CD133+ precursor cells CD133+/CD44+/CD24+ cancer stem cells in human pancreatic tumor xenografts [182]. Low-dose metronomic treatment with Gemcitabine significantly reduces tumor spheres

and peripheral blood levels of CEPs in hepatocarcinoma xenografts, whereas it did not affect the tumor spheres when administered at MTD [183]. A recent article showed that Methylglyoxal (MG) exerts remarkable activity in decreasing the size and numbers of mammospheres formed by breast cancer cell lines, caused by reducing the CD44+/CD24- cell population. In addition, metronomic doses of MG sensitize breast cancer cells to Doxorubicin and Cisplatin, similarly targeting CSC and non-CSC populations, reducing mammospheres forming efficiency and inducing apoptosis or necroptosis of breast cancer cells [184].

Metronomic chemotherapy and metastasis

Metastasis formation is a multistep process through which cancer cells disseminate from the primary tumor site to surrounding tissues and to distant organs [185]. To develop metastases, cancer cells undergo some intracellular changes that allow them to migrate into surrounding tissues, invade, transit, and survive in blood vessels, extravasate, and colonize the new site [186]. Some of these changes include the activation of migration pathways, such as the focal adhesion kinase (FAK) pathway, the secretion of matrix metalloproteinases (MMPs) for matrix degradation, and the acquisition of mesenchymal-like features through a process named epithelial-to-mesenchymal transition (EMT) [187]. Metastasis formation is also supported by the tumor microenvironment in which cancer stem cells, endothelial cells, immune cells, and cancer-associated

fibroblasts (CAFs) reside, promoting the spread of tumor cells [188].

Cytotoxic agents used in MTD-chemotherapy do not target metastases, which are responsible for 90% of cancer-related death [185]. In some studies, metronomic chemotherapy has shown good control of metastasis by directly inhibiting tumor cells and modulating the tumor microenvironment with less toxicity compared to MTD. For instance, metronomic Docetaxel affects the migration and invasion capacity of both endothelial and prostate cancer cell lines at the same drug concentration by targeting hnRNP K (heterogeneous nuclear ribonucleoprotein K) [189]. Metronomic administration of Gemcitabine as a single agent or, in combination with Sunitinib, has a significant effect in reducing metastasis formation in aggressive orthotopic models of pancreatic cancer due to its cytotoxicity on both cancer and endothelial cells and the inhibitory effect on supporting tumor microvasculature [190]. The green tea *Camellia sinensis* water extract combined with metronomic doses of the potent third-generation nitrogen-containing bisphosphonate Zoledronate has antiproliferative, anti-migration, and anti-invasion abilities through the activation of apoptosis and inhibiting MMPs in mouse breast cancer 4T1 cells [191]. Long-term metronomic treatment with CTX has more antitumor activity in rat models of hepatocellular carcinoma (HCC) than the respective MTD-treatment because of its more potent antiproliferative, antiangiogenic, and antimetastatic effects. In particular, metronomic CTX significantly suppressed *in vivo* spontaneous

pulmonary metastasis from HCC via MMPs' activity inhibition [192]. Metronomic CTX used as monotherapy or combined with Celecoxib or Docetaxel showed an increase in median survival time with low toxicity in murine mammary adenocarcinoma models by inhibiting tumor growth and lung metastasis formation [193,194]. CTX combination therapy is more effective as an antitumor therapy than monotherapy, probably due to the decrease in VEGF concentration, induced by the combination with Docetaxel and Celecoxib, and the increase in tumor cell apoptosis activated after the combination with Docetaxel [193,194]. Moreover, Muñoz et al. showed the *in vitro* inhibition of the invasiveness of highly metastatic TNBC cells by the metronomic administration of UTF plus CTX, suggesting potential antimetastatic effects of this treatment [195].

Metronomic chemotherapy and tumor dormancy

From the clinical observation that cancer recurrences often occur decades after surgical resection of the primary tumor, the concept of tumor dormancy emerged [196]. Tumor dormancy is defined as the arrest of tumor growth in the primary (primary dormancy) or in metastatic (metastatic dormancy) site due to a balance between cancer cell proliferation and cell death [196,197]. From a therapeutic point of view, it can be associated with a delay in the development of relapses [198].

Tumor dormancy can be due to angiogenic dormancy, immune-surveillance, or cellular dormancy [199]. Tumor growth needs a complex blood vessel network to provide energy sources to sustain cancer cells' active proliferation. Indeed, it has been

demonstrated that preventing neovascularization tumor dormancy can be induced [200,201]. Therefore, metronomic chemotherapy could be a valid therapeutic approach to induce tumor dormancy because of its pro- and anti-angiogenic factors modulation activity. Indeed, as reported above, metronomic doses of Capecitabine, CTX, Methotrexate, Docetaxel, and Thalidomide can decrease VEGF, PDFGF, FGF levels and to increase TPS-1 in both clinical and pre-clinical models, inhibiting tumor neo-angiogenesis.

The immune system represents another critical part involved in controlling tumor mass growth: immunes system cells, specifically $CD8^+$ T lymphocytes, can recognize tumor cells and induce cell death via apoptosis or can keep them in a dormant state [196]. However, cancer cells can evade immune system surveillance, sustaining tumor mass or metastasis growth. Metronomic chemotherapy using certain drugs such as Cyclophosphamide can also induce tumor dormancy by enhancing immune system response through T-reg inhibition and dendritic cell maturation promotion.

Tumor cell dormancy is characterized by minimum cell proliferation, minimum death, and reversibility [196]. Tumor cell dormancy can be promoted by the tumor microenvironment signals or by the induction of quiescence or autophagy [199,202]. Moreover, some conditions have recently been suggested for senescence reversibility, which can induce tumor cell dormancy [202]. Through anti-angiogenic and immunostimulatory effects and the direct stimulation of cellular senescence and autophagy,

metronomic chemotherapy could in theory induce tumor cell dormancy (figure 7). However, further studies are needed to confirm the role of metronomic chemotherapy in tumor dormancy induction.

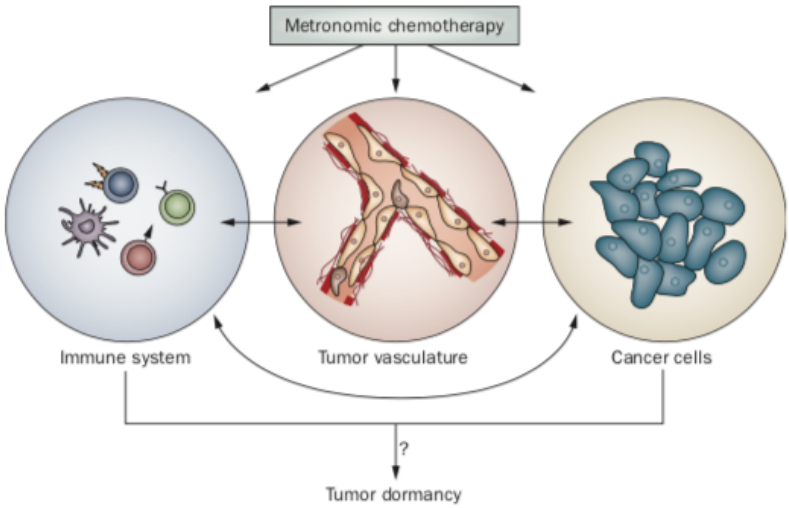

Figure 9. Mechanisms by which metronomic chemotherapy can induce tumor dormancy. *Frequent and low doses of drugs inhibit angiogenesis and improve immune system response against tumors, reducing cancer cells' proliferation rate. Moreover, metronomic chemotherapy slows down tumor cell proliferation activating autophagy and senescence. All these events contribute to induce and protract tumor dormancy, which contributes to postponing the onset of relapses* [199].

Resistance and metronomic chemotherapy

Metronomic chemotherapy regimens have shown promising activity in many clinical trials, even if some small studies have shown limited efficacy of the metronomic [203,204] or the onset of resistance within a few months [205]. Mechanisms by which resistance to metronomic chemotherapy emerged are different. Unlike cancer cells, endothelial cells are genetically more stable

and less prone to develop chemotherapy resistance. However, recent studies have shown that tumor endothelial cells (TECs), which line the inner layer of blood vessels of tumor-stromal tissue, exhibit cytogenetic abnormalities [206], which can lead to the onset of resistance mechanisms [207].

Among resistance mechanisms to chemotherapy, the expression of drug efflux pumps is the most common. For instance, ECs acquire resistance to metronomic treatment with Paclitaxel by expressing the P-glycoprotein via the VEGFR2 and AKT activation [208]. Nevertheless, both endothelial and tumor cells express high drug efflux pumps after MTD-chemotherapy compared to the metronomic one [209].

Another resistance mechanism to metronomic chemotherapy described is the induction of severe hypoxia. It has been shown that metronomic chemotherapy induces severe tumor hypoxia, which stimulates the expression of pro-angiogenic factors, restoring tumor angiogenesis [210]. Mavroeidis et al. showed the AKT pathway's involvement in resistance to metronomic chemotherapy-induced hypoxia. Indeed, the addition of the AKT inhibitor V to metronomic Vinorelbine restores the antiproliferative and pro-apoptotic effect of metronomic treatment in severe hypoxia conditions in HUVECs [139].

Moderate activation of the autophagy is involved in resistance to metronomic Cyclophosphamide of prostate cancer PC-3 cells: indeed, PC-3 cells resistant to metronomic treatment show a lower autophagy rate than the sensitive control. The resistance to metronomic Cyclophosphamide can be reverted by treating

PC-3 resistant xenografts with the autophagy inhibitor chloroquine [211].

Additional resistance mechanisms acquired in response to continuous low dose chemotherapy were found and involved the vascular mimicry, vascular remodeling, decreased vascular dependency, and others [205].

Although resistances may occur after metronomic chemotherapy, tumors remain sensitive to MTD-chemotherapy. Emmenegger et al. demonstrated that PC-3 cells resistant to metronomic treatment with Cyclophosphamide retain sensitivity to Cyclophosphamide, Docetaxel, and Doxorubicin in *in vitro* studies and to MTD Cyclophosphamide *in vivo* [212]. Moreover, using both a variant of the TNBC cell line MDA-MB-231 and the prostate cancer PC-3 resistant to metronomic Cyclophosphamide, they have shown that resistance to metronomic therapy results through other mechanisms than those involved in MTD resistance [212,213]. On the other hand, development of MTD-therapy resistance may be overcome by switching the treatment schedule in metronomic protocol, as shown with Cyclophosphamide by Browder et al. [118]. Kim JT et al. also showed that metronomic administration of Temozolomide overcame the chemoresistance that developed to a conventional treatment, by increasing apoptosis of tumor cells and reducing neo-angiogenesis and tumor growth in orthotopic glioma models [137]. Altogether, these preclinical observations suggest that resistance may be overcome by changing the drug dose and treatment schedule.

Scope of the thesis

Chapter 2: Metronomic combination of Vinorelbine and 5Fluorouracil is able to inhibit triple-negative breast cancer cells. Results from the proof-of-concept VICTOR-0 study.

In this study, we analyzed the effect of the metronomic administration of 5-Fluorouracil (5-FU) and Vinorelbine (VNR) on triple-negative breast cancer (TNBC) cell lines versus the conventional (STD) administration. A significant anti-proliferative effect was observed on cells treated with metronomic administration of single drugs and combined, compared to the standard treatment. Moreover, we addressed the molecular mechanisms of cell death activated by treatments.

Chapter 3: Metronomic administration of 5-Fluorouracil plus Vinorelbine inhibits both endothelial and triple-negative breast cancer cells regrowth and migration via FAK/VEGFR2 downregulation and autophagy/apoptosis activation.

This study investigated the effect of the metronomic and conventional (STD) administration schedule of 5-FU plus VNR on migration and viability of endothelial and triple-negative breast cancer (TNBC) cell lines. In particular, we showed that the metronomic regimen with 5-FU plus VNR completely inhibits the regrowth of new colonies of endothelial and TNBC cells compared to standard treatment. Both treatments strongly reduce endothelial and TNBC cells' migration, albeit through different mechanisms. Metronomic treatment is cytotoxic on endothelial cells inducing apoptosis, whereas TNBC cells shift

the modality of cell death from apoptosis, induced by standard, to autophagy. Moreover, we showed that metronomic treatment with 5-FU plus VNR is more effective than STD regimen in preventing neo-angiogenesis.

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Chapter 2

Metronomic combination of Vinorelbine and 5Fluorouracil is able to inhibit triple-negative breast cancer cells. Results from the proof-of-concept VICTOR-0 study

Maria Grazia Cerrito[1], Marco De Giorgi[1], Davide Pelizzoni[1,2], Sara Maria Bonomo[1], Nunzio Digiacomo[1,2,] Arianna Scagliotti[1], Cristina Bugarin[4], Giuseppe Gaipa[4], Emanuela Grassilli[1], Marialuisa Lavitrano[1], Roberto Giovannoni[1], Paolo Bidoli1[,2] and Marina Elena Cazzaniga[1,2,3]

[1] Department of Medicine and Surgery, University of Milano-Bicocca, Monza 20900, Italy
[2] Oncology Unit, ASST Monza, Monza 20900, Italy
[3] Phase 1 Research Centre, Monza 20900, Italy
[4] M. Tettamanti Research Center, Pediatric Clinic, University of Milano Bicocca, Monza 20900, Italy

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Abstract

Triple Negative Breast Cancer (TNBC) is an aggressive neoplasia with median Overall Survival (OS) less than two years. Despite the availability of new drugs, the chance of survival of these patients did not increase. The combination of low doses of drugs in a metronomic schedule showed efficacy in clinical trials, exhibiting an anti-proliferative and antitumor activity. In Victor-2 study we recently evaluated a new metronomic combination (mCHT) of Capecitabine (CAPE) and Vinorelbine (VNR) in breast cancer patients showing a disease control rate with a median Progression-Free Survival (PFS) of 4.7 months in 28 TNBC patients. Here in Victor-0 study, we examined the effect of mCHT vs standard (STD) schedule of administration of different combinations of 5-Fluorouracil (5FU), the active metabolite of CAPE, and VNR in TNBC cell lines MDA-MB-231 and BT-549. A significant anti-proliferative activity was observed in cells treated with metronomic vs STD administration of 5FU or VNR alone. Combination of the two drugs showed an additive inhibitor effect on cell growth in both cell lines. Moreover, after exposure of cells to 5FU and VNR under mCHT or conventional schedule of administration we also observed a downregulation of chemoresistance factor Bcl-2, changes in pro-apoptotic protein Bax and in cleaved effector caspase-3 and increased expression of LC3A/B autophagy protein. Our results therefore suggest that molecular mechanisms implicated in apoptosis and autophagy as well as the cross-talk between these two forms of cell death in MDA-MB-231 and BT-549 cells treated with 5FU and VNR is

dose- and schedule-dependent and provide some insights about the roles of autophagy and senescence in 5FU/VNR-induced cell death.

Introduction

TNBCs are a specific subtype of epithelial breast tumors that are immunohistochemically negative for the protein expression of estrogen receptor (ER), progesterone receptor (PR) and do not show overexpression/gene amplification of HER2 [1].

TNBCs account for about 10–20% of all breast cancers and are associated with a very bad prognosis, even in early stages of disease: after radical surgery, median time to relapse is approximately 18 months and median OS is less than 24 months. Despite the big efforts aiming to improve this clinical scenario and to understand the molecular basis of breast cancer biology little has really changed in the last decades for these patients [2]. Rest periods between two consecutive cycles of chemotherapy administered at Maximum Tolerated Dose (MTD) is necessary to allow recovery from toxicities. Unfortunately, there is evidence not only of re-growth of tumour cells but also of growth of selected drug-resistant clones [3]. To improve the therapeutic index of chemotherapy it is necessary to modify the choice of drugs or to change the way of administration. In this scenario, mCHT - which refers to regular administration of conventional chemotherapy drugs at low, minimally toxic doses, with no prolonged break periods [4] - could represent a promising therapeutic option for advanced breast cancer patients.

Recently, it has been shown that mCHT has an important stabilizing effect on cancer growth (including chemotherapy-resistant disease) and confers prolonged clinical benefits by improving at the same time the quality of life of cancer patients by avoiding severe toxicity [5–8].

Likewise, many studies have demonstrated that the tumor response to metronomic schedules is due both to antiangiogenic/immuno-stimulatory effects and to direct effects on tumor cells themselves. Therefore, mCHT can be defined as a multitargeted therapy, able to strike both tumor cells and the surrounding microenvironment [9].

Different authors [10, 11] have explored the use of mCHT in TNBC patients, reporting a wide range of Overall Response Rate (ORR, 9–44%) and median PFS of approximately 10 months.

Metronomic combination of VNR and CAPE has been recently studied in 80 advanced breast cancer patients [7], of whom 28 were TNBC, suggesting a promising activity in terms of Clinical Benefit Rate and PFS.

All these results observed in the clinical practice are little supported by pre-clinical data, mainly for what concerns the combination of different agents, all given in a metronomic way.

In order to identify and describe which kind of biological processes are implicated in determining the results observed in the clinical practice here, we evaluated the antiproliferative and cytotoxicity effects of 5FU and/or VNR given either in STD or in metronomic schedule, on MDA-MB-231 and BT-549 cells.

Results

Metronomic administration of 5FU and VNR significantly inhibits human MDA-MB-231 and BT-549 breast cancer cell growth.

The effect on cell viability of 5FU and VNR, alone or in combination, either given in STD or in mCHT administration, was investigated using the MTT assay. For these studies, MDA-MB-231 and BT-549 cells were treated with 5FU or VNR at the indicated dose, for 4 or 96 hours to simulate the conventional (4h) or metronomic (96h) dosing protocol.

A significant anti-proliferative activity was observed in both cell lines treated with metronomic administration of 5FU or VNR compared to STD treatment (Figure 1A, 1C).

Concentrations of drugs provoking 50% cell growth inhibition (IC50) were calculated from curves derived by plotting cell viability (%) versus drug concentration (nM). The reading values were converted to the percentage of the control. The IC50 of single-dose of 5FU administration with the metronomic schedule was more than 20 times lower in both cell lines compared to standard treatment (MDA-MB-231:8500 nM vs 180000 nM; BT-549: 9000 nM vs 200000 nM). The IC50 of VNR at 96h was a couple orders of magnitude lower in MDA-MB-231 and BT-549 cells treated with the metronomic schedule in comparison to the exposure with VNR at conventional concentrations (0.92 nM vs 70 nM and 0.95 nM vs 70 nM respectively) (Figure 1B and 1D, Table 1).

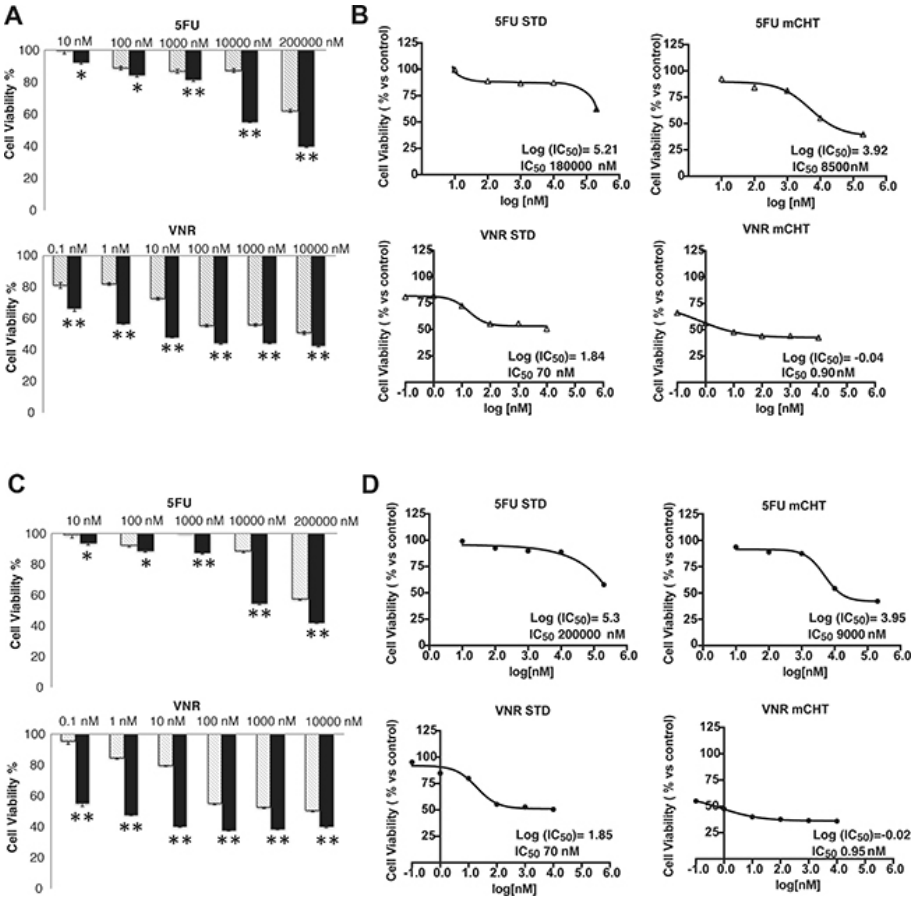

Figure 1. Metronomic administration of 5FU and VNR induced significant growth inhibition in human MDA-MB-231 and BT-549 breast cancer cells. *(A) MDA-MB-231 and (C) BT-549 cells were treated with different concentrations of 5FU and VNR for 4h (STD) or 96h (mCHT), respectively reported in grey and black. The dose-response curves of MDA-MB-231 (B) and BT-549 (D) were used to calculate IC50 value. Cell viability was investigated using the MTT assay and expressed as a percentage relative to the untreated control cells. The experiment was repeated 4 times with at least 8 replicates per sample. *p < 0.05 vs untreated **p < 0.01 vs untreated. Results are means ± SD of three measurements (P < 0.05).*

		IC_{50} Single treatment		IC_{50} Combo treatment		Chou index $CI = (D_1/Dx_1) + (D_2/Dx_2)$
MDA-MB-231	STD	5FU	180000 nM	5FU+VNR	80000 nM + 30 nM	0.9
		VNR	70 nM			
	mCHT	5FU	8500 nM	5FU+VNR	4500 nM + 0.5 nM	1
		VNR	0.92 nM			
BT-549	STD	5FU	200000 nM	5FU+VNR	100000 nM + 35 nM	1
		VNR	70 nM			
	mCHT	5FU	9000 nM	5FU+VNR	4500 nM + 0.50 nM	1
		VNR	0.95 nM			

IC_{50} value for the combo treatment was calculated from curves showed in Figure 2 and Chou Index was calculated using the formula $(D_1/D_{X1}) + (D_2/D_{X2})$, in which D_1 and D_2 are the IC_{50} of 5FU and VNR in the combination treatment while D_{X1} and D_{X2} are the IC_{50} of the 5FU and VNR in the single treatment.

Table 1. 5FU and VNR concentrations used for combination treatment of MDA-MB-231 and BT-549 cells in the STD and mCHT schedule.

The combination ratio was calculated using the IC50 ratio of the single drugs, so that the contribution of the effect for each drug in the mixture would be the same. The results are summarized in Figure 2 which shows the combination index (CI) of the IC50. Synergistic, additive, or antagonistic effects were defined using the CI method of Chou-Talalay [12]. As shown in Table 1, co-incubation of 5FU with VNR showed additive effects on both cell lines with CI values in the range of 0.9 and 1.0.

To investigate the mechanisms underlying the effects on cell proliferation of VNR and 5FU, alone or in combination, under STD or mCHT protocols, we examined cell cycle distribution patterns in MDA-MB-231. FACS analysis indicated a significant decrease of G0/G1 population in 5FU-, VNR- and combo-treated vs. untreated cells under STD protocol whereas a significant decrease was observed only in VNR-treated vs. untreated cells under mCHT protocol. A variable but significant increase in apoptotic cells treated with VNR either alone or in combination with 5FU vs. untreated cells, was

observed both in standard and metronomic procedure (Supplementary Figure 1).

5FU and VNR can induce in TNBC cells either apoptosis alone or in parallel with autophagic cell death, depending upon their schedule of administration.

Recent studies have reported that activation of autophagy upon drug treatments can induce cell death either independently of or in parallel with apoptosis and necrosis [13]. To verify whether autophagy and/or apoptosis are triggered in cells treated with 5FU and VNR we assessed, by western blot, autophagic and apoptotic markers (Figure 3). In MDA-MB-231 and in BT-549 cells, upon exposure to 5FU and VNR alone or in combination in STD treatment, we observed an increased protein expression of microtubule-associated protein 1A/1B-light chain 3 (LC3A/B), a major constituent of the autophagosome that segregates the target protein/organelle and then fuses with lysosomes to form autolysosomes where the contents and LC3 are degraded [14–16]. A significant increase of LC3A/B expression was observed also upon exposure to 5FU and VNR, alone or in combination, under the mCHT schedule of treatment. These data indicate that the administration of 5FU and of VNR under mCHT protocol, in particular when they were given simultaneously, activated autophagy. Autophagy is interconnected with apoptosis by several molecular nodes of crosstalk, including Bcl-2/Bax and caspases [17].

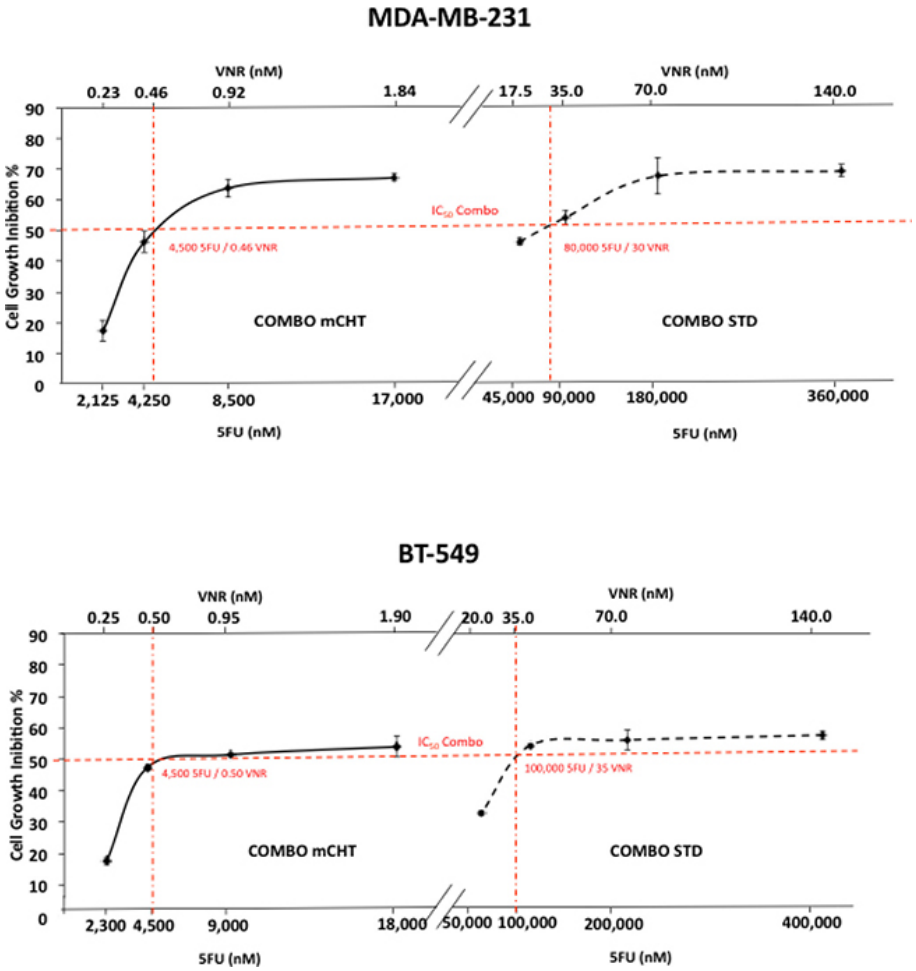

Figure 2. Metronomic administration of 5FU and VNR in combination induced significant growth inhibition in human MDA-MB-231 and BT-549 breast cancer cells. *Representative dose-response curve performed on MDA-MB-231 (A) and BT-549 (B) treated with the following drug combination: 1) 2x IC50 (5FU) + 2x IC50 (VNR) 2) IC50 (5FU) + IC50 (VNR) 3) ½ IC50 (5FU) + ½ IC50 (VNR); 4) ¼ IC50 (5FU) + ¼ IC50 (VNR); cells were treated for 4 h (STD) or 96 h (mCHT) and their number evaluated by MTT assay. The reading values were converted to the percentage and compared to untreated control. The simple two-point method uses 2 data points bracketing 50% inhibition of proliferation (red lines) to estimate the IC50. The experiment was repeated 3 times with at least 8 replicates per sample.*

We therefore examined the expression of the anti-apoptotic Bcl-2 protein and the pro-apoptotic Bax in MDA-MB-231 and in BT-549 cells treated with 5FU or VNR alone or their combination, under the two different protocols. We show that all these treatments, with the exception of 5FU and VNR in BT-549 cells under STD treatment, significantly decreased Bcl-2 protein expression compared to untreated cells. Furthermore, increased Bax expression was induced in BT-549 by all treatments which is consistent with induction of apoptosis. Interestingly, the increased expression of Bax correlated with up-regulation of cleaved caspase-3 expression when BT-549 cells were exposed to STD treatments, and to a lesser extent, under mCHT regimen. In the case of MDA-MB-231 up-regulation of cleaved caspase-3 clearly correlate with Bax induction, even though to a different extent, upon VNR exposure (under both, STD and mCHT, schedules) and when 5FU was given mCHT. In all other cases cleaved caspase-3 levels were increased in absence of Bax induction. Notably, cleaved caspase-3 levels are significantly lower in both cell lines under mCHT schedule (but for 5FU-treated MDA-MB-231 cells) compared to STD treatments. These results suggest that treatments with 5FU and VNR can induce either apoptosis alone or in parallel with autophagy in MDA-MB-231 and in BT-549 cells depending upon their schedule of administration.

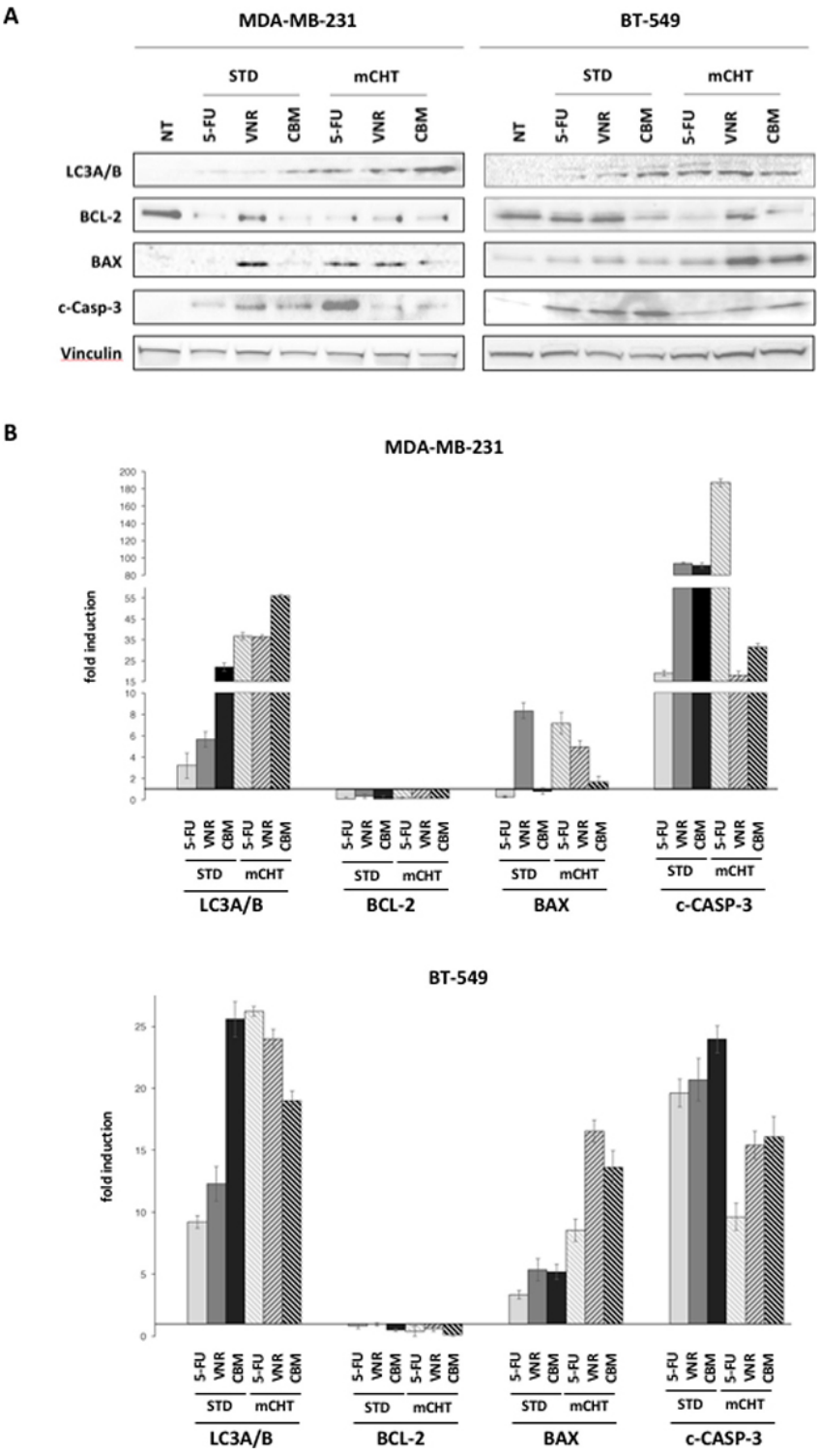
A
MDA-MB-231
BT-549
STD
mCHT
NT
5-FU
VNR
CBM
LC3A/B
BCL-2
BAX
c-Casp-3
Vinculin
B
MDA-MB-231
fold induction
LC3A/B
BCL-2
BAX
c-CASP-3
BT-549

Figure 3. 5FU and VNR can induce either apoptosis and/or autophagy in TNBC cells depending on the schedule of their administration. (*A) Upper panel: representative Western blot of MDA-MB-231 and BT-549 exposed to 5FU and VNR alone (IC50 single drug) or in combination (IC50 combo) for 4h (STD) and for 96h (mCHT). (B) Quantification of the protein expression as evaluated by densitometry. Protein levels were normalized to the corresponding Vinculin loading control. Error bars represent mean ± SEM, n = 3.*

Metronomic treatment of MDA-MB-231 and BT-549 cells resulted in induction of autophagy.

To corroborate the evidence of an autophagic process induced by 5FU and VNR in TNBC cells we immunostained treated cells with an antibody specific for LC3.

Untreated cells showed perinuclear actin and tubulin filaments uniformly distributed along the perimeter of the cell. VNR and 5FU, either alone and in combination, caused a rearrangement of the cytoskeleton. Moreover, in all three metronomic schedules of treatment (5FU, VNR and 5FU + VNR) there was an increase in LC3-positive punctate dots in perinuclear and cytoplasmic region, (pointed out by white arrows in Figure 4) indicating that induction of autophagy is dependent from the schedule of treatment of 5FU and VNR. Indeed, the formation of autophagosomes was not evident in cells under standard schedule of treatment, where only a weak, diffuse presence of the cytoplasmic form of LC-3 was detected. In addition, apoptotic cells that are shrunken with condensed cytoplasm (indicated by yellow arrows in Figure 4) were observed upon DAPI staining in cells treated with VNR under both STD and mCHT schedule.

DNA damaging drugs, including 5FU, have been shown to induce, besides apoptosis and autophagy, also cellular senescence [18, 19].

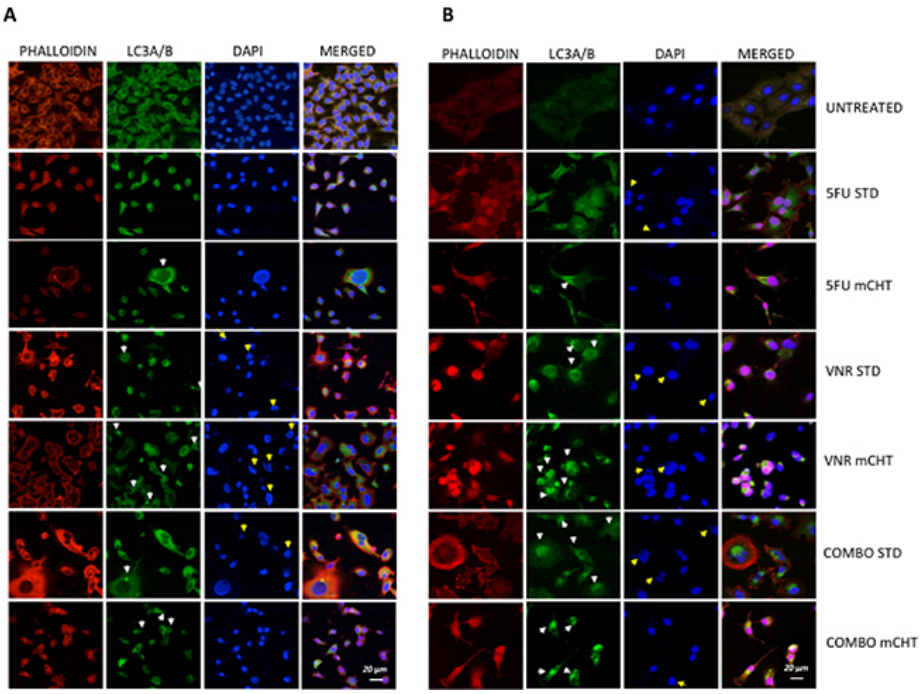

Figure 4. Increased autophagy response in MDA-MB-231 and BT-549 cells treated with 5FU and VNR in metronomic schedule. *(A) MDA-MB-231 and (B) BT-549 cells were exposed to 5FU and VNR alone (IC50 single drug) or in combination (IC50 combo) for 4h (STD) and for 96h (mCHT). Anti-LC3A/B was detected by a FITC-conjugated secondary antibody, TRITC-conjugated phalloidin was used to stain actin and nuclei were counterstained with DAPI before acquiring images by confocal microscope (Biorad Laboratories, Hercules CA, USA). White arrows indicate autophagosomes fully formed in the cytoplasm of the cells upon exposure to VNR alone or in combination with 5FU in metronomic schedule. The Yellow arrows indicate apoptotic cells that are shrunken with condensed cytoplasm.*

To reconcile the fact that in MDA-MB-231 cells mCHT administration of combined therapy seems less effective than STD administration in inducing both apoptosis and autophagy and to provide a possible mechanistic basis of the observed growth suppressive effect we tested SA-β-gal-activity (a marker of senescence) after treating the cells with 5FU and VNR, alone or in combination, under STD or mCHT protocols. Rare or few senescent cells were found after STD administration of 5FU and VNR, respectively. At variance, a significant increase of senescent cells was observed after mCHT administration of VNR alone and in combination with 5FU (Supplementary Figure 2).
On the whole, these data indicate that induction of apoptosis, autophagy and senescence all contribute to the therapeutic effect of metronomic schedule.

Discussion

The rapid development of new therapeutic drugs that target specific molecular pathways involved in tumor cell proliferation or apoptosis offers an extraordinary prospect to achieve a very high degree of specificity associated with lower toxicity [20]. Indeed, molecularly targeted agents often diverge from traditional cytotoxic agents due to their administration schedules and routes, their toxicity profiles and their antitumor activity. For some aspects, the development of metronomic chemotherapy is quite similar to that of targeted agents since the tumor response to metronomic schedule is due not only to antiangiogenic and immune-stimulatory effect, but also to a direct anti-cancer activity

and could therefore be considered a multi-target therapy itself in contrast to conventional MTD or STD dose [21]. In addition, the mechanism of action of some anticancer agents can significantly differ when they are given metronomically or by conventional schedules as reported by Harstrick et al. [22] who compared short and long exposure of human cancer lines to 5FU. Their results show that the inhibition of thymidylate synthase, a key rate-limiting enzyme of DNA synthesis, became more important when the treatment was prolonged. Other chemotherapy agents can show different mechanisms of action when administered according to a metronomic or a STD schedule. Remarkably, in women with breast cancer, re-treatment with metronomic CAPE can lead to a response after standard CAPE dosing has failed [23].

These different mechanisms of action may be the result of different effects on cell death [24]. While anticancer drugs usually kill cancer cells via apoptosis, low-dose mCHT can induce different types of cell death. For instance, Cortes et al. [25] reported that low doses of actinomycin D inhibited proliferation and induced apoptosis in vitro, as well as tumor regression in vivo, in a p53-dependent manner in a model of subcutaneously implanted neuroblastoma. However, a pan-caspase inhibitor only partially inhibited cell death induction, suggesting that the treatment could activate an apoptosis-independent cell death pathway. Bocci et al. [4] reported that the combination of metronomic topotecan and pazopanib significantly enhanced antitumor activity compared to monotherapy with either drug and

prolonged survival, even in the advanced metastatic survival setting, with a marked decrease in tumor vascularity, proliferative index, and the induction of apoptosis.

In breast cancer, among the drugs used as single agents, CAPE and VNR are those supported by the greater amount of data. CAPE is an orally administered fluoropyrimidine carbamate, which was approved by FDA as a single agent for metastatic breast cancer patients. VNR is a semi-synthetic vinca alkaloid, active in a variety of cancers [26]. In breast cancer, oral VNR has been widely studied in metronomic regimens, with encouraging results [21]; for example, in the recent clinical study Victor-2, that evaluated a new metronomic combination (mCHT) of CAPE and VNR in metastatic breast cancer patients, the efficacy and safety of the metronomic combination of both drugs in an unselected group of patients has been shown [27].

Data emerged so far from pre-clinical studies and in vitro models, performed in both the adjuvant and the metastatic setting [28, 29], indicate that the metronomic combination of two different drugs allows to use doses of the single drugs that are much lower than those required by both the standard schedule or the single-agent metronomic administration [10].

On the clinical side, a great number of Phase II studies have been published starting from mid-2000s, showing an increasing interest of clinicians in mCHT: among the 80 publications selected for the systematic literature analysis by Lien and colleagues, 21 trials covered the topic of breast cancer involving 1135 patients. The authors identified 107 treatment regimens

with at least one metronomic drug, being Cetuximab, Capecitabine, etoposide and VNR the most frequently used. The mean Response Rate (RR) of the pooled treatment regimens was 26%, with a mean Disease Control Rate (DCR) of 56.3%. Median duration of response was 4.6 months on average. This systematic literature review, even if not focused on breast cancer patients and TNBC in particular, confirmed that severe side-effects are rare, being observed in less than 5% of patients and the treatment-associated fatalities are very rare (0.4%) too, despite the fact that most study patients had an advanced disease, often refractory to often multiple prior conventional systemic therapies.

Even if international guidelines suggest the use of sequential, single agent regimens for the treatment of advanced breast cancer patients, they recommend the choice of combination regimens particularly for advanced TNBC ones, thus recognizing for these patients a strong clinical need for more aggressive strategies.

More recent trials, some of them conducted as single institution pilot experiences, tested different and more active drugs, mainly VNR and CAPE, reporting percentages of ORRs of approximately 50% and “Complete Bed Rest” of 77–80%.

In the in vitro study, called Victor-0, we focused our attention on the effect of 5FU and VNR in TNBCs given that they represent an important clinical challenge because they do not respond to endocrine therapy or other available targeted agents and have a poor response to STD chemotherapy as well. The metastatic

potential in TNBCs is similar to that of other breast cancer subtypes, but these tumors are associated with a shorter median time to relapse and death [10].

In particular, using MDA-MB-231 and BT-549 cells as a model, we sought to investigate the cellular and molecular effects of mCHT vs. STD schedule of administration of different combinations of 5FU and VNR, in an attempt to elucidate the underlying mechanisms of their antiproliferative activities. We found that the exposure of MDA-MB-231 and BT-549 cells to 5FU/VNR inhibited the growth of cells at nanomolar concentrations and induced expression of cell death modulators such as Bax and cleaved caspase-3 and of autophagy such as LC3A/B. Moreover, we observed increased activity of SA-β-gal in cells treated with 5FU/VNR under mCHT. To investigate the mechanisms involved in 5FU- and VNR-induced growth suppression, we carried out flow cytometric analysis. Biziota et al. [30], reported that these drug concentrations did not have an obvious effect on the cell cycle. In our cellular model metronomic treatment showed lesser modification on cell cycle than STD administration of drugs. Conversely, the percentage of cell death increased significantly after treatment with VNR in both standard and metronomic schedules and also when cells were under exposure of both 5FU and VNR. These results suggest that VNR alone or in combination with 5FU induces a cytotoxic effect on MDA-MB-231. A recent study has reported that activation of autophagy upon drug treatment can induce cell death either independently of or in parallel with apoptosis [13]. Autophagic

cell death is mainly a morphologic definition (i.e. cell death associated with autophagosomes/autolysosomes), therefore, to understand whether autophagy was involved in the death of MDA-MB-231 and BT-549 cells treated with 5FU and VNR, we evaluated the punctate pattern of distribution of LC3A/B, structural proteins of the autophagosomal membranes widely used as biomarkers of autophagy. In all three mCHT schedules of treatment (5FU, VNR and 5FU + VNR) LC3A/B puncta signal was observed in the perinuclear region and throughout the cytoplasm. STD instead caused a strong rearrangement of the cytoskeleton not associated with the LC3A/B clustering typical of autophagosome membrane formation. In addition, we also observed that VNR induces apoptosis cell death either in STD or mCHT schedule of treatment in both cell lines. Even though morphological studies cannot prove a causative relationship between the autophagic process and cell death they are suggestive of a correlation between the two phenomena.

Shimizu S et al. [31] indicated that cytotoxic stimuli activate autophagic death in cells that are protected against apoptosis, such as those expressing antiapoptotic Bcl-2 or those lacking both Bax and Bak. In our in vitro mCHT vs STD models we observed that all treatments decreased Bcl-2 protein expression compared to untreated cells. Furthermore, increased Bax expression was elicited by all treatments which is consistent with induction of apoptotic cell death. Indeed, the increased expression of Bax correlated with up-regulation of cleaved caspase-3 levels in 5FU mCHT treatment whilst in VNR mCHT

and in combo mCHT we observed a lesser increase of cleaved caspase-3 in both cell lines. We also evaluated whether treatments with 5FU and VNR could modulate LC3A/B expression in MDA-MB-231 and BT-549 cell lines. We found a significant increase in LC3A/B expression in cells upon exposure to 5FU and VNR alone that was further increased after co-exposure to 5FU and VNR under mCHT. Finally, we observed induction of cell senescence upon exposure to 5FU and VNR under mCHT way of administration. Cellular senescence is traditionally considered as a tumor-suppressive mechanism that irreversibly blocks cellular proliferation in response to a variety of stresses such as DNA damage, telomere attrition or cancer therapy [32]. Nevertheless, senescent cells have also been shown to promote neoplastic transformation and their autophagy-mediated elimination has been found to delay tumor growth [33]. In our study we found that cells treated with VNR alone or in combination with 5FU under mCHT express high levels of LC3A/B, are SA-β-gal positive and show low levels of cleaved casp-3 suggesting that autophagy and cellular senescence contribute more than apoptosis to the growth suppressive effect triggered by metronomic therapy. On the other hand, the major contribution to the growth suppressive effect on the STD regimen seems to rely on the induction of apoptosis and to a lesser extent to the triggering of autophagy. Thus, our results indicate that depending on the modality of administration of chemotherapy, TNBC cells respond differently by favoring senescence/autophagy vs apoptosis or concomitant induction of

both. The findings that the activation of senescence, autophagy, and apoptosis are dose- and schedule-dependent and that these processes contribute to a different extent to the therapeutic outcome are particularly relevant taking into account that cancer cells are frequently defective at or become resistant to apoptosis: in these cases, triggering cellular senescence and autophagy could represent an alternative pathway to suppress cell growth and promote cell death. Accordingly, it has been shown that several anticancer agents induce autophagic cell death in cell lines and animal models and finally promote tumor regression [32, 34].

In conclusion, our data give novel insights and help to understand which molecular mechanism involved in the cell death of TNBC are triggered by the different chemotherapeutic treatments and/or schedules, even though much remains to be discovered in terms of the cross-talk between signals that mediate autophagy and apoptosis [35]. Therefore, improving our understanding of the mechanisms and relationships between conventional drugs, metronomic chemotherapy, and autophagy in the clinical setting is an important research topic. Such an approach will allow us to develop novel anticancer treatments that target signal transduction pathways related to cancer cell death.

Materials and methods

Materials. Human breast cancer cell line, MDA-MB-231 was purchased from American Type Culture Collection (ATCC, VA,

USA). BT-549 cells were a kind gift from Dr. Luisa Lanfrancone (European Institute of Oncology, Milano, Italy). Cells were maintained in RPMI-1640 medium (Gibco, NY, USA), supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/ml penicillin and 100 mg/ml streptomycin (Euroclone). The cultures were incubated at 37° C in a humidified atmosphere with 5% CO2. Cells were passed every 2–3 days to obtain exponential growth. Cells were cultured for a maximum of 4 weeks before thawing fresh, early passage cells. Cells were routinely tested for the presence of Mycoplasma by Hoechst stain.

5FU (Fluorouracil Teva®) was from San Gerardo Hospital (Monza, Italy). Vinorelbine ditartrate salt hydrate (VNR) was purchased from Sigma-Aldrich and then resuspended in DMSO following the manufacturer instructions. Methyl thiazolyl tetrazolium (MTT) was purchased from Sigma-Aldrich.

MTT assay. Cell viability was assessed using the MTT assay. MDA-MB-231 and BT-549 cells were plated at a density of 1500 cells/well in 96-well plates [36]. The following day, the medium was replaced with a growth medium containing either 5FU (10–200000 nM) or VNR (0.1–1000 nM). In the 96-h experiment, to simulate the metronomic dosing protocol, we replaced it with fresh medium containing the appropriate drug concentration every 24 h, as reported by Biziota et al. [37]. To simulate the conventional administration protocol, which included exposure of MDA-MB-231 and BT-549 to 5FU or VNR for 4 h, with a washout period with drug-free medium for consecutive 92 h.

At the indicated time points, 1 mg/ml MTT was added to each well and cells were incubated for 3 hours. Then, plates were centrifuged at 2000 rpm for 10 minutes and cells were lysed with 100% Ethanol. The absorbance of formazan salt was measured at 540 nm using Infinite 200 Pro microplate reader (Tecan).
Results were expressed as a percentage of control cell proliferation and IC50 values were determined using Prism6 software (GraphPad Software Inc., La Jolla, California, USA).

Drugs combination studies. The IC50 values obtained from single-drug cell viability assays were used to design subsequent drug combination experiments. We added to cells drugs at the following concentrations: 1) 2× IC50 (5FU) + 2× IC50 (VNR); 2) IC50 (5FU) + IC50 (VNR); 3) ½ IC50 (5FU) + ½ IC50 (VNR); 4) ¼ IC50 (5FU) + ¼ IC50 (VNR). The results obtained in the MTT assay were analyzed for synergistic, additive, or antagonistic effects using the combination index (CI) method of Chou-Talalay [12]. A CI was then determined using the equation: (D)1/(Dx)1+ (D)2/(Dx)2 which indicates that for x% inhibition of the dose, Dx, the combined additive effect for the sum of the fractional doses of each drug, (D)1/(D)2 and (D)2/(Dx)2 should be equal to unity. Instead, when CI < 1 the interaction is considered synergistic, when CI > 1 indicated antagonism.
Three independent experiments were performed with eight replicates per condition.

Flow cytometry. For flow cytometry analyses, MDA-MB-231 cells were seeded in 100 mm dishes at a density of 500000

cells/dish. The day after, cells were serum starved for 24 hours. Then, cells were treated with the IC50 concentrations of VNR and/or 5FU under metronomic and conventional protocols. At 96 hours of incubation, cells were detached by trypsinization and 2 × 106 cells were fixed with cold 70% Ethanol for 30 minutes. Then cells were washed with PBS and incubated with 20 ug/ml Propidium Iodide (Sigma Aldrich) and 0.2 mg/ml RNAse A (Life Technology) for 2 hours. Propidium Iodide incorporation was analysed with a FACSaria flow cytometer (Becton Dickinson).

Immunofluorescence. MDA-MB-231 and BT-549 cells were plated in an eight-chamber slide (Nunc) at a density of 2000 cells/well. The day after, cells were treated with the IC50 concentrations of VNR and/or 5FU under metronomic and conventional protocols. At 96 hours of incubation, cells were washed with PBS and fixed with 1:1 methanol-acetone (Sigma Aldrich) for 10 minutes at –20° C. Then, cells were washed three times with PBS and blocked with 3% BSA in TBS-Tween at room temperature for 1 hour. Cells were incubated at 4° C overnight with the following primary antibodies: anti-LC3A/B XP Rabbit mAb (1:100 Cell Signaling). The day after, cells were washed with 0.1% Triton x-100 TBS and beta actin was stained by incubation with Alexa fluor 555 Phalloidin (1:150; Invitrogen). After washing with PBS, nuclei were stained with DAPI (Sigma Aldrich). Stained cells were imaged by using a confocal microscope (Biorad Laboratories, Hercules CA, USA) equipped with a Krypton/Argon laser and a red laser diode. Noise reduction was achieved by “Kalman filtering” during acquisition. All

experiments were repeated at least three times and representative micrographs are shown in the Figures.

Western blotting. MDA-MB-231 and BT-549 cells were seeded in 100mm dishes at a density of 500000 cells/dish. The following day, cells were treated with the IC50 concentrations of VNR and/or 5FU under metronomic and conventional protocols for 96 hours as described above. Cells were trypsinized and lysed in an ice-cold RIPA buffer supplemented with 5 µg/ml aprotinin, 5 µg/ml leupeptin and 1 mM phenyl methyl sulphonyl fluoride. Protein concentration was measured by the BCA method (Sigma Aldrich). 30 µg of proteins were loaded and electrophoresed through 4–20% Tris-Glycine gels in Tris-Glycine running buffer (all were Novex, San Diego, USA) for 2 hrs at 100 volts. Then proteins were transferred to the nitrocellulose membrane by using the iBlot system (Invitrogen) following manufacturer's instructions. Membranes were blocked with 5% milk solution for 1 hour and incubated at 4° C overnight with the following primary antibodies: monoclonal rabbit anti-LC3A/B XP (1:1000 Cell Signaling), monoclonal mouse anti-human Bcl-2 (1:500, Cell Signaling), polyclonal rabbit anti-human Bax Ab (1:500, Santa Cruz), monoclonal mouse anti-human cleaved-Caspase 3 5A1E Cell Signaling (1:500, Zymed, CA, USA), Vinculin (1:5000, Sigma-Aldrich). After three washes with 0.05% PBS Tween, membranes were incubated at room temperature for 1 h with appropriate secondary antibody diluted in 5% nonfat dry milk in TBST. After three washing with 0.05% PBS-Tween, membranes were incubated with "Pierce™ ECL Western Blotting Substrate"

for 5 minutes and proteins were detected using G:BOX Chemi System device (SynGene; Cambridge, UK). Immunoblotting was repeated at least three times with consistent results.

Senescence-associated β-galactosidase activity assay. Senescence-associated β-galactosidase (SA-β-gal) activity was measured with a β-galactosidase staining kit (Sigma- Aldrich) according to the manufacturer's protocol. Briefly, cells were treated with the IC50 concentrations of VNR and/or 5FU under metronomic and conventional protocols as described above. After 96 hours TNBC cells were washed with PBS, fixed and stained with the β-galactosidase reagent. Cells were incubated overnight at 37° C without CO_2 and then observed under a microscope. SA-β-gal positive blue-stained cells were counted per each observed field and the results reported as mean of percentages of cells per treatments (magnification 60X).

Statistical analyses. Results were indicated as mean ± standard error of the mean (S.E.M.) or mean ± standard deviation (S.D.). Statistical analysis was performed using SPSS 17 software. Student's t-test was used to compare data between two treatment groups. Differences between more than two experimental groups were determined with one-way analysis of variance (ANOVA), and when significant differences among groups were found, a post hoc analysis (Tukey test) was used. A value of $p < 0.05$ was considered statistically significant.

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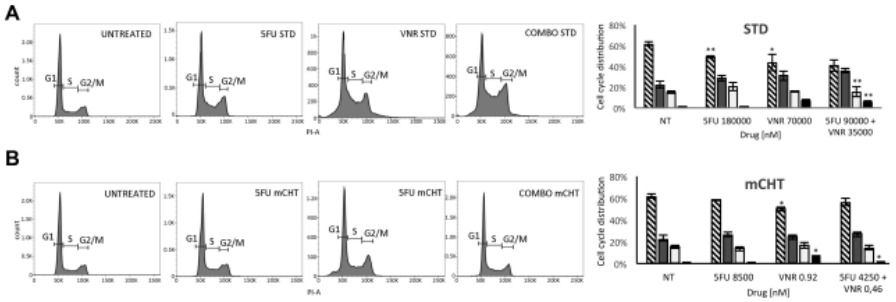
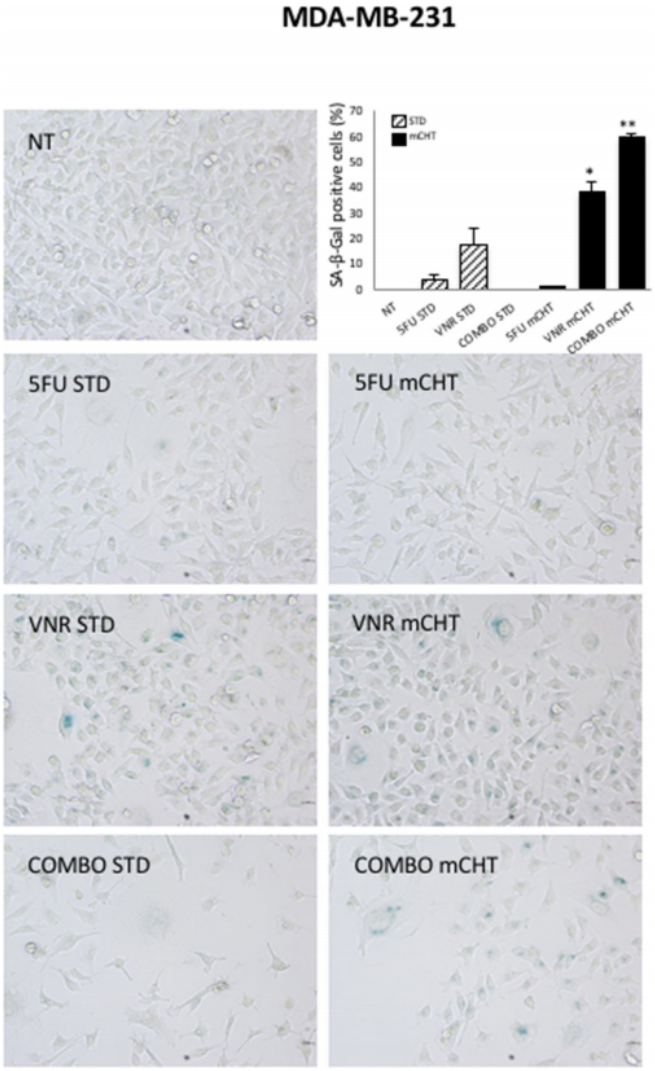
Supplementary materials



Supplementary Figure 1. Changes in cell cycle phase distribution following 5FU and VNR treatment. *(A) MDA-MB-231 cells were seeded in DMEM supplemented with 10% fetal calf serum, and 24 h before treatment were synchronized in the cell cycle by serum starvation. Cells in serum-free medium were exposed to 5FU and VNR alone or in combination for 4 h (STD regimen) or 96 h (mCHT regimen) before cell cycle analysis was performed by FACS after propidium iodide DNA staining. (B) Cell cycle distribution after STD or mCHT treatment with 5-FU and VNR alone or in combination. *p < 0.05 vs. untreated (NT), **p < 0.01 vs. untreated (NT).*



Supplementary Figure 2. Metronomic administration of 5FU and VNR induced a significant increase of the SA-βGal positive MDA-MB-231 cells. *(A) Representative images of SA-β-GAL-stained blue-senescent MDA-MB-231 cells treated with 5FU and VNR alone (IC50 single drug) or in combination (IC50 combo) for 4 h (STD) and for 96 h (mCHT). (B) The ratio between the SA-β-galactosidase positive blue-stained cells and total nuclei was calculated for each observed field and the results are reported as the percentages of senescent cells per treatment. Error bars represent mean ± SEM, n = 3. * p < 0.05 vs VNR STD, **p < 0.01 vs COMBO STD.*

Chapter 3

Metronomic administration of 5-Fluorouracil plus Vinorelbine inhibits both endothelial and triple-negative breast cancer cell regrowth and migration via FAK/VEGFR2 downregulation and autophagy/apoptosis activation

Scagliotti A.[1], Grassilli E.[1], Cazzaniga M.E.[1,2], De Giorgi[1], Lavitrano M.[1] and Cerrito M.G.[1]

[1] Department of Medicine and Surgery, University of Milano-Bicocca, Monza 20900, Italy
[2] Oncology Unit, ASST Monza, Monza 20900, Italy
[3] Phase 1 Research Centre, Monza 20900, Italy
[4] M. Tettamanti Research Center, Pediatric Clinic, University of Milano Bicocca, Monza 20900, Italy

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Abstract

Background Maximum tolerated dose standard-of-care chemotherapy is the only option for triple-negative breast cancer (TNBC) patients, who eventually succumb to their desease due to distant metastases. Recently, metronomic chemotherapy showed advantages in treating TNBC leading us to investigate the anti-metastatic and anti-angiogenic potential of metronomic 5-Fluorouracil plus Vinorelbine (5-FU+VNR) on endothelial cells (ECs) and TNBC cells.

Methods MTT, colony, Transwell and scratch *in vitro* assays were used to evaluate viability, colony formation and migration capability after standard and metronomic treatment with 5-FU+VNR. Molecular changes triggered by standard and metronomic treatments were evaluated by western blot analysis.

Results 10-fold lower doses of 5-FU+VNR given metronomically *vs.* standard are effective in inhibiting cell proliferation and survival of both ECs and TNBC cells. Although both standard and metronomic treatments strongly affect the migration of ECs, only the latter dramatically block TNBC cell migration. Metronomically-treated TNBC cell-derived conditioned medium also strongly affect EC migration. In both ECs and TNBC cells, either standard or metronomic schedules of treatment disrupt FAK/VEGFR signaling. Whereas only metronomic treatment is cytotoxic on ECs inducing apoptosis, it switches the modality of cell death from apoptosis (as induced by standard treatment) to autophagy in TNBC cells.

Conclusions Metronomic administration of 5-FU+VNR is more effective in controlling cell proliferation/survival and migration of both ECs and TNBC cells compared standard administration and causes a strong anti-angiogenic effect. Our data suggest that the stabilization of tumor growth observed in TNBC patients treated with a metronomic 5-FU+VNR therapy schedule is likely due not only to direct cytotoxic effects but also to anti-invasive and anti-angiogenic effects.

Keywords: Metronomic chemotherapy - triple negative breast cancer - endothelial cells - cell migration - FAK - VEGFR2.

Abbreviations

5-FU	5-Fluorouracil
c-mCHT	Conditioned medium – metronomic chemotherapy
c-NT	Conditioned medium – untreated
c-STD	Conditioned medium – standard chemotherapy
ECs	Endothelial cells
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FDA	Food and drug administration
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
HUVECs	Human umbilical vein endothelial cells
LC3AB-I/II	Light chain 3 AB-I/II
mCHT	Metronomic chemotherapy

MMP2	Matrix metalloproteinase 2
NT	Untreated
OS	Overall survival
PR	Progesterone receptor
STD	Standard chemotherapy
TIMP2	Tissue inhibitor of metalloproteinases 2
TNBC	Triple negative breast cancer
UFT	Uracil plus tegafur
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VNR	Vinorelbine

Introduction

Triple-negative breast cancer (TNBC) is an aggressive histological subtype of breast cancer characterized by the lack of estrogen receptor (ER) expression, progesterone receptor (PR) expression, and lack of amplification/overexpression of human epidermal growth factor receptor 2 (HER2). Accounting for about 12-17% of all breast carcinomas [1], TNBC is more aggressive than other breast tumors, and it is often correlated with short disease-free survival (DFS) and overall survival (OS) [2,3]. Chemotherapy remains the primary therapeutic option for TNBC patients because neither endocrine therapies nor HER2-targeted agents can be used in this subtype of breast cancer. Several studies have shown that TNBC express high levels of intratumoral vascular endothelial growth factor (VEGF) [4] and display VEGF gene amplification compared to non-TNBCs [5],

suggesting an angiogenic dependency of TNBCs and thus a potential sensitivity to anti-angiogenic factors. Despite numerous drugs having been approved for anti-angiogenic therapies, their success has been quite limited, providing only a short pause in tumor growth before the onset of drug-resistance, thus often allowing for only a modest survival benefit [6]. In addition, many cancers can gain access to blood supply through vascular co-optation, thus evading the need for tumor angiogenesis [7].

Angiogenesis is a multistep process that involves different players, i.e. tumoral cells, immune system and endothelial cells (ECs), and the balance of anti-angiogenic and pro-angiogenic stimuli is the main regulatory mechanism of the process. During cancer progression, the tumor microenvironment disrupts this balance in favor of stimuli that promote the proliferation and migration of ECs [8] which are among the principal players in angiogenesis; in fact, their responses to extracellular stimuli such as VEGF are essential during growth and subsequently in the adult age as well [9]. Among different VEGF receptors, VEGFR2 has been identified as the principal mediator of many physiological and pathological consequences of VEGF on ECs, including proliferation, migration, survival and vascular permeability [10]. One of the downstream signaling mediators following VEGFR2 activation is focal adhesion kinase (FAK) [11], which is crucial for EC migration. Indeed, activated FAK is recruited to new focal adhesions where it phosphorylates paxillin thereby leading to the cytoskeletal rearrangements necessary for ECs to migrate [12]. Other than in ECs, FAK also plays a role in

cancer cells: it has been shown that high FAK expression in breast tumors is associated with more aggressive tumor types such as lymphovascular invasion and triple-negative phenotype [13]. In addition, Pan et al. indicated FAK as a prognostic marker in patients with TNBC [14] thus suggesting that novel combinations of drugs targeting FAK may be useful in patients that progress or fail to respond [15].

Microtubule-targeting drugs, such as taxanes, or vinca alkaloids, such as Vinorelbine (VNR), are commonly used in the therapy of TNBC [16,17]. Moreover, the addition of Capecitabine to standard chemotherapy (STD) can result in significant improvements in both DFS (HR 0.82, P = 0.004) and OS (HR 0.78, P = 0.004) in patients with early-stage TNBC [18] and provided significant benefits in the metastatic setting, the two agents being highly synergistic [19,20]. STD chemotherapy is usually given at maximum tolerated doses for several cycles, and necessitates prolonged drug-free breaks between successive drug administrations. Metronomic chemotherapy (mCHT) refers instead to the minimum biologically effective dose of a chemotherapeutic agent given as a continuous regimen with no prolonged drug-free breaks. This schedule seems to have a direct cytotoxic effect on cancer cells and an effect on the tumor microenvironment, likely by inhibiting tumor angiogenesis [21,22]. In the early 2000s, several preclinical reports showed an anti-angiogenic activity of some anti-tumor agents when administrated frequently and at low-doses for prolonged periods of time [23–25]. In *in vivo* models of hepatocellular carcinoma

cyclophosphamide given in a metronomic fashion (mCHT) significantly reduced tumor growth and metastasis compared to STD drug administration, thus showing anti-proliferative, anti-angiogenic and anti-metastatic properties of the drug [26].

Many clinical trials using a metronomic schedule are ongoing [27,28], and so far, the results show a strong stabilization of cancer growth along with improvement of the quality of life of cancer patients due to a reduction of the toxic side effects [29]. A clinical study with 80 advanced breast cancer patients, of whom 28 were TNBC, has shown an improvement of the clinical benefit rate and progression-free survival after the metronomic administration of VNR plus Capecitabine [30].

Previously, we demonstrated that the mCHT administration of 5-Fluorouracil (5-FU) - the active metabolite of Capecitabine - plus VNR can induce apoptosis and autophagy in TNBC cells at lower doses compared to the STD administration [31]. In the present study, we evaluated the effects of mCHT administration of 5-FU+VNR on ECs, and we report that this schedule of treatment strongly affects cell proliferation and survival even at 10-fold lower doses than the STD treatment. We also report that the combination of 5-FU+VNR strongly impairs EC migration and tube formation, as well as TNBC cell migration, via downregulation of the VEGFR2/FAK circuit. Finally, we show that a direct cytotoxic effect -via apoptosis induction - is triggered in ECs differently from what was reported for TNBC cells [31].

Methods and Materials

Cell lines

HUVECs were a kind gift from Prof. Adriana Albini (IRCCS MultiMedica, Milan, Italy) and were cultured in Endothelial Growth Medium-2 (EGM™-2 Medium, Lonza, Basel, Switzerland) supplemented with EGM™ SingleQuots™ Kit (Lonza, Basel, Switzerland). HUVECs were maintained in culture until passage 6. Human TNBC cell line MDA-MB-231 was purchased from American Type Culture Collection (ATCC, VA, USA) and cultured in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (Euroclone), 100 units/ml penicillin and 100 mg/ml streptomycin (Euroclone). Cells were routinely tested for the presence of Mycoplasma by Hoechst stain.

Cell treatments

HUVECs were plated at 2000 cells/well in 96-well plates coated with 0.25 µg/mL of Human Collagen Type I (Millipore Merck, Darmstadt, Germany). The following day cells were treated with increasing doses of 5-FU (Fluoruracil Teva®, obtained from San Gerardo Hospital, Monza, Italy) and VNR (Vinorelbine Ditartrate Salt Hydrate, Sigma-Aldrich). mCHT protocol: drug-containing medium was replaced every 24 hours up to 96 hours. STD protocol: after 4 hours of treatment, drug-containing medium was replaced with drug-free medium and this change was repeated every 24 hours up to 92 hours.

Cell viability assay

At the end of single and combined treatments, MTT (Methyl thiazolyl tetrazolium, Sigma-Aldrich) was added to each well at the concentration of 1 mg/ml. After 3 hours of incubation, cells were centrifuged at 2000 rpm for 10 minutes and lysed with 100% Ethanol. The values of absorbance of the formazan salt were measured at 540 nm with Infinite 200 Pro microplate reader (Tecan) and expressed as the percentage of the untreated control. IC_{50} values were calculated with Prism5 software (GraphPad Software Inc., La Jolla, California, USA). Graphs represent the average of 3 independent experiments ± standard deviation (SD).

The IC_{50} values obtained from single-drug treatments were used to design drug combination experiments as reported by Chou-Talalay [32]: cells were treated with 2X IC_{50}5-FU + 2X IC_{50}VNR, IC_{50}5-FU + IC_{50}VNR, ½ IC_{50}5-FU + ½ IC_{50}VNR and ¼ IC_{50}5-FU + ¼ IC_{50}VNR.

Colony formation assay

HUVECs and MDA-MB-231 cells were treated with the respective IC_{50s} of 5-FU+VNR under mCHT or STD schedule. At the end of treatments, surviving cells were trypsinized, counted and seeded at low density (1500 cells/well) in 6-wells plates. Medium was replaced every 3 days with fresh medium. After 10 days, colonies were fixed and stained with 1% crystal violet in 35% ethanol for 40 minutes. Images were acquired using G:BOX XT4 Chemiluminescence and Fluorescence Imaging System (Syngene, Cambridge, UK). The number of colonies was

counted with ImageJ Software (Wayne Rasband National Institutes of Health, USA) and reported as percentage of untreated control ± SD. Images are representative of three independent experiments.

Scratch assay

HUVECs and MDA-MB-231 cells were seeded at $2x10^5$ cells/well in 12-wells plate. The day after, confluent cells were scratched using a 200 µl pipette tip and washed twice with PBS. Cells were photographed immediately after injury (T_0) and then treated with the respective IC_{50s} of STD and mCHT protocol. At the end of treatments, pictures were taken and the change of the scratch wound size was evaluated by comparing the photos from time 0 to the 96h (the last time point) to obtain the measure of each scratch closure based on the distances that are measured by ImageJ software (Wayne Rasband National Institutes of Health, USA). Images are representative of three independent experiments.

Transwell Boyden chamber assay

HUVECs and MDA-MB-231 cells were treated with the respective IC_{50s} of 5-FU+VNR under mCHT or STD schedule. At the end of the treatments, surviving cells were detached with 0.25% Trypsin-EDTA solution, resuspended in serum-free DMEM, and counted within 3 to 5 min of mixing within trypan blue. Cells were seeded ($1x10^4$ HUVEC and $4x10^4$ MDA-MB-231) in 100 µl of serum-free DMEM in the upper chamber of 6.5 mm Transwell® chamber with 8.0 µm pores size polycarbonate

membrane filters (Corning Costar, Corning, NY). Then 600 µl of DMEM containing 10% FBS was added in the lower chamber as chemoattractant. After overnight incubation, cells remained in the upper surface of the membrane filter were removed with a cotton swab; cells migrated and adhered onto lower chamber were fixed with 3.6% formaldehyde for 20 min, permeabilized with 100% methanol and stained with crystal violet. Cells were counted using ImageJ Software (Wayne Rasband National Institutes of Health, USA) and reported as percentage of the untreated control ± SD. Images are representative of three independent experiments.

Tube formation assay

HUVECs were treated with the respective IC_{50s} of 5-FU+VNR under the mCHT or STD schedule. At the end of the treatments, cells were trypsinized, counted and seeded at $2x10^4$ cells/well in 96-wells plate previously coated with 80 µl of BD Matrigel™ matrix HC phenol red-free (BD Biosciences). After the 24h incubation, images were taken and the total length of the tubes and the total meshes area were measured using ImageJ software (Wayne Rasband National Institutes of Health, USA) and reported in graphs as percentage of untreated control ± SD. Images are representative of three independent experiments.

Indirect co-culture

Indirect co-culture was used to evaluate whether treated TNBC cells could affect endothelial cells via releasing factors in the medium. Conditioned media were collected from MDA-MB-231

cells treated with the IC_{50} of 5-FU +VNR under STD or mCHT schedule. The drug-containing medium of each time point of treatment was collected, mixed, centrifuged, and used to treat HUVECs. HUVECs cultured with conditioned medium from MDA-MB-231 pre-treated with STD and mCHT protocols are indicated, as c-STD and c-mCHT, respectively. HUVECs cultured with conditioned medium harvested from untreated MDA-MB-231 cells were used as control (c-NT). Scratch test, Transwell migration and colony formation assays were performed following incubation time with the two conditioned media.

Western Blot

HUVECs and MDA-MB-231 cells were treated with the respective IC_{50s} of 5-FU+VNR under the mCHT or STD schedule and lysed with RIPA buffer (HEPES 50 mM, pH 7.5, NaCl 500 mM, DTT 1 mM, EDTA 1 mM, 0.1% NP40) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy). Protein concentration was measured by the Bradford method (Sigma Aldrich). 25µg of proteins were loaded onto 10% NuPAGE Tris-Glycine protein gels or 4-12% NuPAGE Bis-Tris protein gels (Novex, San Diego, USA) and run for 2 hours at 100 V in Tris-Glycine Running buffer or MES Running buffer (Novex, San Diego, USA). Proteins were transferred to nitrocellulose membrane (Invitrogen) by the iBlot system, following by 1hour blocking solution with 5% BSA and incubation with the following primary antibodies: anti-pFAK (Y397) (1:1000, Cell Signaling), anti-FAK (1:1000, Cell Signaling), anti-VEGFR2 (1:1000, Cells Signaling), anti-VEGF (C-1) (1:500, Santa Cruz), anti-pERK

(pT202/Y204) (1:1000, Cell Signaling), anti-ERK (1:1000, Cell Signaling), anti-cleaved caspase3 (1:1000, Cell Signaling), anti-LC3AB (1:1000, Cell Signaling), anti-βactin (1:5000, GeneTex). After three washes with 0.05% PBS Tween, membranes were incubated at room temperature for 1 h with appropriate secondary antibody diluted in 5% nonfat dry milk in TBST. After three washing with 0.05% PBS-Tween, membranes were incubated with "Pierce™ ECL Western Blotting Substrate" for 5 minutes, and images were acquired using G:BOX XT4 Chemiluminescence and Fluorescence Imaging System (Syngene, Cambridge, UK).

Statistical analysis

Data are presented as means ± standard deviation (SD) of three independent experiments. The significance of results was determined with the Student's t-test. Values with $p<0.05$ are considered statistically significant. * means $p<0.05$, ** means $p<0.001$ and *** means $p<0.001$.

Results

Metronomic administration of 5-FU+VNR is more effective than the respective standard protocol in reducing HUVEC viability

To investigate the antiproliferative effects of STD versus mCHT administration of 5-FU+VNR on HUVECs, we treated cells with increasing doses of 5-FU or VNR for 4 hours (STD) or 96 hours (mCHT) and evaluated cell viability, compared to the untreated control cells, by MTT assay.

When HUVECs were exposed to the STD schedule, we observed the greatest inhibition only at the highest concentrations of the drugs tested (50μM for 5-FU and 100 nM for VNR), whereas only a modest effect was observed at lower concentrations.

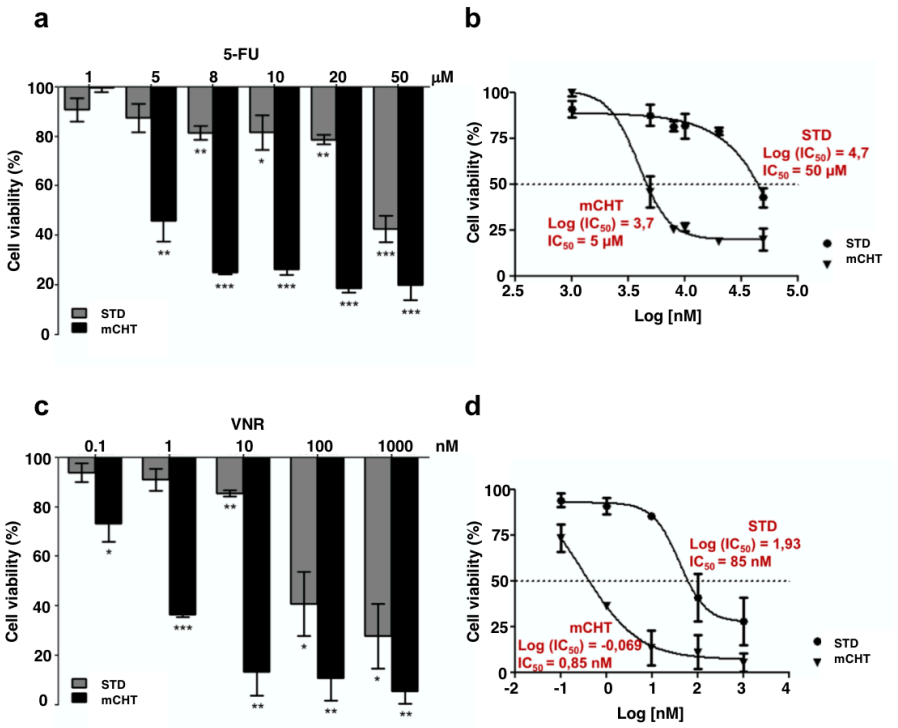

Fig.1 Metronomic administration of 5-FU or VNR strongly reduces HUVECs viability when using concentrations much lower than those used in STD treatment. *MTT assay performed on HUVECs at the end of STD or mCHT treatments with increasing doses of 5-FU* ***(a)*** *or VNR* ***(c)****. Dose-response curves were used to calculate the IC50 values of 5-FU* ***(b)*** *or VNR* ***(d)*** *treatments. Values represent the average±SD of three independent experiments and are expressed as the percentage of viability of treated vs. untreated cells *p <0.05; **p <0.01; ***p <0.001*

On the contrary, a strong antiproliferative effect was already observed at lower concentrations (5μM for 5-FU and 1nM for VNR) when HUVECs were exposed to mCHT 5-FU+VNR (Figs. 1a and 1c).

Indeed, compared to the STD administration of each drug, the IC_{50} of mCHT administration was about 10 fold lower for 5-FU (5μM *vs*. 50μM, $p<0.01$ *and* $p<0.001$) and 100 fold lower for VNR (0.85nM *vs*. 85nM, $p<0.001$ *and* $p<0.01$), as indicated by the dose-response curves in Figs. 1b and 1d.

Notably, the strong effects achieved by lower doses of chemotherapy are particularly relevant from the clinical point of view, since lowering the dose of the drugs, means less toxic side effects for patients.

Next, we performed an MTT assay to evaluate the cytotoxic effects of 5-FU+VNR given in combination (5-FU+VNR) on HUVECs. We found that the metronomic administration of 5-FU+VNR had a higher antiproliferative activity as compared to the STD administration (Fig. 2). The IC_{50s} 2.7μM 5-FU+0.48nM VNR *vs.* 25μM 5-FU+42nM VNR, respectively (Table 1), i.e. 9-fold less 5-FU and 87-fold less VNR are required in mCHT *vs.* STD treatment to achieve 50% inhibition of HUVECs. Therefore, mCHT administration allows to significantly reduce dose of the drugs compared to STD treatment, in line with what we previously reported for TNBC cell lines [31] (Table 1).

Altogether these data indicate that metronomic administration of 5-FU+VNR, strongly decreases HUVEC cell viability at concentrations much lower than those used for STD treatment.

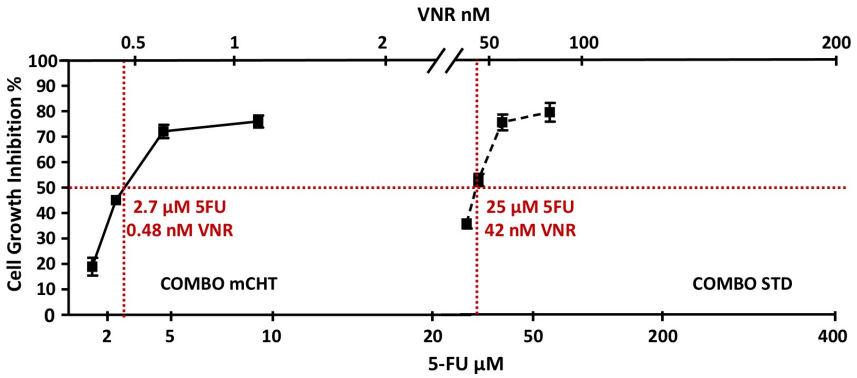

Fig.2 Metronomic treatment with the combination of 5-FU+VNR significantly reduces HUVEC viability compared to the standard protocol. *The dose-response curve obtained from MTT assay performed at the end of STD or mCHT treatment with the following concentrations: ¼ $IC_{50(5\text{-}FU)}$+ ¼ $IC_{50(VNR)}$; ½ $IC_{50(5\text{-}FU)}$+ ½ $IC_{50(VNR)}$; $IC_{50(5\text{-}FU)}$+ $IC_{50(VNR)}$; 2x $IC_{50(5\text{-}FU)}$+ 2x $IC_{50(VNR)}$. Increasing doses of 5-FU are reported on the lower x-axis and increasing doses of VNR are reported on the upper x-axis. The simple two-point method was used to estimate the IC_{50s} (reported in red) from 2 data points that induce 50% proliferation inhibition (red lines). Values represent the average±SD of three independent experiments and are expressed as the percentage of viability of treated vs. untreated cells*

	IC_{50}	
	STD	mCHT
HUVEC	5-FU+VNR	
	25 µM + 42 nM	2.7 µM + 0.48 nM
MDA-MB-231	5-FU+VNR	
	80 µM + 30 nM	4.5 µM + 0.5 nM

Table 1 The combination of 5-FU+VNR given under metronomic protocol affects the viability of HUVECs and TNBC cells in the same range of doses. *Comparison of the IC_{50} values obtained in Fig. 2 treating HUVECs with 5-FU+VNR given mCHT or STD with those we previously reported for MDA-MB-231 cells [31]*

Metronomic combination of 5-FU+VNR suppresses colony formation ability of both HUVECs and TNBC cells

Tumor relapses often occur after STD, due to proliferation of surviving cancer cells and the restoration of damaged tumor vessels. To determine whether HUVECs and MDA-MB-231 cells retain the capacity of proliferating after mCHT or STD treatment we replaced the medium with drug-free complete medium and measured colony formation after 10 days.

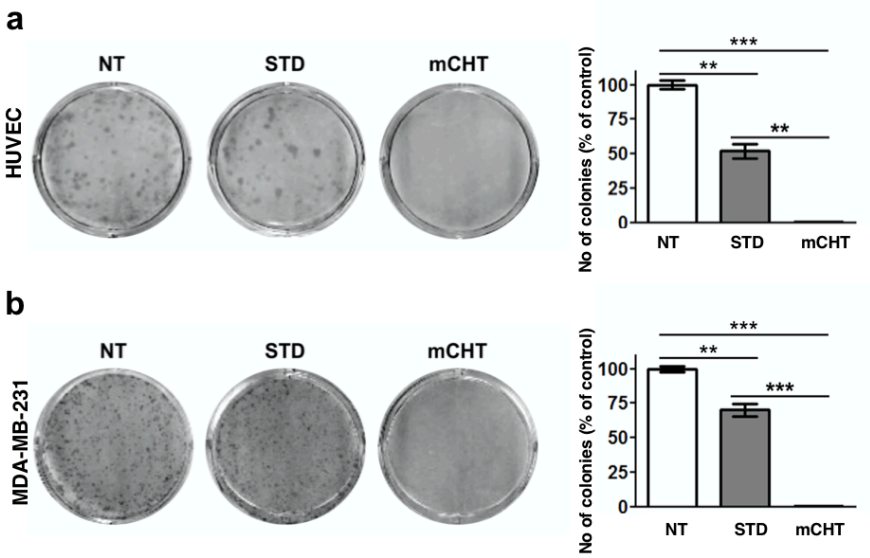

Fig.3 Metronomic administration of 5-FU+VNR is more effective than standard treatment in reducing HUVECs and TNBC cell colony formation. *Representative images of colony formation assay performed at the end of STD or mCHT combination treatments of HUVECs* ***(a****) and MDA-MB-231 cells (****b****). Colonies formed after 10 days without drugs were stained with crystal violet and counted. On the right of each panel: number of colonies grown after treatments quantified as a percentage of untreated controls. Values represent the average±SD of three independent experiments. **p <0.01; ***p <0.001*

Compared to untreated control cells, HUVECs' capability to form colonies is reduced to half by 5-FU+VNR given STD (51%, $p<0.01$) whereas it is completely suppressed by the mCHT administration ($p<0.001$) (Fig. 3a).

Similarly, 5-FU+VNR-treated MDA-MB-231 cells ability to form colonies was reduced to 70% ($p<0.01$) under STD protocol, whereas it was completely abolished under mCHT schedule ($p<0.001$) (Fig. 3b).

These findings indicate that, even though ECs are more sensitive than TNBC cells to 5FU+VNR given at STD, as usually observed with many drugs, both cell types are extremely sensitive to the metronomic combination.

Metronomic combination of 5-FU+VNR strongly reduces cell migration of both HUVECs and TNBC cells

Cell migration is a critical process in tumor progression for both new vessel formation and metastasis propagation. Cell migration was evaluated through the scratch test on HUVECs and MDA-MB-231 cells treated with the respective IC_{50s} of 5-FU+VNR given under the STD or mCHT schedule. The migratory ability of HUVECs is significantly reduced by the administration of 5-FU+VNR under both STD and mCHT protocols. Indeed, about 66% ($p<0.01$) and 85% (*ns*) of the scratched areas are still open at the end of STD and mCHT administration of 5-FU+VNR, respectively, as shown in Fig. 4a.

In contrast, MDA-MB-231 cells' migratory ability is unaffected by the STD treatment, whereas it is significantly reduced under

mCHT schedule with 5-FU+VNR, (2% *vs.* 60% of the scratched area; p<0.001 and p<0.01) (Fig. 4b).

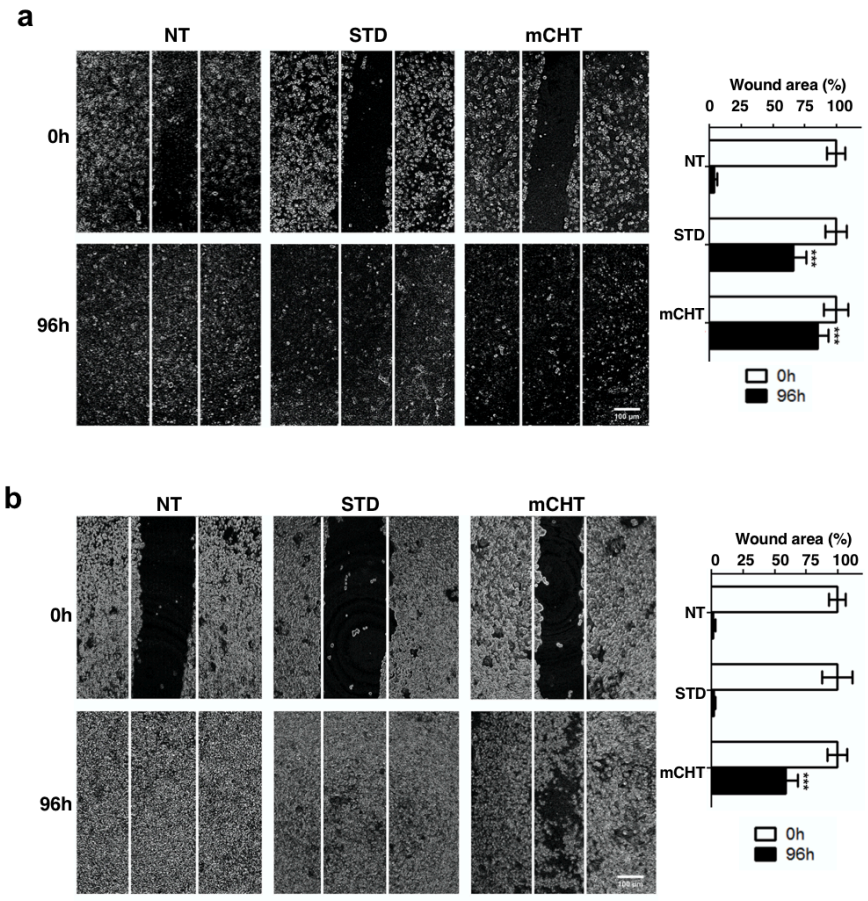

Fig.4 Metronomic administration of 5-FU+VNR is more efficient than standard protocol in inhibiting wound closure of HUVECs and TNBC cells. *Representative images of scratch test performed on HUVECs (**a**) and MDA-MB-231 cells (**b**) before (0h) and 96h after STD or mCHT treatment with 5-FU+VNR. The area of the still open wound after 96hs is quantified as a percentage of the initial scratch. Values represents the average±SD of three independent experiments, **p <0.01; ***p <0.001*

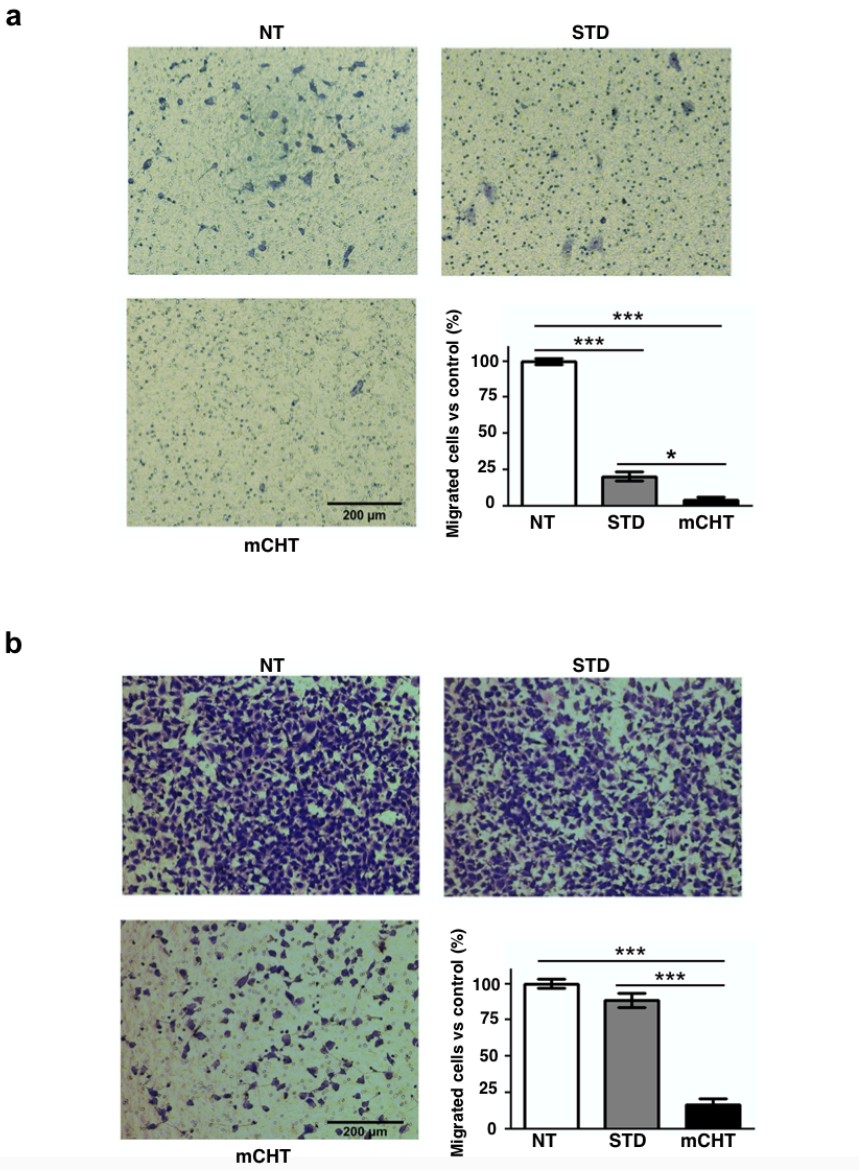

Fig.5 Metronomic treatment with 5-FU+VNR suppresses HUVECs and TNBC cell migration is more efficient than standard administration. *Representative images of the Transwell assay performed on HUVECs (**a**) and MDA-MB-231 cells (**b**) at the end of STD or mCHT combination treatments. Migrated cells were stained with crystal violet, counted and graphically expressed as a percentage of the untreated control. Values represents the average±SD of three independent experiments, **p <0.01; ***p <0.001*

Next, we examined the migratory capability of HUVECs and TNBC cells under mCHT and STD treatments by using the Transwell assay system. Migrated cells were evidenced by crystal violet staining and then counted with Image J, as shown in Fig. 5. The number of HUVECs migrated across the membrane is strongly reduced by 5-FU+VNR given as STD (20.3% *vs*. NT, *p <0.001*) and mCHT (3% *vs.* NT, p <0.001) (Fig. 5a). Importantly, the metronomic administration of 5-FU+VNR resulted in less migrated cells than the standard administration (p<0.05). On the contrary, the migration of MDA-MB-231 cells was strongly suppressed only when the drug combination was given as mCHT; in fact, compared to the untreated control, the percentage of migrated cells is 16% (p <0.001) after mCHT treatment *vs.* 88% after STD (Fig. 5b).

To further characterize how cell migration may be affected by the 5-FU+VNR treatment, we analyzed Matrix metalloproteinase 2 (MMP2) and Tissue inhibitor of metalloproteinases 2 (Timp2) expression, whose activities are essential for matrix degradation during neo-angiogenesis and metastasis formation [33]. Both STD and mCHT administration of 5-FU+VNR similarly reduced MMP-2 expression and strongly upregulated its inhibitor TIMP-2 in HUVECs (Fig. 6a). Conversely, we did not observe evident effects on TIMP-2 and MMP-2 expression by either the mCHT or STD on MDA-MB-231 cells (Fig.6b).

Altogether, our data show that the combination of 5-FU+VNR reduces cell migration both in HUVECs and TNBC cells.

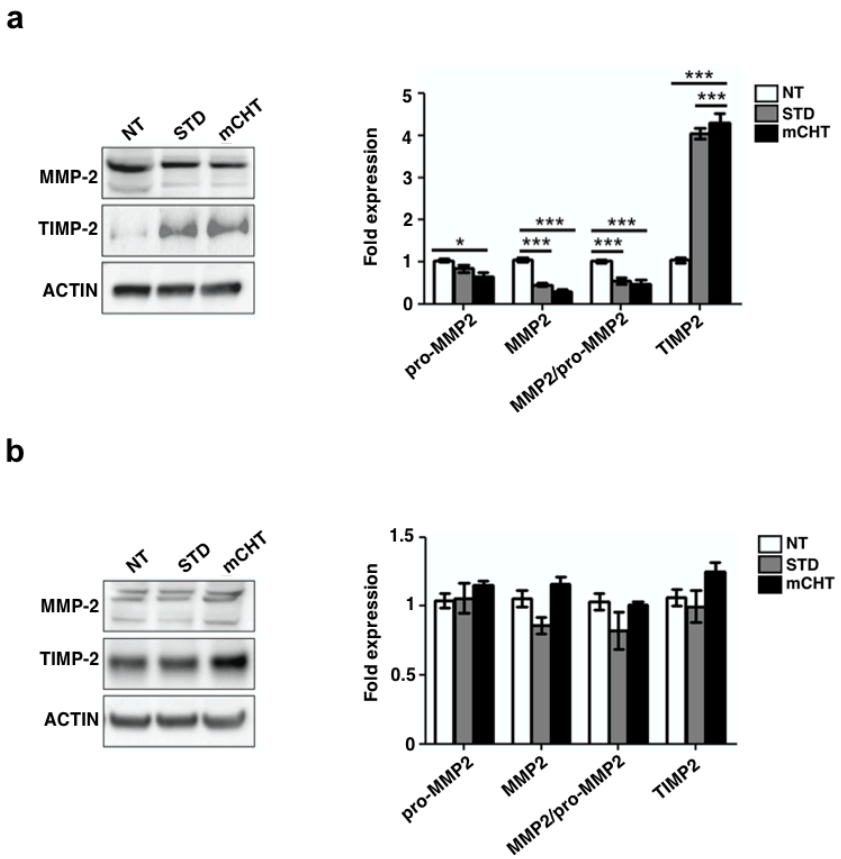

Fig.6 Both metronomic and standard administration of 5-FU+VNR affect TIMP-2/MMP2 expression only in HUVECs. *Representative western blot of HUVECs (**a**) and MDA-MB-231 cells (b) treated with mCHT (96hs) or STD (4hs) 5-FU+VNR. On the right of each panel: quantifications of protein expression levels were normalized to the loading control actin, compared to the untreated control ± SD of three independent experiments is reported. *p <0.05; ***p <0.001*

Metronomic and standard treatments with 5-FU+VNR disrupt FAK/VEGFR2-mediated signaling in HUVECs and TNBC cells and elicit different cell death modalities

Then, we sought to investigate the molecular mechanisms by which 5-FU+VNR STD and mCHT treatments affect HUVECs

and MDA-MB-231 cells motility, proliferation and viability. FAK phosphorylation as well as expression levels are reduced by both STD and mCHT treatment in both cell lines (Figs. 7a and 7b). FAK is involved in the regulation of angiogenesis via the transcription of VEGF and VEGFR2 [11,34,35]. In agreement with the observed downregulation of FAK expression and activation, both STD and mCHT administration of 5-FU+VNR resulted in reduction of VEGFR2 expression both in HUVECs and MDA-MB-231 cells. Differently, VEGF is downregulated by STD and mCHT treatments in HUVECs (Fig. 7a), but not in MDA-MB-231 cells (Fig. 7b). Proliferation, migration, and survival are strictly controlled also by ERK activity [36]. In both HUVECs and MDA-MB-231 cells, we observed that ERK phosphorylation is slightly reduced by STD administration of 5-FU+VNR whereas it is modestly increased by mCHT. Interestingly, mCHT treatment increased total ERK levels both in HUVECs and in MDA-MB-231, whereas STD treatment increased ERK expression only in MDA-MB-231 cells. Increased levels of ERK expression and phosphorylation, may be related to cell growth arrest and apoptosis activation [37]**.** Accordingly, we found caspase-3 cleaved in HUVECs only after mCHT treatment (Fig. 7a) and in MDA-MB-231 cells after both STD and mCHT treatments (Fig. 7b). Several studies suggest the involvement of ERK also in autophagy activation [38,39]. In HUVECs, both STD and mCHT treatments strongly downregulated the autophagy marker LC3AB-I and slightly induced its processing to LC3AB-II which is indicative of autophagosome formation (Fig. 7a).

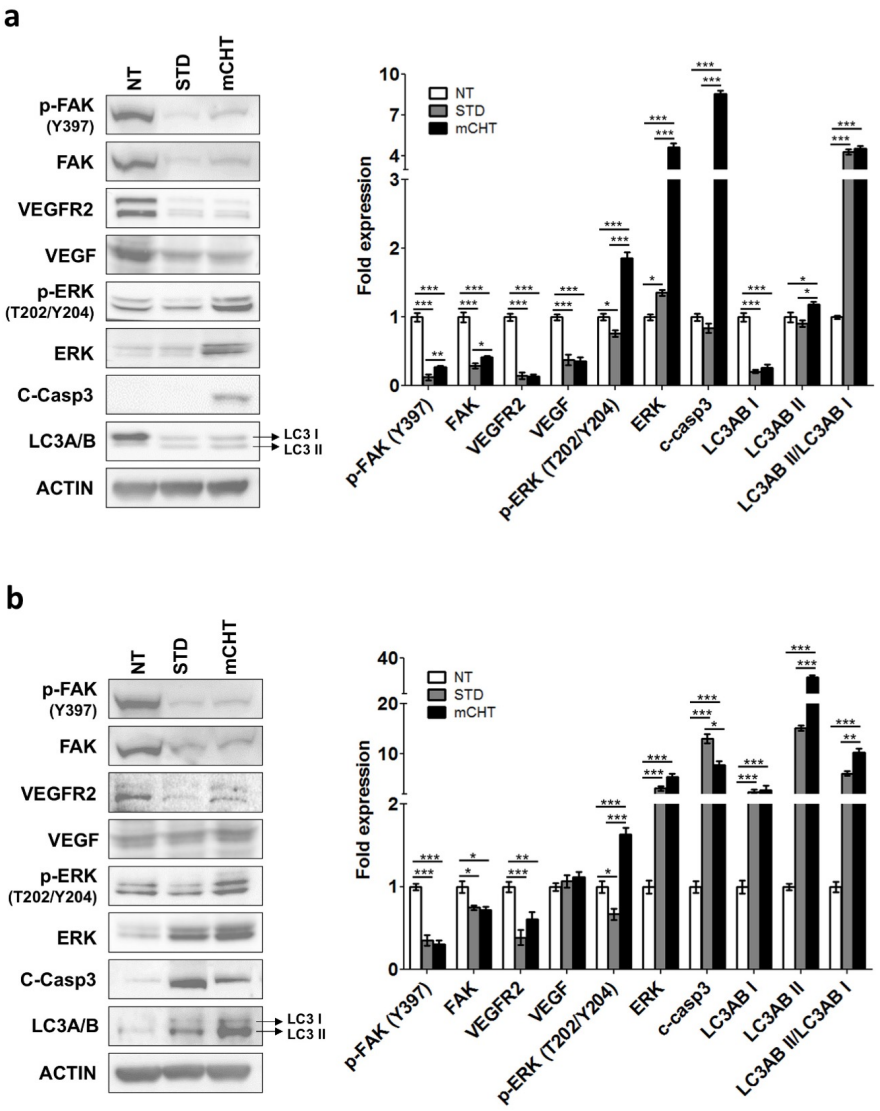
a
NT
STD
mCHT
p-FAK (Y397)
FAK
VEGFR2
VEGF
p-ERK (T202/Y204)
ERK
C-Casp3
LC3A/B
LC3 I
LC3 II
ACTIN
Fold expression
NT
STD
mCHT
p-FAK (Y397)
FAK
VEGFR2
VEGF
p-ERK (T202/Y204)
ERK
c-casp3
LC3AB I
LC3AB II
LC3AB II/LC3AB I
b
NT
STD
mCHT
p-FAK (Y397)
FAK
VEGFR2
VEGF
p-ERK (T202/Y204)
ERK
C-Casp3
LC3A/B
LC3 I
LC3 II
ACTIN
Fold expression
NT
STD
mCHT
p-FAK (Y397)
FAK
VEGFR2
VEGF
p-ERK (T202/Y204)
ERK
c-casp3
LC3AB I
LC3AB II
LC3AB II/LC3AB I

Fig.7 Metronomic and standard treatment with 5-FU+VNR downregulate FAK/VEGFR2 axis and differently activate apoptosis and autophagy in HUVECs and TNBC cells. *(**a**) Representative western blots of HUVECs treated with STD or mCHT 5-FU+VNR. On the left: Quantification of protein expression levels compared to the untreated controls. Actin was used as a loading control. Values represents the average±SD of three independent experiments, *p <0.05; **p <0.01; ***p <0.001. (**b**) Representative western blot of MDA-MB-231 cells treated with STD or mCHT 5-FU+VNR. On the left: Quantification of protein expression levels compared to the untreated controls. Actin was used as a loading control. Values represents the average±SD of three independent experiments, *p <0.05; **p <0.01; ***p <0.001*

In MDA-MB-231 cells, both treatments induced LC3A/B-I and its complete processing to LC3AB-II (Fig. 7b).

Overall, these data show that 5-FU+VNR, either given STD or mCHT, strongly downregulates the VEGF/FAK signaling; however, only the metronomic administration protocol results in cytotoxicity in both cell types even though with different modalities.

Metronomic treatment with 5-FU+VNR is more effective than standard treatment in disrupting neo-angiogenesis

In the context of tumor growth, not only EC migration and survival is crucial, but also the ability of ECs to form new vessels. To assess whether the 5-FU+VNR treatment can also affect neo-angiogenesis, we performed a tube formation assay on HUVECs treated with 5-FU+VNR given STD or mCHT (Fig. 8). As shown in Fig. 8a-c, both the total tube length and the total meshes area are mildly reduced by STD as compared to control cells. On the contrary, the mCHT administration of 5-FU+VNR resulted in 50%

reduction of total tube length and meshes area as compared to control cells. These data show that the mCHT schedule is much more effective in disrupting neo-angiogenesis than the STD schedule.

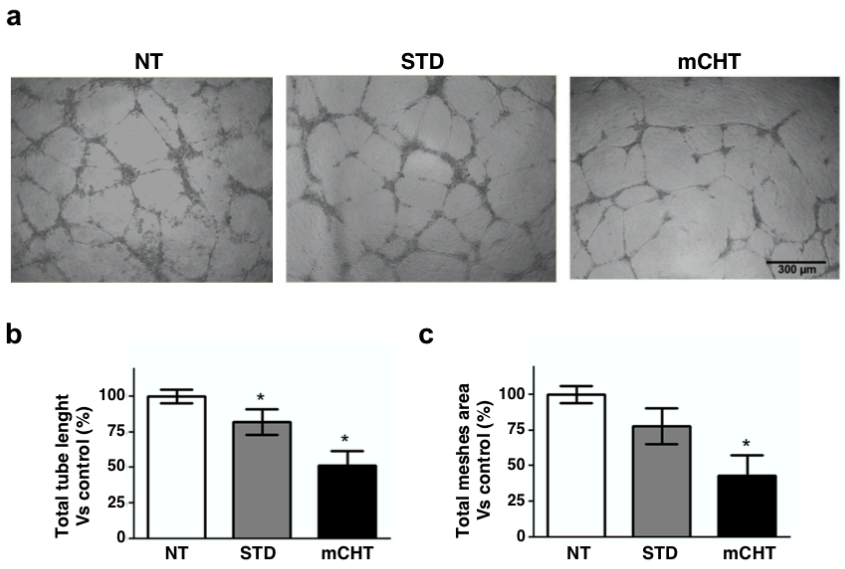

Fig.8 Metronomic administration of 5-FU+VNR is more active than standard treatment in impairing HUVEC neoangiogenesis. *(**a**) Representative images of tube formation assay performed on HUVECs after STD or mCHT treatment. Total tube length (**b**) and total meshes area (**c**) were quantified by ImageJ software and graphically represented as a percentage of the untreated control. Values represent the average±SD of three independent experiments, *p <0.05*

Conditioned medium from TNBC cells treated with 5-FU+VNR under mCHT schedule inhibits HUVECs migration and abolish clonogenic survival

The interactions between tumor and its microenvironment are crucial for tumor formation, progression and the development of metastasis; in particular, the crosstalk between cancer cells and

ECs, participates in promoting neo-angiogenesis and cell motility [40]. Therefore, we investigated the effects of the conditioned medium harvested from TNBC cells treated STD or mCHT FU+VNR. We found that the medium of both STD- and mCHT-treated MDA-MB-231 cells, modulates migration and clonogenic survival of HUVECs (Fig. 9).

The scratched area is still open 96 hours after the incubation with conditioned medium derived from

both STD- and mCHT-treated MDA-MB-231 cells (Fig 9a), being about 40% in size ($p<0.001$) or 90% ($p<0.01$) of the initial scratched area, respectively. Furthermore, the conditioned medium from treated-MDA-MB-231 cells significantly inhibited HUVEC migration, as shown in fig. 9b. In fact, the percentage of migrated HUVECs is reduced to 15 % of the control (*p<0.001)* after incubation with conditioned medium derived from STD-treated TNBC cells and to 2.5% (*p<0.001*) after incubation with conditioned medium derived from mCHT-treated TNBC cells (Fig. 9b).

Finally, HUVEC clonogenic ability was markedly reduced in the presence of conditioned medium derived from STD-treated TNBC cells (about 20% of control, $p<0.001$, Fig 9c).

Notably, the conditioned medium derived from mCHT -treated TNBC cells completely abrogated clonogenic growth of HUVECs ($p<0.001$) *vs.* control and c-STD).

Overall, these data show that both STD and mCHT treatments induce TNBC cells to release factors contributing to suppress

migration and survival of ECs and, again, show that mCHT schedule is more effective in doing so.

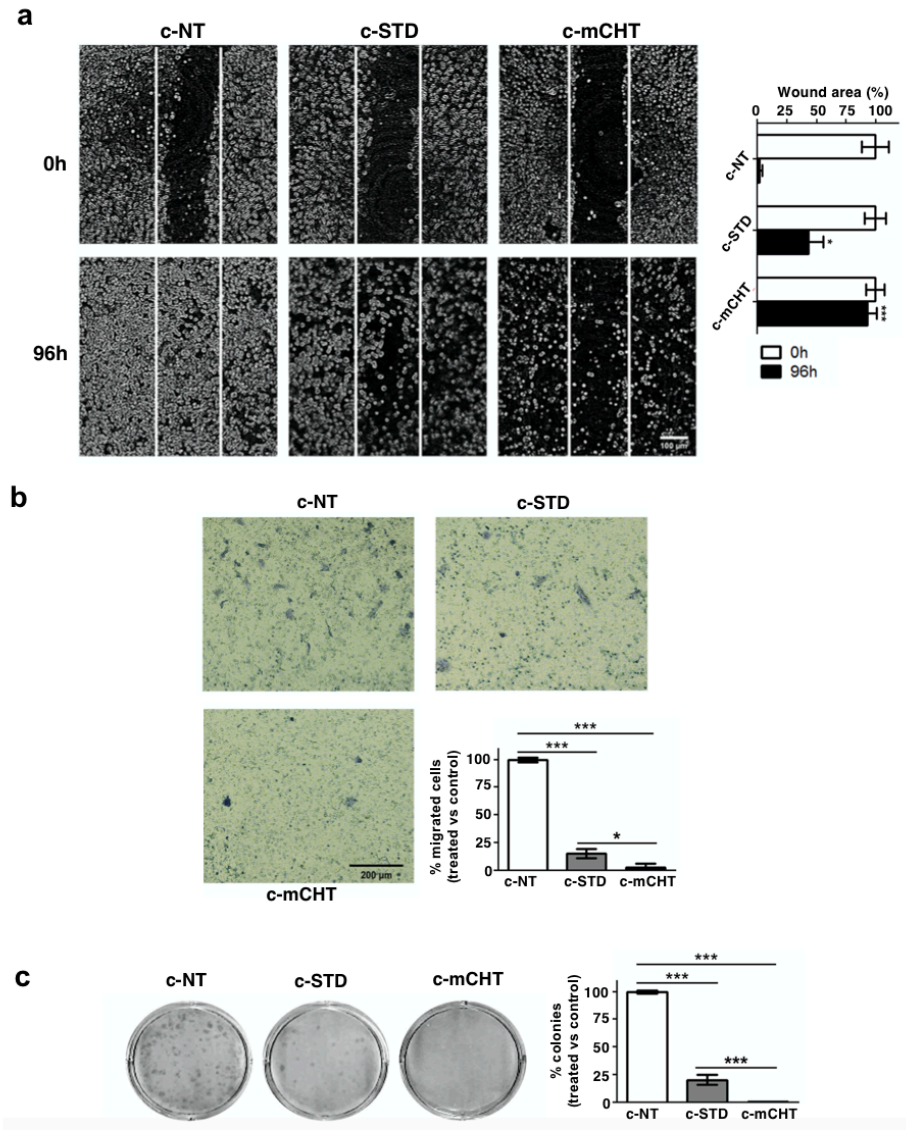

Fig.9 Conditioned medium from metronomically 5-FU+VNR-treated MDA-MB-231 cells suppresses HUVEC migration and colony formation. *(**a**) Representative images of scratch test performed on HUVECs before (0h) and 96h after treatment with conditioned medium from MDA-MB-231 cells treated with 5-FU+VNR given STD (c-STD) or mCHT (c-mCHT). Conditioned medium from untreated MDA-MB-231 (c-NT) was used as control. The area of the still open wound after 96 hs is quantified as a percentage of the initial scratched area. Values represents the average±SD of three independent experiments, **p <0.01; ***p <0.001. (**b**) Representative images of Transwell assay performed on HUVECs at the end of the treatment with c-STD or c-mCHT media. Migrated cells were stained with crystal violet, counted and graphically expressed as percentage of untreated control (c-NT). Values represent the average±SD of three independent experiments, **p <0.01; ***p <0.001. (**c**) Representative images of colony formation assay performed on HUVECs at the end of the treatment with c-STD or c-mCHT media. Colonies formed after 10 days without drugs were stained with crystal violet and counted. On the right: number of colonies grown after treatments quantified as a percentage of untreated controls. Values represent the average±SD of three independent experiments, ***p <0.001*

Discussion

Despite advances in cancer treatment, metastases remain the main cause of death in most cancer patients, including those with TNBC [41–43]. TNBC is one of the most aggressive tumors [44], and the standard treatment with chemotherapy usually does not inhibit metastasis formation. Indeed, among more than 200 FDA-approved drugs, very few have anti-metastatic activity [45], which is evident only when administrated under metronomic protocol [25]. Metastasis formation is a complex process requiring the formation of new blood vessels through which metastatic cancer cells spread to other anatomic sites [46]. Proliferation and

migration of both tumor and endothelial cells are crucial for metastasis development. To evaluate whether mCHT can exert anti-angiogenic/anti-metastatic activities, we investigated the effects of mCHT combination of 5-FU and VNR on HUVECs and TNBC cell proliferation and migration compared to the STD treatment.

First, we demonstrate that 5-FU and VNR given mCHT as single agents, or in combination, have a strong anti-proliferative effect on ECs, similar to what we previously reported for TNBC cells [31]. Importantly, this effect is achieved using doses that are about 100-fold lower than those given STD (Figs. 1 and 2). In addition, the IC_{50} of 5-FU+VNR is similar in both HUVECs and MDA-MB-231 cells when given mCHT, differently from what seen when the combination of drugs is given as STD: in this case, a 3-fold higher dose of 5-FU is needed to kill 50% of MDA-MB-231 *vs.* HUVECs (Table I). The strong anti-proliferative effect on both tumor cells and ECs using a much lower amount of single drugs is likely to account for the reduction of the toxic side effects observed for mCHT, compared to the STD regimens, as observed in several clinical trials [27,28,47,48]. Moreover, our evidence showing that the combination of 5-FU+VNR given mCHT is active on both HUVECs and TNBC cells within the same range of doses suggest that this protocol is, at the same time, both anti-tumoral and anti-angiogenic. These findings are particularly relevant for the clinical practice: they indicate that using a metronomic combination of 5-FU and VNR could affect both tumor and vascular endothelial cells without additional

therapies, like monoclonal antibodies. Our data are in line with the literature, which defines metronomic chemotherapy as a therapy simultaneously targeting tumor and endothelial cells [49]. Importantly, we observed that both HUVECs and MDA-MB-231 cells retain clonogenic capability following STD treatment. On the contrary, re-growth is completely abolished by the mCHT treatment (Fig. 3), suggesting a cytostatic *vs.* a cytotoxic effect of the STD and mCHT protocols, respectively. These data are in line with what reported in the clinical setting [50], where relapses occur more frequently after the STD protocol than mCHT. A better control of recurrences and metastasis has been observed after mCHT and significantly long periods of clinical benefit (Complete + Partial + Stable Disease $\geq$ 24 weeks) have been reported, but only in small early clinical studies [51,52]. This interpretation is further supported by the migration assay (Figs. 4 and 5). We observe that only the mCHT combination of 5-FU+VNR strongly inhibits cell motility of both ECs and TNBC cells. When administered as STD, 5-FU+VNR significantly reduces the migration of ECs but not TNBC cells. Accordingly, the mCHT administration results in ~50% of total tube length and total mesh area compared to ~20% observed after STD administration (Fig. 8). Several studies reported an anti-migratory effect of some antitumoral agents when given metronomically, such as ceramide analogs, docetaxel, the 5-FU prodrug UFT (uracil plus tegafur) plus cyclophosphamide [53–55].

Tissue inhibitor of metalloproteinase 2 (TIMP-2) plays an inhibitor role in cell migration and proliferation by blocking the matrix degradation activity of metalloprotease 2 (MMP-2). High TIMP-2 levels in ECs and tumor cells are associated with a poor invasiveness both *in vitro* and *in vivo* [56–58]. Our data suggest that 5-FU+VNR inhibits cell migration upregulating TIMP-2 after both STD and mCHT administration in HUVECs (Fig. 6a) but not in TNBC cells. (Fig. 6b). The strong reduction in the number of cells found at the bottom of the transwell (Fig. 5b) together with the lack of closure of the wound (Fig. 4b) and the induction of both apoptotic and autophagic markers (Fig. 7b) indicate that a cytotoxic effect rather than an inhibitory effect on cell migration is exerted by mCHT on TNBC cells.

FAK's high expression and phosphorylation levels are associated with cancer progression and metastasis by promoting, at least in part, tumor and endothelial proliferation and migration [59]. In particular, FAK also promotes neo-angiogenesis by upregulating pro-angiogenic factors, such as VEGFR2 and VEGF [11,34,35]. We observed that the combination 5-FU+VNR strongly suppresses the levels of total and active FAK as well as VEGFR2 in HUVECs and TNBC cells regardless of the modality of administration (Fig. 7). Despite less FAK expression and activation, TNBC cells are still able to migrate after the STD administration of 5-FU+VNR (Figs. 4b and 5b). We then investigated the MAPK pathway, which is also involved in cell migration by analyzing ERK expression and activation levels. In both HUVECs and MDA-MB-231 cells, ERK

phosphorylation is slightly diminished after STD treatment, whereas it increases after mCHT treatment. Surprisingly, total ERK levels are differently affected by the treatments: in fact, ERK expression was strongly increased after 5-FU+VNR given mCHT in both cell types and also after STD treatment in MDA-MB-231 cells. Several reports showed that ERK also has a kinase-independent activity and that different expression levels can lead to either cell proliferation or cell growth arrest [60,61]. Moreover, Hong et al. demonstrated that ERK overexpression, associated with its phosphorylation, results in cell growth arrest and caspase-dependent apoptosis activation [37]. Consistent with the observed ERK induction, we found caspase-3 cleaved in HUVECs after mCHT treatment and in MDA-MB-231 cells after both STD and mCHT administration of 5-FU+VNR. Aberrant ERK activity is also associated with autophagy induction [38] as a result of ER stress, DNA damage or oxidative stress induced by anticancer therapies in several tumors, including breast cancer [39,62,63]. Accordingly, we observed that 5-FU+VNR given mCHT strongly induced the expression and conversion of the autophagic marker LC3A/B-I to LC3A/B-II in MDA-MB-231 cells (Fig. 7b), suggesting an ongoing autophagic activity [64]. On the contrary, the STD administration results only in a slight induction and conversion of LC3A/B-I to LC3A/B-II in the same cells (Fig. 7b). These data suggest that the mCHT treatment causes cell death mostly by autophagy, whereas the STD treatment induces apoptosis in MDA-MB-231 cells [31]. In HUVECs, both kinds of treatment strongly suppressed LC3A/B-I expression. However,

we can still detect LC3A/B-II suggesting ongoing autophagic activity (Fig. 7a). Since autophagy activation has also been associated with inhibition of cell migration inhibition in different tumors [65–67], we cannot exclude this process's involvement in the anti-migratory effect seen in HUVECs and TNBC cells after mCHT administration of 5-FU+VNR.

Cancer development is promoted not only by tumor cells directly but also via interaction with microenvironment elements, which in turn strongly influences tumor progression and metastasis formation and the clinical outcome [68]. Therefore, we used an indirect co-culture system to study whether STD or mCHT treated-TNBC cells influence HUVECs migration and colony formation ability.[69–71]. In this experimental setting, mCHT appears to be significantly more efficient than STD protocol in inhibiting ECs migration and survival (Fig. 9): in fact, only conditioned medium from mCHT-treated TNBC cells completely suppresses transwell migration (Fig. 9b) and colony formation (Fig. 9c). These results are in agreement with the clinical situation, where the regenerative capability of damaged tumor vasculature has been described after STD therapy, despite high doses of drugs employed [72], and reinforces our finding about the effectiveness of the mCHT schedule in disease control. Notably, our results indicate that the combination of 5-FU+VNR acts on ECs directly and via factors released from treated-TNBC cells. The factors released by STD and mCHT treated-TNBC cells that can strongly reduce and abolish, respectively, migration and clonogenicity of HUVECs are under investigation.

In summary, we showed that the combination of 5-FU+VNR administered mCHT is more effective in simultaneously inhibiting EC and TNBC cell migration as well as regrowth compared to the STD schedule of treatment.

In conclusion, our pre-clinical data offer a way to interpret how the therapeutic effect of the metronomic administration of 5-FU plus VNR is mediated, i.e., by targeting both TNBC and endothelial cells. In particular, our findings that only metronomic administration completely block colony re-growth, affect both ECs and TNBC cells migration and tube formation, strongly indicate that the stabilization of tumor growth observed in TNBC patients treated with mCHT is likely due not only to direct cytotoxic effects but also to anti-metastatic and anti-angiogenic effects. Therefore, taken together with other published data, our findings confirm the multimodality mechanism of action of mCHT and support the rationale for its use in TNBC patients, where the dual targeting of the tumor and its vasculature at the same time results in better therapeutic outcome.

Further confirmations in the clinical setting are urgently needed through randomized trials to assess the role of mCHT in the treatment's algorithm of TNBC patients. Additionally, even though these data are limited to the TNBC *in vitro* model, inhibition of angiogenesis and suppression of migration should represent relevant endpoints to be assessed in different subtypes of breast cancer, i.e., HR+ after cell-cycle inhibitors (CDK 4/6) or in those tumors characterized by the loss of endocrine-sensitivity.

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Chapter 4

Conclusions and future perspectives

Summary

Triple-negative breast cancer (TNBC) represents a class of aggressive tumors with a higher rate of mutations than other breast cancer subtypes, distant metastases, and poorer outcomes. Characterized by the lack of ER, PR, and HER2 amplification, hormone therapy is not effective in TNBC treatment, and due to the high molecular heterogeneity, the use of target therapies is limited. Maximum tolerated dose (MTD) chemotherapy remains the standard of care for patients with TNBC. The MTD's effectiveness in managing the TNBC is often only transitory and can cause severe side effects, accompanied by an inadequate long-term response. Distant recurrences remain, therefore, the first cause of death in TNBC. A key role in tumor relapses is played by neo-angiogenesis because it allows the regrowth of tumor vasculature damaged by chemotherapy, leading tumor cells to spread in other tissues. Therapeutic antibodies against proangiogenic factors, such as VEGF or VEGFR2, or TKI inhibitors, such as Sunitinib or Sorafenib, have been developed, but their association with chemotherapy agents increases toxicity. Thus, new therapeutic strategies that target tumor vasculature during anticancer treatment are needed.

Metronomic chemotherapy, defined as the constant administration of low toxic doses of antitumor agents without prolonged drug-free breaks, has shown strong anti-angiogenic properties due to the inhibitory effect of the proangiogenic factors and by promoting the anti-angiogenic ones. In addition to the

effect on endothelial cells, the stimulating effect on the immune system and the direct activity on tumor cells has also been highlighted, indicating metronomic administration as a multitarged therapy.

In this thesis, the effect of the metronomic (mCHT) administration of 5-Fluorouracil (5-FU) plus Vinorelbine (VNR) on TNBC cell lines and endothelial cells HUVECs compared to the standard treatment (STD) was investigated using *in vitro* assays. In particular, we have shown that the mCHT administration of 5-FU and VNR, both in single and in combination, affects HUVECs and TNBC cells at doses significantly lower than the STD one. Moreover, the inability to form new colonies at the end of the treatment highlights the cytotoxic activity of the mCHT combination of 5-FU+VNR in both HUVECs and TNBC cells. On the contrary, the STD administration of 5-FU+VNR cannot completely block the colony formation neither in HUVECs nor in TNBC cells. Despite the low doses used, mCHT 5-FU+VNR is more effective than the respective STD protocol in inhibiting cell migration of HUVECs and TNBC cells and in preventing HUVEC tube formation. Using an indirect co-culture to simulate the crosstalk between TNBC cells and HUVECs, it has also been shown that the medium conditioned by TNBC cells treated with mCHT 5-FU+VNR completely blocks HUVEC migration and colony formation. Moreover, the 5-FU+VNR mCHT protocol inhibits the FAK/VEGFR2 axis in HUVECs and TNBC cells independently of drug administration timing. In contrast, cell death modality in HUVECs and TNBC cells depends on the

treatment schedule. The metronomic combination promotes HUVECs' apoptosis, whereas it switches the modality of cell death from apoptosis, induced by standard treatment, to autophagy in TNBC cells, activating senescence.

In conclusion, metronomic administration of 5-Fluorouracil plus Vinorelbine is more efficient than the standard protocol in inhibiting cell proliferation, migration, and colony re-growth of HUVECs and TNBC cells, suggesting that the metronomic dose and schedule of drug administration may provide better control of tumor growth and relapses, if the *in vitro* results also apply to the *in vivo* setting.

Conclusions

TNBC accounts for 10-20% of invasive breast cancer diagnoses and is associated with poor clinical outcomes [1]. The TNBC subtype is highly heterogeneous and is characterized by the absence of ER, PR, and HER2 amplification [2]. Despite advances in cancer treatments, TNBC patients do not benefit from hormone or targeted therapies. Although considerable efforts have been invested in studying TNBC, the understanding of the disease remains limited [3]. Novel and effective therapies for the treatment of this type of tumor are urgently needed.

Metronomic chemotherapy, referred to the chronic administration of low doses of chemotherapy drugs without prolonged rest periods [7], is currently in clinical trial for TNBCs and other tumors and, to date, has shown some promising efficacy [4], mostly in clinical II trials, but also recently in some randomized phase III trials, e.g. the CAIRO III study on colorectal cancer [5] and a pediatric rhabdomyosarcoma trial [6]. Unlike standard MTD chemotherapy, which mainly targets only tumor cells, metronomic chemotherapy acts on the tumor microenvironment because of its antiangiogenic, immune-stimulatory and direct antitumoral effects. For these different modes of action, metronomic chemotherapy is defined as multi-targeted therapy.

My Ph.D. project aimed to investigate the antitumoral, anti-angiogenic and anti-metastatic effects of the metronomic administration of 5-FU and VNR in comparison to the standard

one, by analyzing, in *in vitro* models of TNBC and endothelial cells, proliferation, migration and cell survival.

The results presented in this thesis showed that the mCHT administration of 5-FU and VNR, both in single and combined has a significant antiproliferative activity at lower doses than the high standard dose in endothelial cells and in the two TNBC cell lines studied. Notably, mCHT treatments with 5-FU and VNR affect endothelial and TNBC cells within the same range of doses both in single and in combination. According to the literature, which defines metronomic chemotherapy as multi-targeted therapy with no severe toxicity [7,8], these results suggest that the mCHT treatment with 5-FU and VNR has, at the same time, antitumor and antiangiogenic effect with lower toxicity on the vascular compartment compared the standard treatment protocols. The high toxicity of the standard chemotherapy dose schedules represents a major limitation of this type of treatment. Indeed, during the drug-free breaks, it is necessary to allow the damaged tissues to recover, but this also allow tumor cells, and damaged tumor vasculature to recover, facilitating tumor relapses [9]. Our data on TNBC and endothelial cells abrogation of clonogenic survival are of utmost importance because they reflect tumor control and the prevention of recurrences. Even in the clinical setting, better control of relapses and metastasis by the metronomic protocol in some studies has been reported [10,11].

Metastasis is responsible for most of cancer-related deaths. The development of metastases requires cancer cells to undergo

some intracellular changes that allow them to migrate, invade, and colonize distant tissues. The administration of 5-FU+VNR under mCHT schedule strongly inhibits TNBC cell migration, whereas the STD administration of drugs does not do so. These results highlights that only the continuous administration of low doses of 5-FU+VNR can block TNBC cell migration, suggesting a more significant effect on reducing tumor cell spread than the respective STD treatment.

However, the formation of metastases depends not only on the tumor cell properties but also on the tumor microenvironment. It plays a crucial role in this process, e.g. new blood vessel formation within the tumor facilitates the spread of migrating tumor cells [12]. Therefore, neoangiogenesis represents a promising therapeutic target in metastasis control. Endothelial cell migration is one of the main steps of metastasis [13]. Although both STD and mCHT administration of 5-FU+VNR strongly affect endothelial cell migration, only mCHT treatment inhibits their tube formation capacity, suggesting a more significant anti-angiogenic effect of this drug combination if administrated in continuous at low doses. Previously studies reported that low doses of drugs, in particular microtubule-stabilizing anticancer drugs such as Paclitaxel and Vinblastine, exert their antitumoral effect through the inhibition of angiogenesis [14,15]. Indeed, through different mechanisms of action from the conventional administration of drugs, Vinblastine and Paclitaxel suppress *in vitro* proliferation, migration and sprouting of endothelial cells [16,17]. Our data support the anti-

angiogenic effect of the frequent administration of low-doses of drugs and, for the first time, assign this effect also at the metronomic combination of 5-FU+VNR.

Several studies reported the antimetastatic effect of metronomic administration of some chemotherapy agents, i.e., Docetaxel [18], Gemcitabine [19], Cyclophosphamide [20–22], through the inhibition of both cancer cell dissemination as well as new vessel formation both *in vitro* and *in vivo*. For instance, metronomic doses of Cisplatin reduce tumor mass in hepatocarcinoma models by inhibiting VEGF and MMP-2 expression [23]. Metronomic doses of Zoledronate combined with the green tea Camellia sinensis water extract has antiproliferative, anti-migration and anti-invasion effect through the inhibition of MMPs and the activation of apoptosis in mouse breast cancer 4T1 cells [24]; metronomic Cyclophosphamide plus Docetaxel decreases VEGF expression levels and increases tumor cell apoptosis [20,21]. Our results suggest that both mCHT and STD administration of 5-FU+VNR affect cell migration by decreasing MMP-2 expression or increasing TIMP-2 in endothelial and, though not significant, in TNBC cells. In addition, in our *in vitro* model, the TNBC and endothelial cell migration and angiogenesis are modulated by drugs independently by the modality of administration, through inhibition of the Focal Adhesion Kinase (FAK) pathway, which reduces VEGFR2 expression in endothelial and TNBC cells and VEGF expression in endothelial cells.

Even though both STD and mCHT treatments downregulate FAK signaling pathway, which is required for cell motility [25,26], its inhibition does not seem sufficient to explain the different migration capacity of TNBC cells after STD and mCHT treatments. Instead, the different cell death mechanisms induced in TNBC cells by STD and mCHT treatments appear to be responsible for the different antitumor and antimetastatic activities of the schedules. Although FACS analysis did not reveal important changes in the cell cycle after STD or mCHT treatment, there was a significant increase in apoptotic cells after STD and mCHT treatment with VNR alone and in combination with 5-FU. Apoptosis activation was also confirmed by western blot analysis, which showed a strong increase of pro-apoptotic markers and decreased anti-apoptotic markers, overall, after the drug combination under STD administration.

Drug-induced apoptosis can also be followed by autophagy activation, which can lead to cell death [27]. The autophagic marker LC3A/B expression is increased in TNBC cells by the mCHT administration of 5-FU+VNR both in single and in combination and only by the STD combination. However, immunostained TNBC cells with LC3 antibody showed a perinuclear and cytoplasmic distribution of LC3 after both single and combination mCHT treatments but not after the STD ones, suggesting that the formation of the autophagosomes depends on the low dose schedule. Other studies suggest the activation of apoptosis and autophagy after the mCHT administration of drugs. For instance, metronomic photodynamic therapy (PDT)

can induce colorectal cancer cell death via long-lasting autophagy activation accompanied by greater activation of apoptosis than the acute treatment [28]. The metronomic combination of Everolimus and Etoposide strongly affects non-Hodgkin lymphoma cell lines by inducing apoptosis and autophagy, together with senescence [29]. Senescence induction has recently been positively associated with cancer treatment response, due to its ability to stop the cell cycle, activate the immune response and induce autophagy [30]. Several studies demonstrated the activation of cellular senescence by metronomic administration of drugs: metronomic doses of hydroxyurea induces senescence in primary neuroblastoma cell lines *in vitro* [31]; metronomic low dose Topotecan treatment of neuroblastoma cells causes senescence through DNA-damage and p21 up-regulation, resulting in tumor regression *in vitro* and *in vivo* [32]. Our results indicated that the mCHT administration of 5-FU+VNR induces cellular senescence because of the increase of senescence-associated (SA) – β – galactosidase activity in TNBC cells, especially in those treated with mCHT VNR alone or combined with 5-FU.

Endothelial cells also undergo different modalities of cell death after STD or mCHT administration of 5-FU+VNR. In contrast to TNBC cells that preferentially activate apoptosis after STD treatment and autophagy after the mCHT one, endothelial cells showed a weak autophagic activity after both the drug administration modalities accompanied by the activation of the apoptotic pathway after the mCHT combination treatment.

Curiously, consistent with the cleavage of caspase 3, ERK expression and phosphorylation are over-induced both in TNBC and endothelial cells. Hong et al. demonstrated that ERK overexpression and phosphorylation result in cell growth arrest and caspase-dependent apoptosis activation [33]. Indeed, ERK also has a kinase-independent activity that directs cells to proliferate, growth arrest, or death based on the level of total protein [34,35]. ERK overexpression is indeed responsible for *in vitro* melanoma cell line death and *in vivo* tumor regression, due to the activation of apoptosis, ER stress and DNA damage signaling pathways [36]. In several types of tumors, including breast cancer, aberrant ERK activity has been correlated to autophagy induction [37] in response to anticancer therapies [38–40]. We suggest that the mCHT administration of 5-FU+VNR strongly stresses TNBC and endothelial cells, stimulating ERK hyperactivation leading to apoptosis rather than autophagy activation to induce cell death. Since autophagy is also related to inhibition of cell migration [41–43], we cannot exclude the autophagic activity's contribution in the inhibition of endothelial and TNBC cell migration induced by the mCHT treatment.

Tumor growth and spread strongly depend on direct and indirect interactions between tumor cells and its microenvironment. For instance, tumor cells stimulate neo-angiogenesis via the release of proangiogenic factors, which induce endothelial cell proliferation, migration, and sprouting. Indirect co-cultures using conditioned media are accepted systems to mimic an *in vitro* tumor microenvironment because they allow studying the effect

of factors released by cancer cells on other cell types that are part of the tumor microenvironment [44,45]. Using an indirect co-culture, we showed that the mCHT administration of 5-FU+VNR can directly abolish endothelial cell migration and colony regrowth and block them indirectly via the modulation of TNBC cells secretomes. The factors secreted by TNBC cells modulated by the mCHT administration of 5-FU+VNR are under investigation.

In conclusion, the data presented in this thesis are in accordance with the current knowledge about the simultaneous effect of the metronomic chemotherapy on cancer and endothelial cells at lower doses than the standard chemotherapy. Moreover, our work shows, for the first time, the anti-proliferative, anti-migratory, and anti-angiogenic activity of the metronomic combination of 5-Fluorouracil plus Vinorelbine, highlighting the underlying potential molecular mechanisms, that help explain clinical anti-tumor benefits observed in clinical trials such as VICTOR-2 in breast cancer patients [46].

Overall, these results lay the foundations for increasing the study of the metronomic combination in the clinical practice, in order to improve the treatment of TNBC patients.

Future perspectives and translational relevance

The role of the microenvironment on tumor growth and metastasis has been established [47]. Cytotoxic drugs combined with immunotherapies or anti-angiogenic therapies have been proposed to target the tumor microenvironment [48], but these approaches are often limited by the high toxicity [47].

Due to its antitumor, anti-angiogenic, and immune stimulatory effect, metronomic chemotherapy is able to target both tumor cells and its microenvironment, but associated with a low toxicity profile [7]. In particular, metronomic chemotherapy can inhibit tumor angiogenesis by directly targeting endothelial cells and modulating angiogenic factors expression. For instance, after metronomic chemotherapy with Capecitabine plus Cyclophosphamide, a decrease of VEGF-A expression levels in serum of metastatic breast cancer patients has been detected [49]. Several alkylating agents and microtubule inhibitors administrated using metronomic protocols increase anti-angiogenic inhibitor thrombospondin 1 (TSP-1) expression *in vitro* and *in vivo* [50]. Metronomic doses of 5-Fluorouracil reduce gastric cell proliferation and *in vivo* tumor growth by inhibiting angiogenesis by decreasing VEGF and PDGF levels in cancer cells supernatant and in the blood of tumor-bearing mice [51].

Based on the literature data about the modulation of angiogenic factors by the metronomic chemotherapy, I would like to investigate which angiogenesis-related factors secreted by

TNBC cells change after the metronomic administration of 5-Fluorouracil plus Vinorelbine in order to understand how TNBC cells influence endothelial cell's proliferation and migration in the indirect co-culture shown in this thesis. I recently started analyzing the secretome of MDA-MB-231 cells treated with the STD or the mCHT combination of 5-FU and VNR by measuring angiogenic proteins level using the Human Angiogenesis Antibody Arrays (AAH-ANG-1000, RayBio®), following the manufacturing instructions.

As shown in figure 1, cells treated with mCHT 5-FU+VNR reduce pro-angiogenic molecules' release to a greater extent than STD treatment. In particular, compared to the untreated control, the mCHT combination of drugs strongly reduces the expression of Angiogenin, bFGF, and VEGF-A, which promote new vessel formation through the induction of endothelial cell proliferation, survival, and migration [52–54]. VEGF-A and bFGF transcription is positively regulated by the FAK/STAT3 pathway [55]. The western blot analysis on TNBC and endothelial cells showed the inhibition of FAK activity after treatments, therefore, we can speculate that the inhibition of FAK by STD and mCHT 5-FU+VNR leads to the downregulation of VEGF-A and bFGF expression. FAK/STAT3 pathway activation may result by the binding of VEGF and bFGF to their receptors [56,57], highlighting that the downregulation of these pro-angiogenic factors blocks the autocrine signaling loop of the pathway [58].

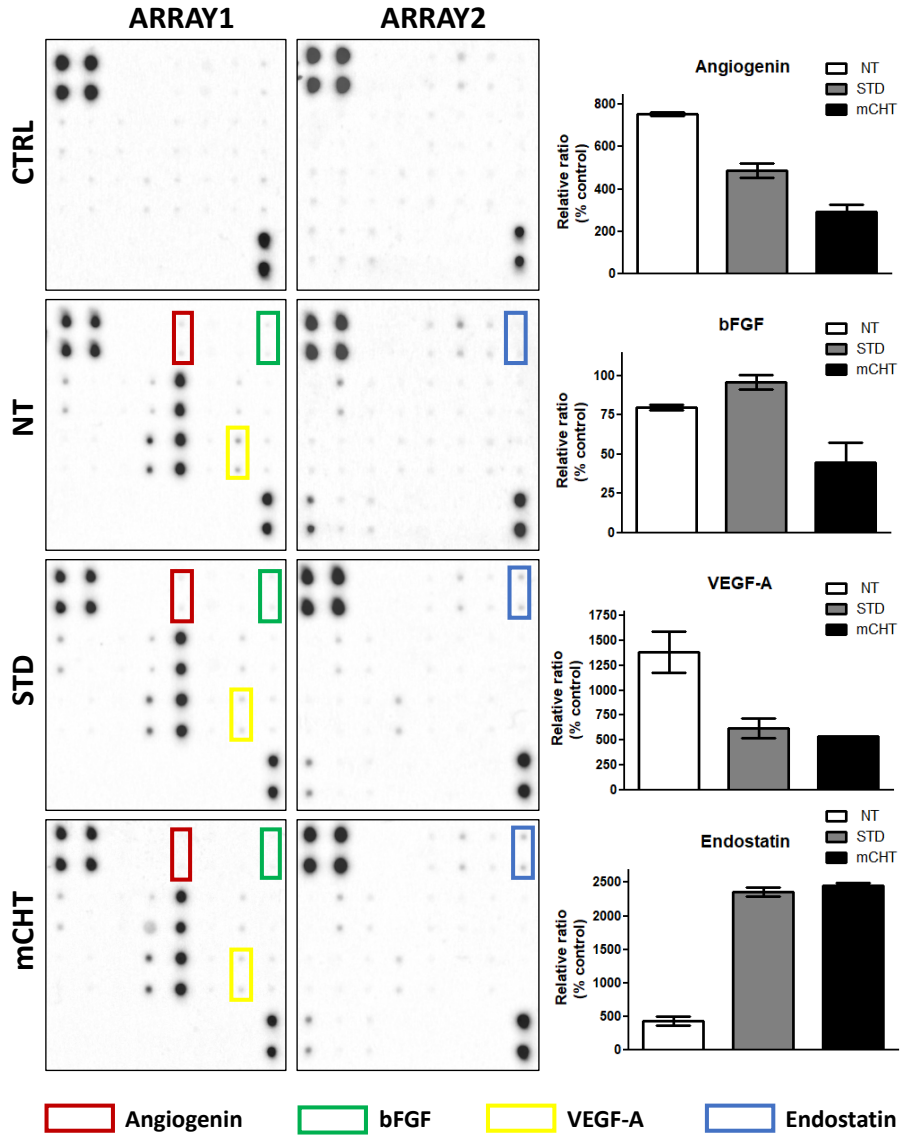
ARRAY1
ARRAY2
CTRL
NT
STD
mCHT
Angiogenin
bFGF
VEGF-A
Endostatin
NT
STD
mCHT
Relative ratio (% control)
Angiogenin
bFGF
VEGF-A
Endostatin

Figure 1. Metronomic administration of 5-FU+VNR strongly modulates the secretion of angiogenic-related factors by TNBC cells. *Representative images of Human Angiogenesis Antibody Arrays (AAH-ANG-1000, RayBio®) performed on cell culture media of MDA-MB-231 untreated or treated with STD or mCHT 5-FU+VNR. 10% FBS DMEM was used as medium control. The proteins more significantly modulated by treatments were quantified on the control using ImageJ and reported in graphs as average±SD of the spots.*

The angiogenic inhibitor Endostatin also blocks FAK pathway, by binding integrins or competing for the binding to bFGF and VEGF receptors [59,60]. A significant increase of Endostatin levels after both STD and mCHT administration of 5-FU+VNR emerged from the array analysis (figure 1), suggesting that FAK inhibition could also be due to the increase of soluble Endostatin. However, further studies are needed to clarify the molecular mechanisms based on the anti-angiogenic effect of mCHT 5-FU+VNR in TNBC and endothelial cells.

In this thesis the anti-proliferative, anti-migratory and anti-angiogenic effects of the mCHT treatment with 5-FU+VNR using 2D single cell and co-culture systems are reported. Although the 2D co-culture systems allow studying cell-cell communications, their use is limited by the inability to detect direct cell-cell interactions, which strongly influence cells to crosstalk within the tumor microenvironment [61]. Thus, the results presented in this thesis have to be confirmed into a system that takes into account all the cell-cell interactions better than the 2D system, such as 3D cell cultures [62]. For this purpose, I would like to generate multicellular tumor spheroids (MCTS) using TNBC and endothelial cells, as reported by Shoval et al. [63], and then

investigate the response of MCTS to mCHT and STD treatments with 5-FU+VNR by measuring MCTS' viability and size. I would also like to evaluate the MCTS' migration capacity, as described by Vinci et al. [64], and the endothelial cell's ability to generate capillary-like structures within the spheroid after treatments, as reported by Amann et al. [65]. The medium of MCTS treated with the mCHT or the STD combination of 5-FU+VNR will also be analyzed to corroborate the angiogenesis antibodies' arrays results.

Despite the 3D cell cultures well represent the solid tumors architecture and well simulate the interactions between cancer cells and the microenvironment [47], to date they cannot recapitulate the complexity of an *in vivo* biological system. Therefore, the data presented in this thesis will be further validated in a preclinical mouse models that better simulate cancer growth, so that the effects of mCHT 5-FU + VNR will be analyzed in a system that has closer relevance to the clinical situation.

In conclusion, all these experiments will help to better predict the antitumor, antimetastatic and anti-angiogenic effects of the metronomic administration of 5-Fluorouracil plus Vinorelbine in the clinical practice in order to improve the clinical outcomes of patients with TNBC.

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Publications

1. Cerrito MG, De Giorgi M, Pelizzoni D, Bonomo SM, Digiacomo N, **Scagliotti A**, Bugarin C, Gaipa G, Grassilli E, Lavitrano M, Giovannoni R, Bidoli P, Cazzaniga ME. **Metronomic combination of Vinorelbine and 5Fluorouracil is able to inhibit triple-negative breast cancer cells. Results from the proof-of-concept VICTOR-0 study.** Oncotarget. 2018 Jun 8;9(44):27448-27459. doi: 10.18632/oncotarget.25422.
2. **Scagliotti A**, Grassilli E, Cazzaniga ME, De Giorgi, Lavitrano M and Cerrito MG. **Metronomic administration of 5-Fluorouracil plus Vinorelbine inhibits both endothelial and triple-negative breast cancer cell regrowth and migration via FAK/VEGFR2 downregulation and autophagy/apoptosis activation.** Under review in Cellular Oncology.