UNIVERSITA' DEGLI STUDI DI GENOVA

SCUOLA DI SCIENZE MEDICHE E FARMACEUTICHE

Corso di Dottorato di Biotecnologie in Medicina Traslazionale

Curriculum di Medicina Traslazionale e di Precisione

IRCCS Ospedale Policlinico San Martino

SSD Breast Unit

CIrculating TUmor CELls (CTCs), circulating tumor DNA (ctDNA) and exosomes (Ex) in breast cancer patients: a prospective study

Tutor: Prof. Lucia Del Mastro

External tutor: Dr. Barbara Cardinali

CANDIDATE

Carlotta Mazzitelli

ACADEMIC YEAR 2019/2020

INDEX

RATIONALE

Despite improvements in the diagnosis and treatment of breast cancer (BC), approximately 30% of women diagnosed in the early stages develop metastatic disease. It is therefore extremely important to determine new biomarkers that can monitor the effectiveness of the therapy, that can allow the early detection of micro-metastases, that enable to predict the response to the treatment and to evaluate intra-tumor heterogeneity. As part of the search for diagnostic, prognostic and possibly therapeutic biomarkers, a very promising field is the analysis of circulating biomarkers (CBM) released from the primary tumor or from the sites of metastases through liquid biopsy, a minimally invasive and repeatable method that has been considered as an approach of diagnostic utility to complement traditional biopsy techniques. In particular, the evaluation and analysis of the mutational profiles of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) in the blood have shown that they can provide useful information in the care of patients with BC.

For this purpose, this three-year PhD project has been mainly focused on the recovery and the characterization of CTCs obtained from BC patient samples by liquid biopsy. The experimental work was mainly performed in the clinical trial CITUCEL, "*CIrculating TUmor CELls (CTCs) and circulating tumor DNA (ctDNA) in breast cancer patients: a prospective study*", a spontaneous translational protocol conducted at the Ospedale Policlinico San Martino (HSM) in Genoa. It is a single-centre translational, observational, interventional, non-pharmacological study which therefore does not provide any type of additional treatment or exclusion from the therapeutic gold standard, more than clinician choice as for clinical practice.

In addition, part of the experimental activity was conducted within the study "*Genetic Barcoding to evaluate the Reliability of Liquid Biopsies in featuring Triple Negative BC (TNBC) and Hormone negative (HR) /HER2 over-expressed BC heterogeneity- BaReLiB*", supported by AIRC Investigator

Grant 2017, in which TNBC and HR-/HER2+ BC heterogeneity is evaluated using liquid biopsy and genetic barcoding in mouse model.

Finally, additional *in vitro* preliminary experiments have been conducted with the purpose to evaluate the effect of the treatment with Ixazomib, alone or in combination with Bendamustine, on different subtypes of BC cell lines.

GENERAL PART

1. INTRODUCTION

Breast Cancer (BC) it is the leading cause of cancer death in women and has the highest incidence rate[1]; most of the time, the disease requires a multidisciplinary approach, or a combination of therapies. Treatment plans often involve a primary therapy, generally surgery or radiation therapy, in addition to an adjuvant and/or neoadjuvant therapy.

In particular, neoadjuvant therapies are delivered before the main treatment, to help reduce the size of a tumor or kill cancer cells that have spread; adjuvant therapies are delivered after the primary treatment, to destroy remaining cancer cells. In general, different biological subtypes of BC display differences in propensity to metastatic spread. Biological subtypes are classified according to the expression of steroid hormone receptors (estrogen (ER) and progesterone (PgR)) and HER2 (human epidermal growth factor receptor 2).

In luminal cancers metastasis is not frequent, but this group is heterogeneous, comprising luminal A and B, with different aggressiveness and treatment options. There is a peak of relapses at about 2-3 years after the surgery, but the rate of metastasis remains relatively high after the peak, because disseminated cells may stay dormant even for as long as 20 years and start to proliferate upon some triggering event.

In tumors HER2 positive (about 20% of cases) prognosis is relatively worse than in luminal BC, although they can be treated with targeted anti-HER2 therapy. Triple negative BC (TNBC), that lack the expression of ER, PR, HER2, which constitutes about 15% of all cases, is heterogeneous itself, comprising different subtypes, including basal like, immune-modulatory, luminal androgen receptor, mesenchymal and claudin low; it is highly aggressive and low survival rates. TNBC tend to disseminate quickly (also with a peak in the first 2-3 years after surgery) and the percentage of metastases is high.

However, in contrast to luminal cancers, the rate of distant metastases in TNBC becomes significantly lower after five years, thus dormancy of disseminated is less common in this subtype[2].

These intrinsic differences in BC subtypes raise several opened questions, such as what the routes to metastasis in BC are, what can be learned about them by analyzing CTCs and how the knowledge gained by CTC analysis may impact therapeutic decisions. There are two main hypotheses describing cancer dissemination: linear progression, according to which, metastatic potential is gained gradually and the whole process proceeds in steps and needs time, and parallel progression, according to which dissemination takes place early on even before clinical manifestation of the tumor. In the linear progression model, the evolving primary tumor gives rise to metastases due to increasingly aggressive and invasive phenotypes of the subclones of tumor cells. This model is in agreement with the postulated role of the epithelial-to-mesenchymal transition (EMT) in cancer and assumes active degradation of the stroma and migration into blood vessels by the motile, invasive single cells that broke loose from the epithelial monolayer[3].

In contrast to that, parallel progression postulates intravasation by passive shedding, which may take place shortly after the angiogenic switch, occurring during the early, pre-invasive stage of tumor development. Although the concept of linear progression is well established, recently many reports support parallel progression hypothesis. Accepting the parallel progression model, even if only for some subtypes or specific cases of BC, calls for a close control of the current diagnostic and therapeutic procedures and has implications for the CTCs research. Early dissemination, occurring before the appearance of any symptoms, precludes prevention, unless precautionary screening of the healthy population is considered, but even in this case, the probability of detecting CTCs in time is negligible[3]. Even if CTCs data could not be used for the prevention of metastasis, they still carry important information.

1.1 Liquid Biopsy

In 2020 a total of 54.976 new cases (equal to 14.6% of all new diagnoses) of BC were diagnosed in Italy[4]. With the exception of cutaneous carcinomas, BC is also the most common cancer in women in Italy, where about one malignant tumor every three (30%) is a BC. Therefore, there is an urgent need to find novel biomarkers able to improve the therapeutic management of BC patients.

Recently, the detection of Circulating Tumor Cells (CTCs), circulating Tumor DNA (ctDNA), Exosome (Ex) and other Circulating Bio-Materials (CBM) in the blood has demonstrated to provide useful information for the clinical management of BC patients: their characterization could identify potential predictive and prognostic biomarkers, through a simple blood test defined as *liquid biopsy*. The liquid biopsy has emerged in the last ten years as an appealing non-invasive strategy to support early cancer diagnosis and follow-up interventions. It represents a diagnostic technique able to trace a tumor molecular profile, both at diagnosis and during the treatment and during the follow up, capturing the changing shape of the tumor. From a single sample it is possible to isolate different circulating macromolecules such as CTCs, Ex, platelets and circulating DNA/RNA, in various body fluids among which the most suitable is represented by the blood, but it is possible to isolate these components also in pleural fluid, urine and cerebrospinal fluid. The biological liquid that is mainly used is plasma, because it is easy to obtain and for this reason is the most widely used. Compared to common diagnostic tools liquid biopsies represent a great improvement, allowing multiple sampling from the same patient at different times, therefore providing the dynamic monitoring of tumor changes not achievable with a single sample and giving insight about the whole tumor (including possible metastasis). Infact, the molecular genetics profile of a tumor changes over time and standard tissue biopsies are not able to represent the overall view of the tumor[5]. Traditionally, routine histological evaluation and immunohistochemical study have an essential role in several aspects of diagnosis in both neoplastic and non-neoplastic

disorders. However, tissue biopsy is an invasive procedure and so is often not easily repeatable, also considering the cost and the risk factors.

It is now clear the chance to get benefits of molecular information about primary tumors by using liquid biopsies and compared the result of liquid biopsy with tumor biopsy. There are still controversies in regards of the fact that liquid biopsy can take the place of tissue biopsy or will support "gold standard" tissue biopsies. Its minimally invasive nature could in principle provide a new insight for malignancy checking without delay, expenses, and risks, possibly at a microscopic stage and before the radiological tests. From the perspective of bio-detection, any biomarkers that are highly associated with tumor growth and metastasis, can be indicators of carcinomas: CTCs, ctDNA, and Ex have broad biomarker potential because they can timely and dynamically represent the tumor's genetic status both for diagnosis and for prognosis applications. Therefore, liquid biopsy could have a better sensitivity and may be more convenient as a cancer diagnosis tool in comparison with the traditional tissue biopsy method.

Although imaging and tissue biopsy are still the standard approach in solid tumor screening and monitoring, it is possible that CTCs detection with imaging combination could be the greatest choice. Infact, the quick diagnostic value of CTCs in primary stages of cancer has been considered several times and, in animal models, it was shown that CTCs were established in the very early stage implying the fact that the tumor cells had spread prior to diagnosis. The exceeding indication showed that the CTCs may be a valuable tool for very early cancer diagnosis. Moreover, many researchers have indicated a positive connection of ctDNA quantity and tumor stage to predict the severity of malignancies and efficacy of treatment[6].

The use of Ex as a predictive biomarker and indicator of treatment response completely relies on its protein and miRNA content. Thanks to the latest advances in molecular technology detection, such as Next Generation Sequencing (NGS), whole-genome shotgun bisulfate

sequencing, and tagged-amplicon deep sequencing, the importance of liquid biopsy has constantly increasing. Most scientists believe that its use in cancer treatment will support the conventional tissue biopsy method instead of replacing it. Furthermore, using a blood test along with tissue will be a breakthrough because, more often than not, the traditional biopsy does not pick enough tissue[7].

Unfortunately, discordant detection results are frequently encountered when those single biomarkers are analyzed; this inconsistency probably stems from tumor heterogeneity as well as varied analytical methodologies.

Several recent publications have revealed that the joint detection of different biomarkers or the use of multiple methods with different principles can significantly improve the sensitivity with which cancers can be differentiated at early stages[8]. A potential solution could be represented by the "integrated liquid biopsy", defined as integrating multiple liquid biopsy biomarkers or detection methods for improved analytical sensitivity and specificity to refine cancer management. Since a single biomarker is inefficient in accurately identifying most cancers, integrated liquid biopsy using multiple markers might be a promising method to facilitate early detection and treatment of cancer. For example, the tendency of ctDNA to degrade rapidly makes its accurate detection particularly challenging: Ex-ctDNA integration may elicit compensated information because resistant cell-originated Ex reveals alterations in the cancer microenvironment, and sensitive cell-originated ctDNA may indicate mutation evolution.

Infact, tumorigenesis, metastasis and tumor evolution are the typical forms of cancer development, and the above-noted biomarkers can reflect the accumulation of either genetic mutations or epigenetic modifications. During early tumorigenesis, ctDNA can be used to assess diseases from just a few million malignant cells before radioscopy. Additionally, both Ex and ctDNA can be released from all composites of cancer niches to trigger

tumor growth and metastasis, suggesting their potential in very early-stage detection and predicting tumor growth and metastasis[9].

To note combination analysis of ctDNA and CTCs has alreadyshown to allow a more reliable classification of patients in risk categories [6b].

Overall, until today, liquid biopsy represents the potential best strategy to overcome the hurdle of tumor heterogeneity and to find out new predictive biomarkers, important for the choice of more personalized treatment strategies.

1.2 CTCs

CTCs are cells that originate from the primitive tumor and that, accumulating genetic and epigenetic mutations, have acquired the ability to migrate into the bloodstream. CTCs are usually a rare finding, not present in all patients, but once identified their presence indicates a negative prognosis. The existence of CTCs in the blood of metastatic prostate cancer patients was reported for the first time by Thomas Ashworth, an Australian physician, in 1869.

In recent studies, the detection of CTCs and ctDNA in the blood have demonstrated to provide useful information for the clinical management of BC. In particular, the count of CTCs has been included among the factors of bad prognosis for the patients with primitive and metastatic mammary tumor in the 8th edition of the American Joint Committee on Cancer (AJCC) cancer staging manual[10]. Until now, metastasis is the most challenging process of tumor and the key cause of cancer-associated death (9 of 10 deaths).

The “seed and soil” hypothesis described that tumor invasion - the intrinsic characteristics of the tumor cells (seeds) and host microenvironment (soil) - are the central elements of the place of tumor development. Watanabe in 1954 by injecting bronchogenic carcinoma cells of mice showed that the CTCs can be the key role players in metastasis. In addition, it was shown that the metastases development, to some extent, depends on the size and

quantity of the CTC clusters. The CTCs are highly heterogenic, and several types of CTCs are suggested, which are given below:

Traditional CTCs: the hallmark of these cells is having large and irregular shapes, with an intact, viable nucleus; they express cytokeratins with epithelial origin and nonhematopoietic origin (they do not have CD45).

Cytokeratin-negative (CK−) CTCs: cancer stem cells passing through the epithelial-mesenchymal transition steps and forming the cytokeratin-negative CTCs. They can be opposed to treatment and are the most potential CTCs subpopulation to invade and form metastasis because they do not have cytokeratins and CD45.

Apoptotic CTCs: the traditional CTCs that undergo apoptosis. They are distinguishable with nuclear fragmentation or cytoplasmic blabbing associated with cell death. The proportion of apoptotic CTCs compared with the traditional CTCs can be a predictor of treatment efficacy.

Small CTCs: recognized as the cytokeratin-positive and CD45-negative cells, similar in size to leukocytes. Notably, the small CTCs have been concerned with progressive disease and differentiation into small cell carcinomas, which often need special therapeutic strategies.

However, the new classification system suggested that CTCs should be characterized by their size and the expression of several markers such as EpCAM. The principles of CTCs isolation, quantification, and characterization is based on the different physical characteristics (magnitude, electric charges) and genetic properties of the CTCs compared with the non-tumor cells[11].

CTCs may undergo to epithelial-mesenchymal transition (EMT) during dissemination. Although normal epithelial cells are immobile, some cancerous epithelial cells may begin expressing proteins associated with

motility and increase expressing proteins involved in forming connections between cells during embryonic development. This transformation produces mobile “mesenchymal” cells that can migrate and form other lesions. Cancer cells that have an EMT phenotype can penetrate and pass-through blood vessel walls and enter the bloodstream. These CTCs eventually attach to endothelial cells of blood vessels and exit the bloodstream, forming new metastatic lesions in other organs.

However, which CTCs subtype exhibits the highest metastasis-initiating activity remains unclear, but epithelial-type CTCs have a higher potential to translate protein and proliferate. Moreover, epithelial-type CTCs had the most metastatic potential and the proportion of epithelial-type CTCs shown associations with distant metastases and prognosis. Hence, immunoaffinity-based enrichment technologies relying on epithelial cell surface markers can underestimate the number of CTCs but may quantify metastatic potential cells. EMT and the acquisition of stem cell properties are related (i.e., induction of EMT leads to the expression of stem cell markers, increased self-renewal, and increased tumor-initiating potential). It is thus important to elucidate the biological characteristics of CTCs [12].

A recent study reported the use of CD44, CD133, and vimentin as expression markers. Moreover, the distribution of these markers in CTCs may be changing during disease progression and treatment.

Not surprisingly, CTCs found in the blood of patients with different BC subtypes have diverse phenotypes. For patients with luminal cancers CTCs were predominantly epithelial, while for other subtypes predominantly mesenchymal. Interestingly, the presence of mesenchymal CTCs was described a predictive factor, regardless of BC subtype. It seems that the hybrid phenotype may be the most predictive. Co-expression of E and M markers in CTCs is associated with cancer progression and metastasis[13].

Although the CTCs population is extremely small when compared to other cell populations in the blood, thus limiting our understanding of their biology and clinical utility, unlike the case of tissue biopsy repeating sampling to obtain more cells is possible. This makes CTCs an ideal and

clinically practical material for investigations of not only basic biological and clinical characteristics of cancer cells but of tumor heterogeneity and drug resistance as well. Due to recent technological advances, proteomic and genomic techniques that require only a small amount of sample are now available. The sheer number of CTCs circulating in the blood of a patient is the simplest and statistically significant factor in assessing the outcome. In BC, CTCs are detected in about 20-30% of early and around 60% of advanced patients. Interestingly, BC subtypes seems not have an impact on absolute CTCs numbers as well as CTCs positivity rates, except for the very high CTCs counts observed more frequently in luminal A and TNBC and the lower prognostic impact for the HER2+ disease. Therefore, more information to compare and assess CTCs numbers reliably are necessary[14].

Furthermore, CTCs numbers can vary in the blood of the same patient and is important to determine factors influencing this fluctuation: simple methodology factors like blood drawing (arterial vs. venous, the time of day, the technique) or clinical factors, including overall patients' status and clinical features, but also the important issue of CTCs mobilization by therapeutic intervention, like radiotherapy, surgery or even biopsy, could affect CTCs recovery.

Given the fact that the standard cut-off is considered to be ≥1 in the (neo) adjuvant setting and ≥5 in the metastatic setting, the enumeration of CTCs represents an effective prognostic and predictive biomarker; it can be explained by the metastatic potential of these cells, but almost certainly it is not always the case[15]. Alternatively, CTC shedding may be associated with the increased aggressiveness of the tumor. Clarification of the factors determining the CTCs release pattern is thus an important goal that can be achieved only by systematic longitudinal studies of CTCs numbers in a large patients' cohorts.

Besides enumeration, new techniques, which enable to efficiently isolate CTCs from patients' blood provide the possibility of a detailed molecular analysis. Genotype analysis of CTCs in BC is tuned to detect the most

common driver mutations and to assess the difference between CTCs and primary tumor. Frequently mutated genes in BC CTCs include TP53 (mostly in TNBC), PIK3CA, ERBB2, KRAS and ESR1. Moreover, mutations in these genes display high degree of intra-patient heterogeneity. Thus, it is important to recognize mutations at the single-cell level, since they may give rise to metastatic clones, while remaining undetectable in the greater part of analysis. Interestingly, the analysis of the receptor status in CTCs from metastatic patients demonstrated that most of CTCs were estrogen and progesterone receptor negative, while primary tumors were receptor positive. This could reflect a mechanism of escape from endocrine therapy. In contrast to that, in receptor-negative BC changes in receptor status between primary tumor and CTCs are usually minimal. Discordance in expression between primary tumor, CTC and metastases was also observed for HER2[16].

It is also worth considering that, when a tumor cell enters the circulation and becomes CTC, these cells are exposed to various stresses associated with this new microenvironment and them reversible re-program themself to cope with new challenge. Alternatively, some protection from these factors can be achieved by cells' clustering, but the origin of CTCs clusters is subject of debate. Originally, it was surmised that CTCs clusters arise by collective invasion and dissemination of oligoclonal groupings of tumor cells, but recent findings revealed that they can be formed by tumor cells aggregation within vasculature. This aggregation is induced by homophilic interactions of CD44 molecule, the receptor for hyaluronic acid. It was demonstrated in mouse models that BC metastases are of polyclonal origin, which is inconsistent with the dissemination and expansion of a single cell but can be easily explained by the dissemination of CTCs clusters[17].

However, CTCs clusters are rare (about 10 times more rare than single CTCs), but have up to 50 times more metastatic potential than single CTCs. Molecular profiling of single and clustered CTCs revealed high expression of

desmosomal junction protein plakoglobin in clusters, which may be due to its function in maintaining strong cell-cell contacts.

Anyway, CTCs analyses give important information with diagnostic and therapeutic relevance and since they represent tumor cells' population currently present in the circulation and, as such, can serve as a source of invaluable information, considering tumor properties, more accurate, recent and relevant than tissue analysis from the primary tumor obtained during surgery or biopsy. Learning from that input, it is possible to modify the treatment and either hold the potential metastases in check, by knowing their biology, or, in more advanced cases, tailor the treatment according to the specific properties and sensitivities of the circulating cells. This can be especially important in case of ER+ BC, which tends to lose responsiveness to the usual treatments. CTCs longitudinal screening may detect this change and flag the necessity for therapy modification. Gaining more knowledge on the issue if some routes of metastasis are more likely in specific subtypes of BC would be invaluable for the CTCs data interpretation. In early BC (EBC) the accumulated data about the biology of CTCs released by the primary tumor are scarce. Tumor cell dissemination has been suggested to occur early in BC progression, even before the tumor has become invasive. A single study on 73 patients with either ductal or lobular carcinoma in situ reported that three patients (4.1%) had 1 CTC per 22.5mL of blood. Prognostic significance of CTCs was also clearly demonstrated in adjuvant setting: analysis of 206 patients enrolled in the same study with known CTC status at 5 years, revealed that CTCs-positivity was found in 7.8% and was associated with a 6-fold increase in recurrence. Prognostic value of CTCs has been demonstrated in metastatic BC by many studies. CTCs were detectable in approximately 60% of MBC patients, and a CTC count ≥5 cells per 7.5mL of blood was associated with significantly worse progression-free survival (PFS) and overall survival (OS, providing evidence for its clinical validity[18].

However, the question if either CTCs number or/and CTCs phenotype may serve as a criterion for therapy decisions in still unsolved. There are

currently insufficient data to support the use of CTCs status as a criterion for therapeutic choice and it still remains to be investigated in prospective trials that show how it can be used for a prediction of therapy response and an improved clinical outcome.

1.3 ctDNA

Circulating tumor DNA (ctDNA) merely refers to fragmented DNA, originates from tumor cells itself. It represents a part of cfDNA /cell free DNA, and has a substantial fluctuant proportion ranging from <0.1% to >90% in cfDNA; in general, cfDNA levels in the blood are elevated in patients with carcinoma compared to healthy individuals. With lots of ctDNA released into the circulatory system by tumor cell apoptosis or necrosis, the quantity of ctDNA could reflect tumor burden in cancer patients. How to detect and analyze ctDNA from the background of normal cfDNA is a big challenge in the development of liquid biopsy application because it is highly fragmented to on average 170bp in length. Moreover, ctDNA may occasionally be present only as a minimal fraction in the circulation compared with the wild-type cell-free DNA (cfDNA) released from normal cells. In healthy individuals, cfDNA originates from hematopoietic cells while in cancer patients is tissue-specific, as the majority of their ctDNA comes from the tumor tissue of origin. The ratio of ctDNA to cfDNA depends on tumor and immunological factors, tumor burden and progression, and blood clearance mechanisms. If found, ctDNA has the potential to capture complete information on genetic alterations, including the somatic single-nucleotide variations present in the tumor and its metastases, and also to longitudinally monitor these mutations throughout the course of patient treatment. In addition, ctDNA is considered to be released from apoptotic and necrotic tumor cells, and its blood circulation half-life ranges from minutes to several hours, which makes it a dynamic, almost real-time, biomarker for cancer monitoring[19].

Targeted NGS, which generates sufficiently high coverage over a certain region, is therefore required, and ctDNA molecular analysis and

identification of these low-frequency variants is important for detection of cancer in its early stages and appropriate diagnosis[20].

Moreover, ctDNA analysis in the clinical setting may guide decision-making and potentially facilitate personalized cancer care. It is able to provide information on the presence of cancer and its genetic composition and is correlated with clinical parameters such as stage, disease burden, recurrence, and response to treatment. In BC, recent studies have indicated the versatile role of ctDNA analysis in determining the genomic profile of tumors, to sensitively and specifically identify preclinical metastases and predict a relapse ahead of its diagnosis, and to assess resistance to targeted therapy or to uncover tumor heterogeneity[21]. In another study, the molecular profile in serial ctDNA samples from BC patients was monitored and it was demonstrated that ctDNA sequencing provides dynamic information on somatic variants with pathogenic effects in tumors. The molecular analysis revealed that patients with lower-grade and luminal A BC subtype and patient with low-grade luminal B subtype had somatic pathogenic variants detected only in their ctDNA prior to surgery. This correlates with a generally better prognosis of the luminal A subtype compared with other BC subtypes. Detection of somatic pathogenic variants in ctDNA only after surgery was associated with patients of unknown HER2 status and luminal B, and unknown luminal type; but these patients had higher-grade (G2 or G3) cancer. While patients with luminal B, HER2+ and grade 3 had somatic pathogenic variants detected in ctDNA only after chemotherapy, restricted patient numbers, lack of characterization and impossibility of analyzing certain samples prevented significant conclusions from these results[22].

In a recent study a PIK3CA and TP53 mutations in matched ctDNA samples before biopsy and tumor biopsy samples in patients with early BC were analyzed. Concordance between ctDNA mutations and biopsy mutations was observed in only 8/29 (27.6%) patients. All patients with concordant mutations had higher-grade disease. Somatic PIK3CA mutations were identified in 19/29 (65.5%) patients. TP53 mutations were identified in

6/29 (20.7%), and 4 patients (13.8) had both PIK3CA and TP53 mutations. Therefore, these analyses could identify tumor heterogeneity, improve the diagnosis of early BC patients, and provide significant information[23]. Similar studies have been performed in ER+ and TNBC and showed ctDNA genomic changes in patients with ER+ metastatic BC (mBC) by hybrid capture-based genomic profiling. The time-matched ctDNA and tissue samples from 14 patients and 89% of mutations in tissues were also present in the in ctDNA, but mutations in ESR1, TP53 and PIK3CA were detected only the in ctDNA samples has been analyzed[24]. This detection of extra mutations in ctDNA could support liquid biopsy use in detecting heterogenous metastatic sites in ER+ mBC. Moreover, a retrospective study using cfDNA to determine somatic copy-number variations in primary and metastatic tumors and cfDNA tumor fractions were conducted in patients with TNBC. The work highlighted that cfDNA tumor fractions ≥10% were significantly associated with worse survival. These results add to the mounting evidence supporting the utility of ctDNA as complementary analysis to tissue biopsy for thorough molecular profiling of tumor variants[25]. Finally, it was demonstrated that next generation ctDNA sequencing is useful for monitoring the changes in somatic variants with pathogenic effects in patients with BC, and this analysis can supplement the results of tumor tissue biopsy. However, in BC, ctDNA analysis has a low sensitivity of detection (<40%), in part due to the low frequency of common mutations in BC. Nevertheless, its detection has been already proved to great value in clinical settings, where liquid biopsies are now starting to be included in the validated tests panel used to detect mutations for target therapies, such as the use of Alpelisib for the treatment of patients with hormone receptor-positive, HER2-, and PIK3CA-mutated advanced or metastatic BC progressed after previous endocrine therapy as monotherapy[26].

1.4 Ex

Ex are 40-100nm diameter membrane vesicles of endocytic origin, which are secreted by various kinds of cells and contain a broad repertoire of

molecules, including nucleic acids (ex., DNA, mRNA, miRNA, long and short noncoding RNA), proteins (ex., cytoskeletal proteins, transmembrane proteins, and heat shock proteins), and enzymes (GAPDH, ATPase, pgk1). Generally, contents of Ex can reflect the nature and status of the cells of origin. The upgrading of specific proteins and nucleic acids implies a degree of specific cellular sorting into Ex.

On the other end of spectrum, Ex have the ability of modulating cellular activities in recipient cells by transferring genetic information[27]. Ex has endocytic origin, which shaped from inward budding of membrane of multi-vesicular bodies (MVB) and released from the cell into the extracellular environment with the plasma membrane. There are a large number of mobile membrane-limited vesicles called extracellular vesicles (EVs) in the extracellular environment, which can be further subcategorized based on their size, biogenesis and release mechanism, and content into Ex, micro-vesicles (MVs), and apoptotic bodies. Ex and MVs have a distinct biogenesis, while the most dramatic difference between Ex and MVs is how they are formed: Ex are formed from inward budding of an endosome resulting in a multivesicular body (MVB), with the plasma membrane, which is secreted by succeeding fusion of the MVB. On the other hand, MVs are released directly by budding from the cellular plasma membrane [28].

Up today, the biomarker research has been mostly focused on Ex analysis, since Ex analysis provides several advantages as liquid biopsy and their application as a potential complement to personalized medicine in some common malignant tumors. Ex exist in almost all body fluids and possess high stability encapsulated by lipid bilayers that can reduce the cost of sample short-term storage and the difficulty of transportation, which greatly enhances the clinical applicability; Ex are secreted by living cells and contain biological information from the parental cells and be more representative than cell-free DNA (cfDNA), which is secreted during necrosis or apoptosis; Ex identification is easier compared to other circulating biomarker, such CTCs, because they express specific proteins

which can be used as markers to effectively distinguish them from other vesicles; finally, Ex can present specific surface proteins from parental cells and even target cells, which can realize the isolation of origin-specific Ex and predict organ-specific.

However, there are still some barriers between basic research and real clinical practice: a standardization of the classification and extraction method for different body liquids is needed and the identification of specific subtypes of EVs is necessary, as different vesicles may exert various biological effects. Therefore, the standardization of protocols and identification methods are required when attempting to widely use Ex in clinical testing; although many molecules carried by Ex have been documented to serve as potential biomarkers, little of them are qualified for application; last but not the least, as potential therapeutic cargo, the biological safety, and targeted efficacy[29] have to be verified.

Recently, research on the role of Ex involved tumorigenesis and cancer progression has grown exponentially, including immune suppression, angiogenesis, cell migration and invasion. As Ex are capable of transferring specific proteins and nucleic acids to recipient cells in the tumor microenvironment or at specific distant sites, Ex could be the tool by which cancer cells can transfer malignant phenotype to normal cells and establish a fertile local and distant microenvironment to help cancer cell growth. Increasingly, Ex are studied for their potential roles as both indicators of BC and a prospective new treatment approach, explained by the fact that they can play important roles in different stages of development in BC[30].

Ex also exert a feedback mechanism regulating the release of Ex from normal mammary, it means that those who are released from BC cells and normal human mammary epithelial cells are regulated by Ex derived from their own cells. Through horizontal transfer of genetic information between BC cells, Ex are supposed to exhibit pleiotropic biological functions, including stimulating tumor angiogenesis, reorganizing the stroma to establish the tumor microenvironment, as well as promoting tumor growth and drug resistance. In details, during initial malignant

transformation, Ex generated by BC cells contain a variety of proteins and RNA species can be transfected between cancer cells as well as cancer and normal cells, conferring a transformed-like phenotype to normal mammary epithelial cells.

Although the exact underlying mechanisms remain to be elucidated, Ex could alter the transcriptomes of target cells and contribute to oncogenic transformation and tumor formation. For example, Ex secreted by a specific BC cell line were capable of transforming normal human mammary epithelial cells into cancer cells. At the same time, in BC cells hypoxia condition is regarded as a major trigger for Ex secretion. This is a transient phenomenon presents at microscopic sites within tumor microenvironment, which is linked to angiogenesis, tumor aggressiveness, treatment resistance and poor outcomes. With a unique ability, cancer cells can survive and grow under hypoxic environments. In these conditions, the effects of Ex on tumor angiogenesis and growth are even more noticeable: hypoxia-induced release of Ex from cancer cells may lead to neoplastic transformation, malignant cell growth and invasion[31].

Since BC therapy has received many restrictions due to explanation of mechanisms responsible for drug resistance, a reliable indicator to predict the therapeutic effect, is essential to achieve more effective and individualized chemotherapeutic treatment of BC patients. Currently, some studies have shown that BC Ex-mediated transfer of genetic information can induce therapy resistance and promote tumor progression, such as extruding hydrophilic drugs from cancer cells. Moreover, it is also implied the roles of Ex in BC chemotherapy or radiotherapy resistance and cancer immunotherapy. Radiotherapy is a significant treatment method in BC. Generally, oxygen is indispensable for radiation cytotoxic to cause DNA damage, thus, BC can be more resistant to radiation therapy under hypoxic microenvironment, and hypoxia could lead to the increased production of Ex. Besides, Ex transferred from stromal to BC cells stimulates antiviral signaling and derived radiation resistance in primary tumor: drug-resistant BC cells may spread resistance capacity and alter chemo-susceptibility in

recipient sensitive cells via intercellular transfer of Ex. Finally, tumor Ex carrying antigens that are targets for anti-tumor antibodies, therefore competitively inhibit the drug activity and contribute to immunotherapy failure[32].

Overall, Ex have been proven to be important regulators in health and disease, especially in tumor biology. It is well known that tumor derived Ex enclosing tumor-specific antigens and nucleic acids can be assessed non-invasive as potential diagnostic and predictive biomarker.

Besides, Ex could potentially be used to identify patients who are likely to develop metastatic disease, and the process of Ex production could yield new targets for cancer therapy. With the development of novel therapeutic strategies targeting or utilizing Ex, it will lead to more effective prevention and intervention strategies in BC therapy. However, it is critical to note that crucial contents of Ex are still not fully elucidated, and as a consequence it is challenging to complete understand the role of Ex in BC progress.

EXPERIMENTAL PART

2. THESIS PURPOSE

The purpose of this thesis is to verify the technical feasibility and possible clinical utility of ***liquid biopsy in BC clinical contest*** at Ospedale Policlinico San Martino.

The work has been carried out in two translational protocols: mainly, a translational study aimed to *detect and characterize CTCs, together with ctDNA*, (CITUCEL trial); part of the work was conducted within a study designed to evaluate which circulating biomaterial obtained by liquid biopsy better represents TNBC and HR-/HER2+ BC *heterogeneity* (BaReLiB Study).

2.1 CITUCEL

This project presents the preliminary results of the translational protocol CITUCEL "*CIrculating TUmor CELls (CTCs) and circulating tumor DNA (ctDNA) in BC patients: a prospective study*".

This is a no profit study and takes the advantage of a close collaboration between different groups at Ospedale Policlinico San Martino: the Molecular Diagnostic Unit (Dr. Zupo/Dr Dono/Dr. De Luca) and the Clinic of Internal Medicine (Prof. Ballestrero/Dr Anna Garuti).

The study was approved on February 4th, 2016 by the Local Ethic Committee and made operative on May 4th, 2016 with administrative resolution no. 0519.

In a period between February 2017 and January 2020, 60 patients with invasive BC were enrolled at the SDD Breast Unit of the Ospedale Policlinico San Martino.

Inclusion Criteria:

- Patients with histological diagnosis of invasive carcinoma undergoing neoadjuvant chemotherapy (stage IIB, III tumors).
- Patients with histological diagnosis of invasive early stage BC at high risk of

relapse (stage IIB tumor) undergoing adjuvant chemotherapy.

- Patients with histological diagnosis of metastatic BC candidates for a first line of treatment.
- Patients with histological diagnosis of metastatic BC progressing to first-line endocrine therapy.
- Patients must have signed informed consent.

Exclusion criteria:

- Patients with histological diagnosis of invasive BC undergoing neoadjuvant therapy for stage IIA (T1N1 or T2N0) neoplasia.
- Patients with histological diagnosis of invasive early-stage BC with low or intermediate risk of relapse (patients not candidates for adjuvant therapy, patients' candidates for adjuvant therapy with luminal A subtype at any stage and those with TNBC or luminal B or HER2+ with N<4).
- Patients with a histological diagnosis of metastatic BC progressing to >1 line of chemotherapy for advanced disease.
- Patients with a histological diagnosis of metastatic BC progressing to ≥2 lines of endocrine therapy for advanced disease.

2.2 BaReLiB

TNBC is defined by the absence of ER, PR and HER2 expression. TNBC are generally more aggressive tumors: patients have a relatively poorer outcome compared with those with other BC subtypes and have a higher risk of both local and distant recurrence.

Nearly, only around 30-40% of TNBC patients achieved a pathological complete response (pCR) after neo-adjuvant chemotherapy. Currently adopted classification considers 4 TNBC subtypes[33].

Similarly, the prognosis of patients with HER2+/HR- BC, a very aggressive subtype, is poorer if they did not achieve pCR after neo-adjuvant therapy. These evidences suggest a marked heterogeneity and consequent differential responsiveness to chemotherapy.

An important issue in BC field is represented by tumor heterogeneity, defined as heterogeneity within the tumor or between the primary tumor and metastatic sites within a same patient. Besides the inter-tumor heterogeneity, the picture is further complicated in the intra-tumor clonal heterogeneity, which may have profound clinical implication.

This study aims to systematically evaluate the reliability of ctDNA, CTCs and Ex (CBM) in featuring the heterogeneity of TNBC or HER2+/HR- BC xenotransplanted mice using an unprecedentedly powerful technique based on genetic barcoding and NGS.

The sensibility and specificity of the individual CBM in representing tumor heterogeneity has not been evaluated yet: to determinate which of those could be the more representative biomarker for the study and treatment of TNBC and HER2+/HR- is therefore of crucial importance.

A systematic analysis of these aspects implies the ability to recognize a large number of genomic assets simultaneously; therefore, it has been so far impractical even in animal models.

The recent development of NGS opened the opportunity to analyze unparalleled amount of genetic materials. This provides a tool to detect a huge number of artificial genetic labels (barcodes) that can be inserted in tumor cells prior xenotransplantation, allowing to systematically monitor tumor clones and CBM released by them.

This analysis will allow to establish how many different clones compose the tumor masses and how large they are. In particular, this project has the propose to exploit genetic barcoding and NGS to directly compare the performance of the different CBM analysis in a mouse model of primary TN or HER2+/HR- BC cells xenografts.

This protocol brings together different expertise within the Institute: a deep experience in clinical practice of BC (Prof. Lucia Del Mastro) and patient CTCs isolation (Dr. Barbara Cardinali).

A long lasting experience in primary culture enriched in TIC and the derived mouse model of cancer (Dr. Antonio Daga); a skilled training in Ex isolation and analysis (Dr. Roberta Tasso); a strong background in molecular and

cancer biology with a special feeling for computational biomolecular analysis and NGS applied to different tissue types (Prof. Paolo Malatesta; Dr. Davide Ceresa).

2.3 Ixazomib

The ubiquitin-proteasome signalling pathway plays a critical role in several processes regulating cellular homeostasis and survival. It represents the main non-lysosomal route for degradation of misfolded, oxidized, damaged proteins. Non-lysosomal mechanisms are responsible for the highly selective turnover of intracellular proteins that occurs under basal metabolic conditions, but also for some aspects of degradation of intracellular proteins under stress.

An important non-lysosomal proteolytic pathway is the ubiquitin system in which proteins are degraded by a 26s protease complex following conjugation by multiple molecules of ubiquitin. The catalytic core of the complex is a 20s protease complex also known as the *proteasome*[34].

MLN9708 (Ixazomib) is a second-generation small-molecule proteasome inhibitor approved for the treatment of multiple myeloma (MM) with a shorter proteasome dissociation half-life compared to first-in-class bortezomib, as well as improved pharmacokinetic, pharmacodynamic and antitumor activity[35].

Furthermore, upon exposure to aqueous solutions or plasma, MLN9708 rapidly hydrolyses to its biologically active form MLN2238.

Ixazomib is active in some of the major apoptotic signalling pathways. In fact, it induces cleavage of caspase-3, an upstream activator of PARP-1 and decreases cyclin D1 and its target protein Cdk6.

In MM cells, apoptosis induced by MLN2238 has been shown to be caspase-dependent, since this compound is able to activate both caspase-9 (intrinsic) and caspase-8 (extrinsic) apoptotic pathways through the involvement of other signalling pathways, such as p53-p21, p53, NOXA, PUMA, and ER stress[36].

A previous preclinical study has shown that in BC cell lines this drug could lead to specific tumor cell death through apoptosis activation caspase-3 and PARP-mediated mechanism[37].

It has been demonstrated the activation of pleiotropic cell death signalling cascades in response to proteasome inhibition. This is likely due to the fact that the majority of cellular proteins undergo degradation through proteasome, and blockade of proteasome negatively affects this normal cellular process resulting in accumulation of unwanted proteins and subsequent activation of multiple cell death signalling[38].

Bendamustine is a drug used for the therapeutic treatment of MM. It is a bifunctional alkylating agent that combines the alkylating properties of 2 chloroethylamine and the antimetabolite properties of a benzimidazole ring. Bendamustine induces cell death by the activation of apoptosis and the DNA damage response, acting as an alkylating agent that induces inter-strand DNA crosslinking and subsequent strand breaks[39].

Until now, the potential therapeutic effect of Ixazomib and/or Bendamustine on BC remains unknown.

A pilot *in vitro* study was developed starting from this data and clinic evidences: a 68-year-old patient who experienced MM and BC was treated at Ospedale Policlinico San Martino with Ixazomib and Bendamustine.

In details:

Clinical case (S. A. 68 years woman)

February 2017: diagnosis of MM

21/2/17: staging PET scan revealed a right breast lesion confirmed by mammography and breast ultrasound (9mm right QSI)

6/3/17: right breast biopsy positive for ductal invasive carcinoma ER 99%, PgR 99%, Ki67 13% HER2 1+; clinical TNM: cT1N0

2/3/17: start induction therapy: MM in UNITO-EMN10 Trial with MLN9708 4 mg orally on days 1,8,15 or desamethasone 40mg orally on days 1,8,15,22 or Bendamustine 75 mq/mq iv on days 1,8

6/7/17: Mammography and breast ultrasound negative for breast lesions.

To treat the patients, priority was given to the treatment for MM. Surgery for BC was delayed and the patient started dexamethasone, Ixazomib and Bendamustine, the MM induction therapy.

Intriguingly, a complete response of BC was observed by mammography and PET scan. It is therefore possible to hypothesize that the clinical response for BC was induced by the treatment with Ixazomib and/or Bendamustine.

This project proposes to determine if Ixazomib, Bendamustine or their combination could be effective in the cure of BC using *in vitro* assays on different BC cell lines and put in evidence if this effect could be a BC subtype specific.

The results of this study could lead to the development of new diagnostic approaches specific for this neoplasia.

3. AIMS

3.1 CITUCEL

Our aims are:

1) To perform a correlation between:
   - the presence of CTCs, ctDNA and the rate of pathological complete response (pCR) in BC patients treated with *neoadjuvant therapy*.
   - the presence CTCs, ctDNA and disease-free survival/overall survival in *(neo)adjuvant therapies*.
   - the presence CTCs, ctDNA and the progression-free survival/overall survival in *first line therapy.*
2) To assess the mutational profiles of primary tumour, CTCs and ctDNA at different time-points for a comparative analysis of these profiles in the *adjuvant, neo-adjuvant and metastatic settings.*

A total of 60 patients were planned to be enrolled in three years. Eligible patients undergo peripheral blood sampling according to specific timings:

- For patients undergoing *neo-adjuvant* CT: at baseline and at the end of neoadjuvant therapy, before surgery
- For patients undergoing *adjuvant* CT: before and after the end of adjuvant chemotherapy.
- For *metastatic BC*: at the time of initiation and at progression to first-line therapy

For all the patient's data were obtained on: tumor characteristics, treatment and follow up.

3.2 BaReLiB

The study aims to:

- Evaluate the reliability of various CBM in featuring the heterogeneity of TNBC.
- Determine the representativeness of tumor mass in the different clones recovered from the different circulating biological biomaterials (CTC, cDNA and Ex).
- Evaluate the proportion between cell number and single clones in the different circulating biological components.
- Evaluate of the lower limit of detection of single clones in the different CBM.
- Establish the gene expression profiles of tumor initiating cells in TNBC subtypes.

3.3 Ixazomib

In vitro experiments have been conducted with the aim to:

- Evaluate of the activity of Ixazomib, Bendamustine or their combination on BC cell lines.

- Characterize the biological mechanism involved in the possible therapeutic efficacy of Ixazomib, Bendamustine and/or their combination.

4. MATERIALS AND METHODS

4.1 CITUCEL

4.1.1. Sample collection and treatment

Enrolled patients underwent a peripheral blood sampling through venous access. Five samples of 6mL each were collected in tubes containing Ethylenediaminetetraacetic acid (EDTA) as anticoagulant, following specific timings. In particular, for patients treated with neoadjuvant chemotherapy, samples were taken before and at the end of neoadjuvant chemotherapy before surgery; for patients undergoing adjuvant chemotherapy, samples were taken before and after the end of the chemotherapy treatment; finally, for metastatic patients, the samples were collected before the beginning of treatment and eventually at the time of disease progression.

Blood samples for CTCs and ctDNA analysis were collected simultaneously and the material was processed in a short time, when possible within an hour from blood withdrawn. The correct timing was ensured thanks to the close collaboration between the research nurse and researchers at the Breast Unit.

- CTCs: the extraction and recovery of CTCs is conventionally carried out from 7.5mL of whole blood. In this study, blood sample in EDTA was stored at +4°C from the time of collection until the first step of the enrichment procedure, the start of which was standardized between half an hour and an hour from blood collection.
- ctDNA: ctDNA extraction is carried out from plasma. Experimentally, plasma was obtained from approximately 12mL of whole blood. Due to the low stability of cfDNA, plasma preparation, based on two consecutive centrifugations at 4°C for 10 min, was performed within two hours of collection. The blood sample was centrifuged a first time at 1600xg in order to separate the plasma from the cellular component; a second centrifugation at 1600xg, on the plasma sample collected in a new conical tube, was carried out to eliminate any residual cellular trace from the isolated plasma. Recovered plasma,

about 4-5mL for each patient, was stored at -80°C until the ctDNA extraction.

4.1.2. Experimental workflow

The analyses of CTCs and ctDNA were carried out in parallel on samples collected simultaneously and processed according to the experimental workflow (shown in Fig. 1), based on the close collaboration between the SSD Breast Unit (patient enrollment, recovery and characterization of CTCs), SC Clinic of Internal Medicine (patient enrollment and ctDNA analysis) and UOS Molecular Diagnostic (mutational analysis of CTCs and tissue).

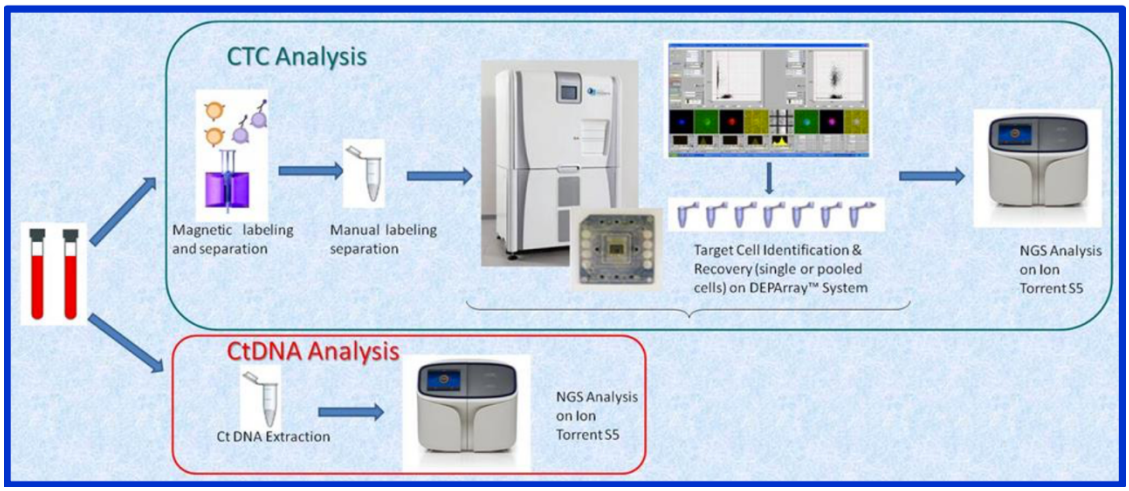

Fig. 1 CITUCEL protocol experimental workflow

4.1.3. CTC enrichment and recovery

CTCs are traditionally defined as larger cells with irregular shape or subcellular morphology positive for citokeratines and EpCAM, which demonstrate epithelial origin, and negative for CD45, indicating the cell is not of hematopoietic origin. However, EpCAM negative CTCs exist, because of the epithelial-to mesenchymal-transition: thus, these CTCs may be missed by currently available assays based on EpCAM positivity. To overcome this limitation, in this study, CTCs have immunomagnetically negative selected from peripheral blood by depletion white blood cells of (WBC) and red blood cells (RBC) using a methodology adapted form Bulfoni

et al. Breast Cancer Research, 2016. Briefly after RBC lysis, CTC were immunonegative enriched on magnetic LD columns (Miltenyi Biotec) by depletion of normal leukocytes and residual RBCs with an immunomagnetic micro-beads cocktails targeting CD45 (antigen expressed on all cells of hematopoietic origin except erythrocytes and platelets) and Glycophorin A (expressed on mature erythrocytes and their precursor). CTCs were collected in the unbound fraction, concentrated, stained for nuclei (with HOECHST 33342) and for EpCAM for epithelial origin, CD44 for mesenchymal and for the pan-leukocyte (CD45) markers antigens. Then CTCs have been fixed, using Inside Stain Kit (Miltenyi Biotec), that is able to achieve mild fixation and permeabilization of cells and leaves their morphological characteristics intact. Enriched CTCs were further selected with a new technology, the DEPArray™ System. DEPArray™ is the only system available on the market capable of visualizing, moving and retrieving small cell populations down to single cells through the use of di-electrophoretic forces on a software-controlled semiconductor chip. The heterogeneous cell populations loaded on the device can be selected identifying target cells through the use of different phenotypic markers detected by fluorescence and isolate them as individual cells by use of di-electrophoretic manipulation that allows their movement within the chip.

Fig. 2 Photograph of the DEPArray™ Instrument at Policlinico San Martino

Finally, CTCs selection, based on morphological and phenotypical analysis is performed in image-based post-recovery analysis: this step is fundamental to identify CTCs, to choose the cells of interest, assess their phenotype, and identify statistically significant marks.

Before analyzing patient's samples, sensitivity and specificity of the enrichment method have been verified by spiking experiments using BC cell lines: MDA-MB-231 and MCF-7.

In particular, whole blood samples have been spiked with different kind and number of tumor cells, in order to verify the power of the enrichment and detection on cell with different phenotypes.

The rate of recovery and purify of the fractions have been obtained from the comparison of the number of spiked tumor cells with the number of recovered tumor cells after the enrichment procedure. The specificity of the method has been assessed comparing the expression of the selected markers on blood cells of healthy donors by FACS analysis.

4.1.4. Isolation, extraction and quantification of cell-free DNA (cfDNA)

The analysis of circulating free DNA (cfDNA) samples from cancer patients is able to provide detailed prognostic and predictive information, data on the response and resistance to treatment and on the evolution of the disease.

In this trial it was chosen to use a multigenic approach in NGS with the use of a single panel capable of investigating multiple variants simultaneously, rather than the Digital PCR Technique, which is often used for these analyses, since it would allow to detect a specific mutation at a time and requires a significant amount of DNA and to know in advance the alteration to be searched for.

CtDNA extraction was carried out with the Maxwell® RSC LV ccfDNA Kit (Promega Corporation) on the Maxwell semi-automatic extraction system as per commercial protocol, starting from 3/5 mL of plasma, on average 4mL. The extracted DNA was quantified in two ways:

1) using the QubitTM dsDNA HS Assay Kit (InvitrogenTM) designed to

be used on the QubitTM 2.0 Fluorometer (InvitrogenTM).

2) through High Sensitivity D1000 ScreenTape system designed for the Agilent TapeStation 2200 platform and capable of quantifying DNA molecules between 35 and 1000bp.

4.1.5. Mutational analysis

Mutational analysis was performed using NGS approach that has been remarkably improved in the last years, providing numerous advantages in recent generations of instrument that are faster, cheaper devices producing better-quality data, requiring less genetic material and less reagents compared with previous ones.

In the CITUCEL protocol, the Ion Torrent platform was used.

In particular the analyses were carried out on an Ion Torrent S5, intermediate between the second and third generation, that is a unique instrument because it uses semiconductors (Ion Chips) as structural element of the supports in which the sample is dispensed for sequencing and uses a detection system not based on easy luminescent reactions to decay, but on potential variations.

Briefly, this analysis involves four different phases:

1) Building the library
2) Preparation of the template / library amplification
3) Sequencing
4) Data analysis

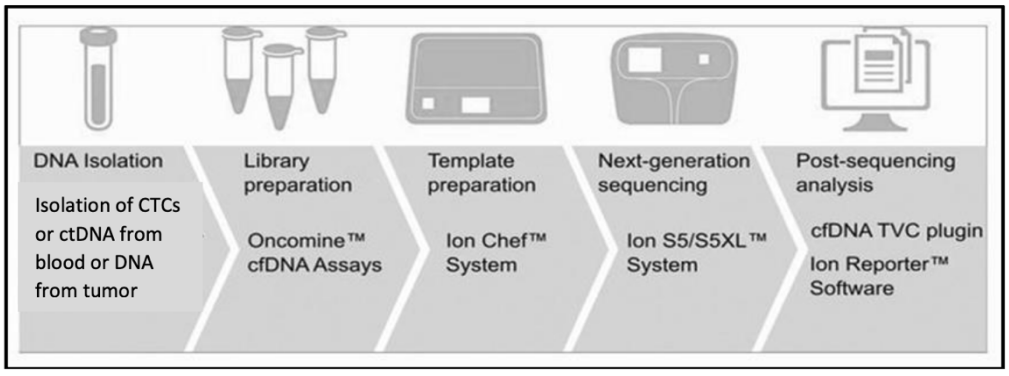

Fig. 3 Mutational analysis phases in NGS

The mutational analysis was performed in NGS using for both ctDNA and CTCs analyses the Oncomine™ Breast v2 cfDNA Assays (OBcfRAv2-ThermoFisher Scientific) panel designed for the detection of somatic mutations in plasma with a limit of detection (LOD) even below 0.1% in genes considered to be of clinical utility in BC.

The panel covers more than 150 hotspot genomic locations distributed across 10 genes: AKT1, EGFR, ERBB2, ERBB3, ESR1, FBXW7, KRAS, PIK3CA, SF3B1 and TP53; includes analysis of copy number variations (CNVs) CCND1, ERBB2, FGFR1.

- CTCs

In this regard, a method designed for the mutational analysis of CTCs through NGS has been optimized and developed in house and recently published[40].

The validated methodology is performed without the pre-amplification step, namely without applying the so-called whole genome amplification (WGA) which allows to obtain sufficient DNA molecules starting from small samples such as those containing a few isolated CTCs, with the aim of eliminating errors usually introduced with such amplification.

The NGS protocol, originally created for the study of free circulating cfDNA, has been adapted for the molecular characterization of CTCs; it is based on the very sensitive technology of "molecular tagging", which allows to intercept even a single DNA molecule within a sample and recognizes a true mutation from a DNA amplification/sequencing error. Detailed description in paragraph 5.1.4.

- ctDNA

As mentioned above, the library size was assessed using the Agilent High Sensitivity DNA Kit by Tapestation 2200 instrument, and the library concentration was determined with the Fluorometer 2.0 using the QubitTM dsDNA HS Assay Kit.

Template preparation was conducted on the Ion Chef System (Thermo) and sequencing conducted on the Ion 540^{TM} chip with the Ion S5 platform to obtain coverage ≥25,000X, as per company specifications.

4.1.6. Tissue analysis

Mutational analyses of the patients' baseline tumor samples were performed in NGS with a dedicated panel based on Ion AmpliSeqTM technology. The panel was designed to study the common alterations found in BC in order to incorporate also the mutations included in the Oncomine Breast V2 panel and includes 291 amplicons distributed in 20 genes (ie, TP53, PIK3CA, ERBB2, ERBB3, ERBB4, ESR1, MCL1, GATA3, PTEN, CCND1, KRAS, AKT1, CDH1, MAP2K4, SF3B1, FBXW7, MAP3K1, PIK3R1, EGFR, and FGFR1).

Genomic DNA was manually extracted from FFPE samples with the instrument QIAsymphony (Qiagen, Milan, Italy).

To improve the extraction yield, the tissue was micro-dissected in order to obtain samples with at least 50% of tumor cells.

The extracted DNA was quantified with the QubitTM 3.0 Fluorimeter and used for the automatic construction of libraries on the Ion ChefTM using the Ion AmpliSeqTM Kit for Chef DL8 (ThermoFisher Scientific, Carlsbad, CA, USA) and the libraries were analyzed on the Ion 520TM Chip.

Sequencing was performed on the GeneStudioTM S5 Torrent platform.

The data were analyzed on the Ion Torrent Suite and the annotation of the variants was done with the Ion Reporter Software (version 5.10, ThermoFisher Scientific, Carlsbad, CA, USA).

4.1.7. Statistical analysis

In order to analyze the differences in CTCs number and CtDNA concentration between the different settings time points, the statistical analysis was conducted with classic tests (X^2 or Fisher, Mann Whitney, Kruskal-Wallis tests), using the Stata SE version 14 software. The differences were considered significant if P-value <0.05.

4.2 BaReLiB

The experimental activity carried out in this PhD project has been focused on the enrichment and recovery of CTCs.

In the BaReLiB study (Fig. 4 represents the experimental workflow) all the experiments regarding the setting up of culture condition, the xenografting of barcoded cells into immunodeficient mice and NGS analysis have been done by Molecular Biology Unit at HSM.

4.2.1 Cell line set-up

MDA-MB-231 cell line (TNBC human cell line) and primary cells derived from patients' resected tumors have been individually transduced by a retroviral library that carries a degenerated barcode and the GFP gene and then xenotransplanted in immunodeficient mice.

Following the development of the tumor, CBM and the tumor mass have been collected.

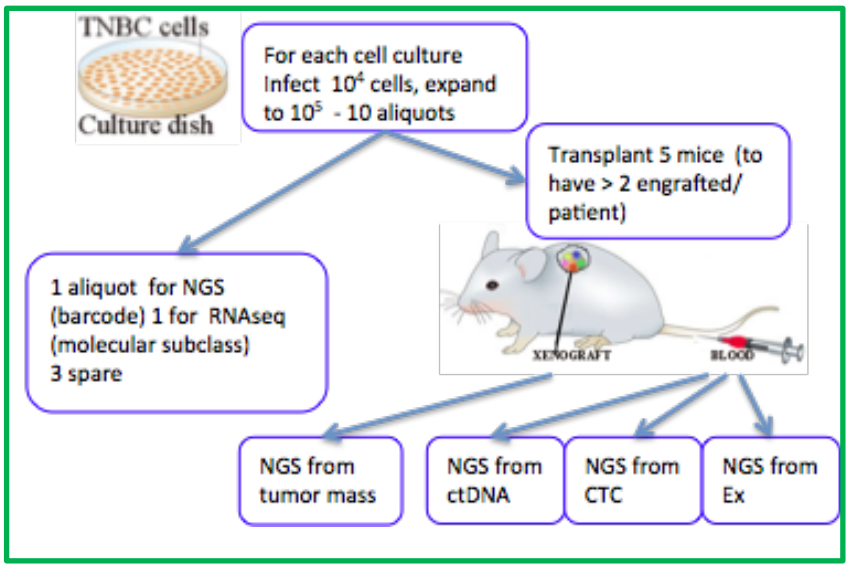

Fig. 4 Experimental workflow

4.2.2. Enrolling TNBC patients, setting up the culture conditions for TNBC cells

Triple negative BC or HR2-/HER2+ Tru-Cut biopsies or post-surgical specimens histologically diagnosed according to the WHO classification, have been provided by the SSD Breast Unit after patients' informed

consent. Tumor fragments collected immediately after surgical resection were mechanically dissociated to obtain single cells. Then cells were grown *in vitro* as mammospheres, using MammoCult™ Medium (StemCell Technologies) and low attachment plates, in order to preserve their stemness and take rate efficiency, with the cell differentiation that results minimized.

4.2.3. Genetic barcoding of TNBC cells with a retroviral library. Xenografting barcoded cells into NOD/SCID mice

With the aim of genetically label single cells of BC cultures with a unique barcode, a library of retroviral vectors expressing Luciferase-EGFP (LUC-GFP) fusion and containing a co-cistronic 22-mer degenerated barcode, with a multiplicity higher than 105, were produced in VSVG packaging cells. At least 10^4 cells from each BC culture were infected and expanded in 10 aliquots.

Among them, 5 aliquots have been resuspended in 50% Matrigel to increase the take rate and xenografted in the interscapular fat pad of 8-12-week-old female NOD/SCID mice under general anesthesia with the aim to obtain at least 3 mice successfully xenografted for each BC patient.

4.2.4. Characterization of xenotrasplantable TNBC samples

One of the remaining aliquot of each BC sample able to engraft in mice was analyzed by NGS, in order to determine the entire set of barcodes actually labelling the original culture.

4.2.5. Recovering of liquid biopsies and primary tumors

At the moment in which the xenotransplanted BC reaches a volume of 1 cm^3, mice were killed, and their blood collected.

Primary tumors were then be micro-dissected with the help of an epifluorescence stereomicroscope, allowing to identify tumor LUC-GFP-positive cells.

- Genomic DNA and ctDNA were extracted respectively from primary tumor and from the blood using commercially available kits.
- The enrichment of CTCs from blood has been performed following the procedure optimized in our laboratory, which takes the advantage of the use of the DEPArray™ System (more details in paragraph 5.2.1).
- Ex have been isolated from plasma obtained from blood by sequential centrifugation, using commercial isolation kits such as the ExoQuick Exosome Isolation Reagent (Qiagen), which enables efficient Ex isolation from as little as 50µl of plasma. Exosomal total RNA and DNA were then extracted using commercially available nucleic acid extraction kit as previously reported.
- Barcodes from the different CBM and from primary tumors have been retrieved by PCR (or RT-PCR for RNA derived from Ex) by using primers annealing on the barcode flanking regions. Successfully amplified material was then processed in NGS.

4.2.6. NGS analysis to identify the barcodes in the primary tumors and in CBM

Amplicons obtained from CBM obtained from few cultured patients' samples were sequenced by NGS and the list of their barcodes compared with that obtained from the primary tumor.

4.3 Ixazomib

4.3.1. Cell lines and culture conditions

In this study MDA-MB-231, MCF-7 an SKBR3, representing different BC subtypes, were used. All the cells were cultured in DMEM medium with 10% of heat-inactivated fetal bovine serum FBS, penicillin (100 I.U/mL) and streptomycin (100 ng/mL) and 1% of glutamine (Sigma, USA).

Cells were cultured at 37 °C in a 90% humidified incubator with 5% CO2. When the cells were 80% confluent, they were sub-cultured to a fresh media. Disaggregation was carried out using a 5 min incubation at 37°C with a 0.05% solution of trypsin in phosphate-buffered saline (PBS).

4.3.2. Chemosensitivity assay

To study the effect of Ixazomib and Bendamustine the different cell lines cells were seeded at different concentration, based on their grown rate. Following overnight adherence, the cells were incubated with the medium alone or with different concentration of Ixazomib (100 - 50 - 25 - 10µM) or with 10µM of Bendamustine, for 24 and 48 hours. Then, 50µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) solution was added to each well and mixed. After 4 hours, the supernatants were removed and 200µL DMSO was added to each well to dissolve the precipitate. The cells viability was estimated by measuring absorbance at 555nm using SPECTROstar nano.

The cell viability percentage was calculated based on the absorbance ratio between cell culture treated with Ixazomib/Bendamustine and the untreated control (percentage of control, %).

5. RESULTS

5.1 CITUCEL

5.1.1. Enrolment and clinical-pathological characteristics of patients

Between February 2017 and January 2020, 60 patients with invasive BC treated at the SDD Breast Unit and at Clinic of Internal Medicine of the Ospedale Policlinico San Martino were enrolled in CITUCEL trial. Each patient, after signing the informed consent, was subjected to a peripheral blood sample by venous access following specific timings as per protocol.

A total of 32 patients were enrolled in the neoadjuvant setting, 22 patients in the adjuvant setting and 6 patients in the metastatic setting.

Table 1 lists the characteristics of patients on neoadjuvant therapy. In this setting, almost all the patients presented ductal carcinoma, with positive estrogen and progesterone receptors and HER2 negative expression. Patients underwent to standard clinical therapy for their histology: the response to the treatment was 57.25%.

Few differences were observed in patients undergoing adjuvant therapy, most often suffering from hormone positive ductal carcinoma as reported in Table 2. It should be noted that the patients undergoing adjuvant therapy had diseases with a higher tumor grade.

The characteristics of metastatic patients are shown in Table 3.

Table 1. Characteristics of patients in neoadjuvant therapy

Neoadjuvant	
NUMBER	32 (100%)
MEDIAN AGE, YEARS (RANGE)	48,5 (29-72)
HISTOLOGICAL TYPE	
DUCTAL CARCINOMA	31 (96,9%)
NOT SPECIFIED	1 (3,1%)
BASELINE ER RECEPTOR STATUS	
POS	23 (71,9%)
NEG	9 (28,1%)
BASELINE PGR RECEPTOR STATUS	21 (65,6%)
POS	11 (34,4%)
NEG	
BASELINE GRADE (G)	
G2	4 (12,5%)
G3	4 (12,5%)
NA	24 (75%)
BASELINEKI 67 BASAL	
<25	10 (31,25%)
>25	22 (68,75%)
BASELINE HER2	
POS	12 (37,5%)
NEG	20 (62,5%)
CLINICAL T	
T1	2 (6,25%)
T2	16 (50%)
T3	8 (25%)
T4	6 (18,75%)
CLINICAL N	
N^+	11 (34,4%)
N0	2 (37,5%)
NA	9 (28,1%)
RESPONSE	
pCR	18 (56,25%)
PR	14 (43,75%)

Table 2. Characteristics of patients in adjuvant therapy

Adjuvant	
NUMBER	22 (100%)
MEDIAN AGE, YEARS (RANGE)	58 (44-79)
HISTOLOGICAL TYPE DUCTAL CARCINOMA	22 (100%)
ER RECEPTOR STATUS POS NEG	 18 (81,81%) 4 (18,18%)
PGR RECEPTOR STATUS POS NEG	 17 (77,27%) 5 (22,72%)
GRADE (G) G2 G3	 11 (50%) 11 (50%)
KI 67 <25 >25	 5 (22,7%) 17 (77,2%)
HER2 POS NEG NA	 5 (22,72%) 16 (72,72%) 1 (4,54%)
pT pT1 (a, b, c) pT2 pT3	 7 (31,83%) 14 (63,63%) 1 (4,54%)
pN pN0 pN+	 7 (31,81%) 15 (68,19%)

Table 3. Characteristics of metastatic patients

Metastatic	
NUMBER	6 (100%)
MEDIAN AGE, YEARS (RANGE)	55 (47-67)
HISTOLOGICAL TYPE DUCTAL CARCINOMA NA	 3 (50%) 3 (50%)
HR RECEPTOR STATUS POS NEG	 5 (83,3%) 1 (16,7%)
HER2 POS NEG	 0 6 (100%)

5.1.2. CTCs analysis in BC patients: phenotypic/morphological characterization and enumeration

CTCs enumeration was performed on the basis of morphological and phenotypic characteristics by two independent investigators.

This identification required a considerable effort in the analysis and evaluation on different samples, as the phenotypic and morphological expression of the CTCs isolated from patients was markedly heterogeneous when compared with the analysis of tumor cell lines (MDA-MB-231 and MCF-7) used in the development and validation of the analysis/recovery method carried out in the laboratory.

The heterogeneity concerned both the expression of the markers and the size of the cells, as it could be appreciated in Fig. 5.

In fact, it can be underlined that most of the patients presented negative cells with respect to the two markers used (EpCAM and CD44): this characteristic was found above all in the early setting where 60% of patients in neoadjuvant and 45% of patients in adjuvant presented EpCAM positive cell and only 30% and 13.5% CD44 positive cells, respectively. On the contrary in 5 out of 6 of the samples analysed from metastatic patients

it was possible to recover EpCAM positive cells; these latter samples generally showed much larger CTCs than the CTCs recovered from candidate patients to (neo)adjuvant therapy. Moreover, almost in all of the patients' samples it has been possible to detect smaller cells, morphologically very similar to WBC, expressing CD44 or negative for all the marker used. Similar cells have been already described in the literature as "non-conventional CTCs" [40b].

Their clinical significance is still to be proven and deserve a deeper evaluation.

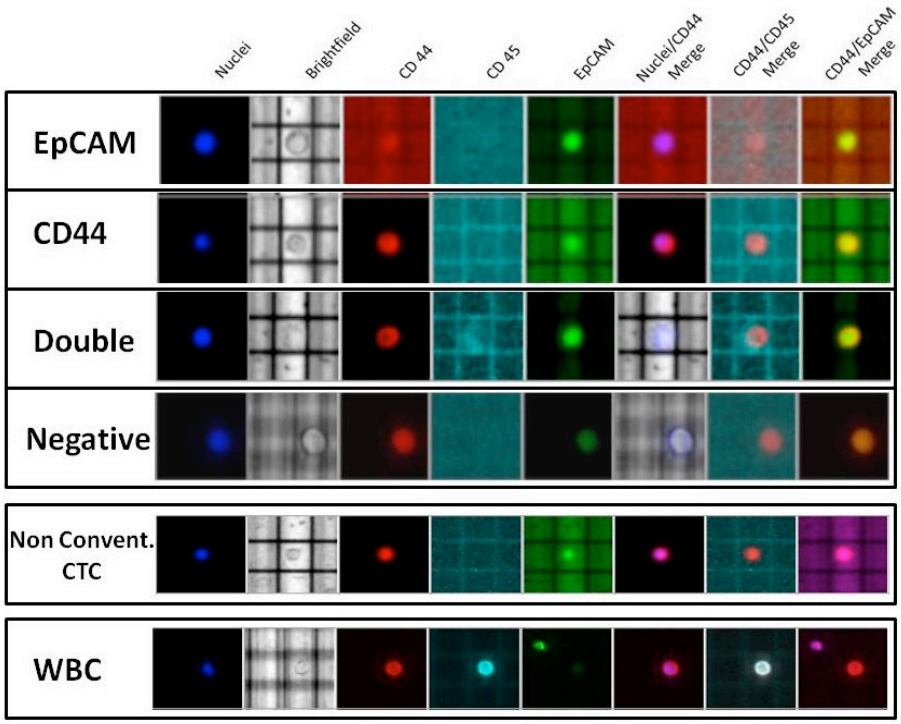

Fig. 5 Examples of CTCs isolated from patients' samples representing the different phenotypes detected in comparison with WBC frankly expressing pan leukocyte marker. A "Non-conventional CTC" is also listed, representing a group of cells lacking both epithelial and often mesenchymal marker and negative for CD45, definitely smaller than "classical" CTCs.

Considering the entire study cohort, the presence of CTCs was demonstrated in 59/60 (98%) patients, with a number of CTCs/patients above the standard cut-off, considered to be ≥1 in the (neo) adjuvant setting and ≥5 in the metastatic setting.

Table 4 shows the results of CTCs count in the different clinical groups of patients. It is possible to appreciate the difference (P=0.03) in the number

of CTCs detected at baseline between the patients treated in the early setting and the metastatic one, in which the number of CTCs is appreciably higher (Fig. 6). The differences between the number of CTCs of the neoadjuvant group versus the metastatic group is significant with P=0.006, differences that remain significant between the adjuvant versus metastatic group (P=0.04).

Table 4. Enumeration of CTCs in the various settings

	Median CTC (Range)		
	Neo	Ad	MTS
Tot	10.5 (6-16)	12.5 (7-21)	24 (19-30)
Neg	7 (4-14)	10 (5-19)	14 (16-21)
EpCAM pos	1 (0-3.5)	0.5 (0-2)	8.5 (4-13)
CD44 pos	0 (0-1)	0 (0-0)	0.5 (0-1)

Finally, no significant difference was found in the number of cells identified at the beginning of therapy between patients treated in the neoadjuvant and adjuvant setting (Fig. 7).

Considering the early settings, the assessment of the change in the number of cells isolated by liquid biopsy before and after chemotherapy treatment surprisingly did not show a significant difference, with a decrease in the number of CTCs for neoadjuvant and adjuvant patients of 50% and 47%, respectively. Considering the neoadjuvant setting, the count of CTCs before systemic treatment correlated with the pathological complete response (Fig. 8): in this case there was a significant correlation (P=0.015) between a lower number of CTCs at onset with complete response, suggesting and confirming the potential prognostic value of this parameter. It should be noted that the most marked difference in the number of CTCs was found in the positive EpCAMs cells which seem to better discriminate the different treatment settings (P=0.023 Neo vs MTS, P =0.035 Ad vs MTS).

No significant correlation was found with the clinical features (dimension, ki67) in all the settings.

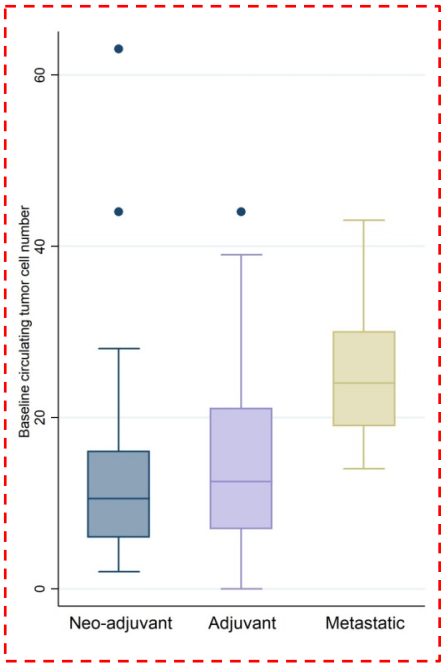

Fig. 6 Number of CTCs found in baseline withdrawals

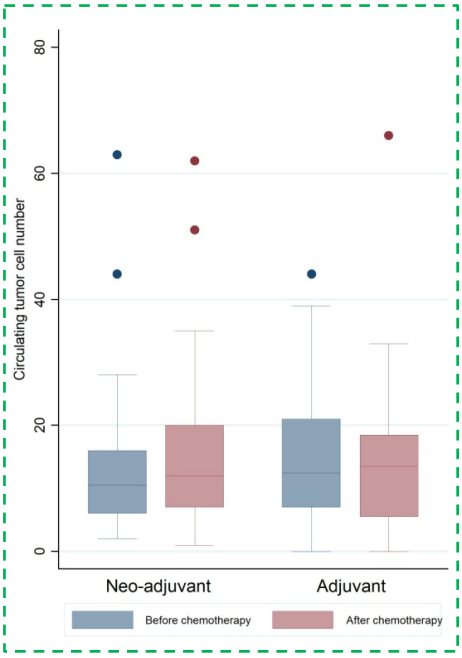

Fig. 7 Correlation between CTC quantities determined pre- and post-therapy

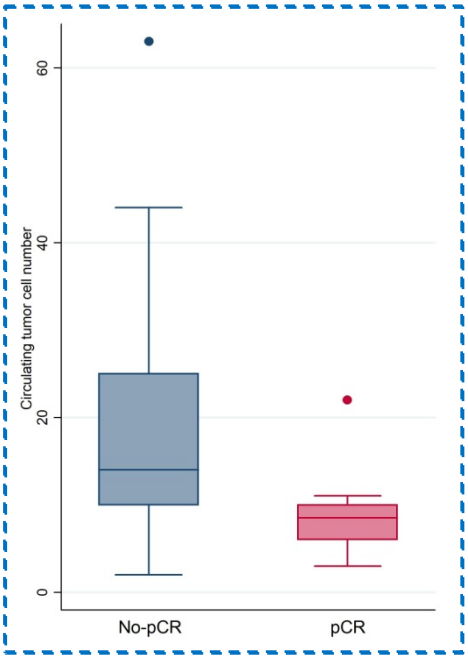

Fig. 8 Correlation between the number of CTCs and pCR

Regarding the cluster's formation, differently from the evidences reported in the literature, their presence was found in most of the patients, both before and after treatment in the early setting and baseline in the advanced setting. Detected clusters were constitute by [CTC + WBC], or by CTCs only, and were mainly composed by two or three cells; WBC + CTCs clusters were detected more often compared to CTCs clusters only (Fig. 9).

I

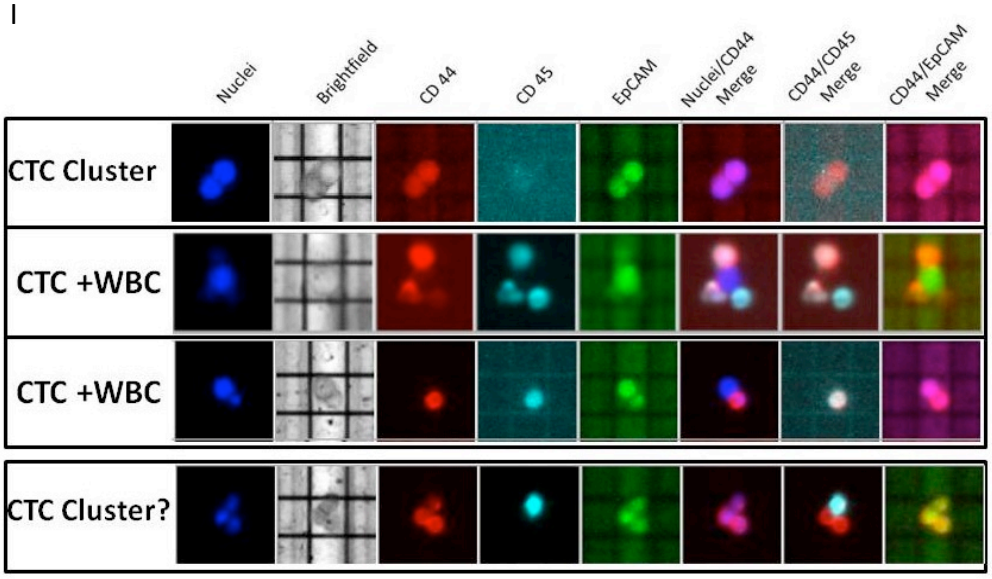

Fig. 9 Examples of cells clusters showing the heterogeneous compositions (CTCs only or CTCs with different number of WBC). Aggregates of small cells, often including WBC, have been also found. At the moment is not possible to confirm their origin.

In many cases CTCs involved were negative for the marker chosen (EpCAM/CD44) in clusters with WBC, while more frequently EpCAM positive were found in cluster formed by CTCs only.

The characterization of these clusters and their correlation with therapy is still ongoing, but it is possible to put in evidence a decrease of clusters number after treatment in both neo-adjuvant and adjuvant patients.

However, the characterization of those clusters deserves a deeper analysis, and the presence of CTCs should be confirmed by downstream mutational data: infact, it is not possible to exclude that their presence could be an artifact due to the fixation process used in the enrichment procedure.

5.1.3. CtDNA analysis: quantification

For all enrolled patients it was also possible to extract cfDNA at the same time as the CTCs, using a specific commercial kit.

Table 5 describes the amount of cfDNA (expressed in pg/µL) determined in the samples tested from 44 patients.

As from CTCs enumeration, it is possible to appreciate the correlation between the concentration of ctDNA with the burden of disease (P=0.013 between the three settings), being significantly higher in the metastatic patient' samples, compared to neoadjuvant (P=0.007) and adjuvants patients (P=0.03) (Fig. 10).

Table 5. Amount of cfDNA in the analyzed samples of 44 patients

	Median ctDNA (Range µg/µL)		
	Neo	Ad	MTS
(Range pg/µL)	230.5 (153-299)	265 (214-511)	1780 (654-1960)

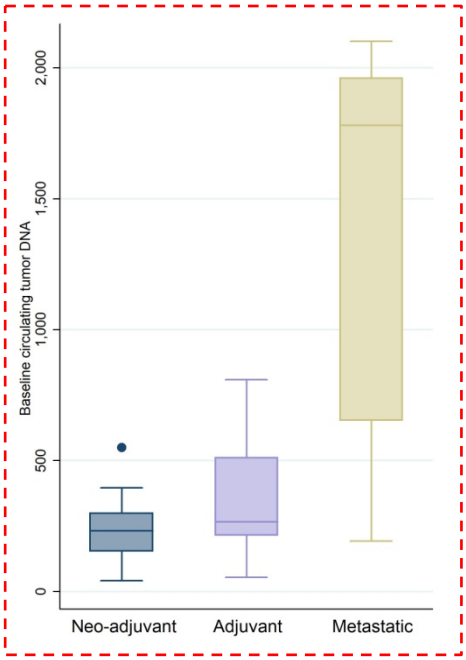

Fig. 10 Amount of ctDNA in baseline samples

No significant correlation was found between the presence of ctDNA and chemotherapy, in regards of detected amount pre- and post-therapy in both settings and to the response to neoadjuvant treatment (Fig. 11).

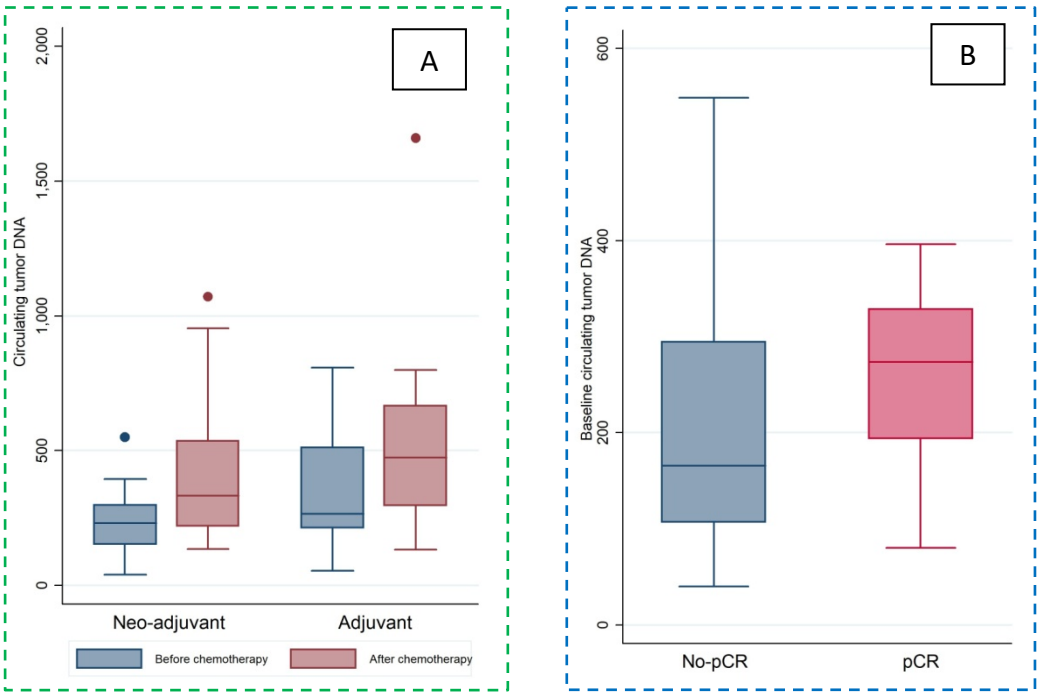

Fig. 11 A. Correlation between the amount of ctDNA before and after therapy; B. Correlation between amount of ctDNA and pCR

5.1.4. New markers cocktail

As previously described, our results showed that most of the detected cells were negative for the markers used and this does not give us the opportunity to characterize them uniquely. Therefore, we have started to develop a more comprehensive labelling cocktails based on multiple markers, both epithelial and mesenchymal, which are not present on white blood cells (WBC) and could help in CTCs characterization. Unfortunately, due to the COVID-19 emergency, it was not possible conclude the development of this methodology, therefore only preliminary results have been obtained on cell lines.

A deep literature review was done to make the best possible choice in markers selection, on the base of their properties and involvement in tumour development: consequently CK, E-Cadherin, MUC1, N-Cadherin, Slug, CD146 and Vimentin, were chosen in addition to EpCAM and CD44, already in use. Those markers should be useful to discriminate three types of CTCs: epithelial, mesenchymal, and epithelial/mesenchymal hybrids.

As regard epithelial markers, CK are particularly useful tools in oncology diagnostics because they provide valuable and already in use markers for epithelial malignancies. Moreover, it is known that E-Cadherin plays a crucial role in maintaining the polarity of epithelial cells[41]. It is reported that cadherin switching (a decrease in E-Cadherin with an increase in N-Cadherin) is a feature of EMT in numerous types of malignant tumor[42]. Furthermore, MUC1 was also added to the cocktails in consideration of the fact that in TNBC was found a potential mechanism by which MUC1 contributes to the metabolic process involved in BC. In particular, MUC1 was selected because it is able to translocate to the nucleus in association with β-catenin, represses E-Cadherin expression, and upregulates expression of the EMT inducers Snail, Slug, Vimentin, and Twist[43].

Regarding mesenchymal markers, N-Cadherin is associated with a heightened invasive potential in cancer. A recent study has demonstrated that overexpression of N-Cadherin in BC correlates with invasiveness as a result of N-Cadherin-mediated interactions between cancer and stromal

cells. The phenotype of BC cell lines was found to undergo dedifferentiation from epithelial to mesenchymal as a result of N-cadherin transfection without a loss of E-Cadherin expression.

Finally, N-Cadherin, as a marker of ongoing EMT, is not expressed in normal epithelial cells, but its expression has been demonstrated in several types of carcinomas. Vimentin is another candidate because it is known to express aberrantly in epithelial cancers. Vimentin+ cells are associated with high grade tumors and increased tumor proliferation. Moreover, Vimentin expression is a rather rare finding in invasive BC and is associated with high tumour invasiveness and chemoresistance.

Furthermore, CD146 is important for endothelial cell migration and angiogenesis[44]. CD146 is expressed at abnormally high levels and is associated with a reduction of E-Cadherin in TNBC. CD146 silencing in TNBC MDA-MB-231 partly reverses its mesenchymal phenotype, implying that CD146-induced EMTs partially explain the mesenchymal and malignant characteristics of TNBC. In addition, increasing evidences have supported the critical role played by tumor angiogenesis in BC progression[45].

Another potentially important marker is SLUG because it is able to regulate the expression of genes responsible for the EMT. Expression of SLUG in cells suppresses E-Cadherin expression, which subsequently reduces intercellular adhesion and increases cell motility properties[46].

Finally, in order to better detect WBC, which do not express CD45 in the same way, two different markers (CD14 and CD16), neutrophils and macrophages specific, were chosen. The presence of elevated levels of CD14+/CD16+ monocytes with tissue macrophage features and the association of this subpopulation with disease is shown in various pathological conditions, including cancers.

The functional significance of CD14+/ CD16+ monocytes is still unclear, but in certain disease states CD14+/CD16+ monocytes are able to promote tumour growth and express the vascular endothelial growth factor receptor (VEGFR)[47].

For this purpose, experiments on WBC, collected from a healthy donor

whole blood, were performed to verify that these markers did not give aspecific results and to confirm the positivity of CD14+/CD16+, the WBC extended marker set. Consequently, the same experiments have been done on different BC cell lines representative of BC cancer subtypes: MDA-MB-231, MCF-7 and SKBR3. The markers expression has been analysed using FACS and confirmed no expression of the WBC expanded marker set (CD14, CD16 plus CD45) on BC cell lines.

In regards of CTCs expanded optimised marker preliminary results (reported in Table 6) showed that SKBR3 cells, a cell line with mesenchymal-epithelial characteristic, are positive for EpCAM, CD44, CK, MUC1, CD146 and Slug, while are negative for N–Cadherin, E–Cadherin, Vimentin, just as was expected. Furthermore MCF-7, epithelial cells, turn out to be positive to EpCAM, CK, MUC1, CD146, Slug and often CD44, while are negative for E–Cadherin, N-Cadherin, Vimentin. Finally, MDA-MB-231 cells, with mesenchymal characteristics, are positive for EpCAM, CK, CD44, CD146 and Slug, while are negative for N-Cadherin, E–Cadherin, MUC1, Vimentin, CD45, CD14 and CD16.

Table 6. Positivity of the different cell lines to new cocktail markers

	APC			EPITELIAL MARKERS					MESENCHIMAL MARKERS			
	CD45	CD14	CD16	EpCAM	CD44	CK	MUC1	E-Cadherin	CD146	Slug	N-Cadherin	Vimentin
WBC	+	+	+	-	+	-	-	-	-	-	-	+
MCF7	-	-	-	+	+	+	+	-	+	+	-	-
SKBR3	-	-	-	+	+	+	+	-	+	+	-	-
MDA	-	-	-	+	+	+	-	-	+	+	-	-

On these bases, this new improved labelling cocktail will be tested in further experiments in the lab on patient samples in parallel with the current method, to check if, the latter actually allows to obtain a more accurate detection and characterization.

5.1.5. Mutational analysis

CTCs translational research is also related to the necessity of improving molecular methods for a more comprehensive and accurate detection of occurring molecular alterations. This could provide a useful tool for early detection of the disease at diagnosis as well as in monitoring patients' response to therapy and progression and help in subsequent therapeutic choices.

To date, the major efforts for molecular characterization of CTCs have focalized in optimizing genetic analysis protocols consisting in a first step of whole genome amplification (WGA), which allows to obtain a sufficient amount of DNA from few starting copies. This procedure is usually downstream combined with sequencing methods as Sanger or NGS and/or other molecular assays, such as comparative genomic hybridization approaches. Therefore, within the CITUCEL trial our researchers group developed and optimized a workflow which completely abolish WGA step for CTCs mutational analysis, taking advantage of a molecular tagging technology applied to NGS. The experiments about NGS analysis have been done at the Molecular Diagnostic Unit of the Institute (Dr. Zupo/Dr Dono/Dr. De Luca). First the protocol was tested on MDA-MB-231 and MCF-7 cell lines isolated by DEPArray^{TM}, used as surrogate BC CTCs. In the preliminary NGS assessment, MDA-MB-231 lysates of one, three, and five cells were added to normal DNA. Then, these DNA mixtures were employed to prepare libraries: two hotspots present in the MDA-MB-231 cell line—the KRAS p.Gly13Asp and TP53 p.Arg280Lys— and one PIK3CA Glu545Lys in MCF-7 - were followed and detected.

Consequently, cells were used as a unique starting genetic material, in the complete absence of normal genomic DNA or WGA, for subsequent library preparation. As this strategy can dramatically decrease the input amount of DNA, a specific adjustment of the NGS protocol was developed.

The entire new procedure presented main technical changes consisting of the following four items: DNA derived only from sorted cells was used as a

starting material for the preparation of libraries; a three-times volume reduction of the reaction reagents was applied for the two PCRs. By applying the optimized NGS protocol, a total of 47 libraries were prepared from nine different cellular pool types. In particular, three pools composed of two, four, and five tumor cells only and four combined pools (4:1, 3:2, 2:3, and 1:4 MDA-MB-231/leukocytes ratios, respectively) were investigated for MDA-MB-231 specific mutations, together with two pools of leukocytes only as negative controls. For MCF-7 analysis, duplicates were considered for all pools, except for pools consisting of two cells only and the 1:4 MCF-7/leukocytes combination, respectively, which were tested in triplicate.

In details, 2µL of Lysis Reaction Mix (Ampli1 WGA kit, Silicon Biosystems, Bologna, Italy) were used. MDA-MB-231 cell lysates were processed together with 5ng of normal genomic DNA from healthy donor blood and MDA/normal DNA mixtures were used for library construction with the OBcfRAv2 (ThermoFisher Scientific, Carlsbad, CA, USA).

Manually prepared libraries were quantified by qPCR assay with an Ion Library TaqManTM Quantitation kit (ThermoFisher Scientific, Carlsbad, CA, USA), diluted to 50–60 pMol and then multiplexed up to 5 samples for automated template preparation on an Ion ChefTM System and loaded on an Ion 530TM Chip (ThermoFisher Scientific, Carlsbad, CA, USA). Sequencing runs were performed on an Ion Torrent GeneStudioTM S5 (ThermoFisher Scientific, Carlsbad, CA, USA), according to the manufacturer's user manual.

In order to obtain an optimized molecular tagging NGS workflow, libraries were prepared using directly the 3µL cell lysates from isolated cells as a source of DNA.

The entire protocol for library preparation with OBcfRAv2 was modified to adjust the amplification of small quantities of DNA input into a small reaction volume. All reactions used for library construction were set-up with a 1:3 dilution reduced volume. Thermal conditions and number of amplification cycles were maintained as the canonical original protocol

suggested. Manually prepared libraries were quantified, diluted, and used for template preparation. The only relevant modification consisted of multiplexing up to 24 amplified libraries on an Ion 520TM Chip (low-coverage sequencing). Run sequencing was performed on an Ion Torrent GeneStudioTM S5, as described above.

A successful sequencing was realized in 46/47 libraries (97.9%), with a 12,272–294,265 range of total reads (median value 83,723). KRAS p.Gly13Asp and TP53 p.Arg280Lys variants were successfully found in the MDA-MB-231 triplicates, as well as PIK3CA Glu545Lys in MCF-7 (except for one combination), regardless of the number of tumor cells present in a pool.

The robustness of this adapted NGS protocol was also demonstrated by the fact that no other mutations than those expected were found and by the absence of any mutations in negative controls. Consequently, this workflow offers the possibility to investigate genetic hotspots from independent pools of few cells simultaneously.

On these bases, this protocol was also validated with CTCs recovered from patients' samples collected within the CITUCEL trial. Thirteen pools of different numbers of CTCs (range: 2-6 cells) were recovered and used for subsequent NGS experiments. Libraries were successfully prepared and sequenced from all the 13 CTCs pools, thus showing that even if this analysis was limited in number, the optimized molecular tagging NGS workflow was robust when applied to "real" CTCs. Quantification of libraries revealed a yield range of 11-107pMol, with an optimal performance (in terms of total produced reads) ranging from 20,633 to 294,305 (results reported in Table 7).

5.1.6. Comparative tissue/CTCs/ctDNA mutational analysis

Then, mutational analysis on circulating biomarkers and related solid tumors was performed on 8 patients (6 patients in neoadjuvant therapy, 1 patient in adjuvant and 1 patient in first line therapy). Table 7 shows the results relating to the mutational analysis

The CTCs analysis was carried out on 13 cell pools (from 3 to 6 cells); where possible, in fact, 2 or 3 pools were analyzed for the same patient: in particular, the analysis was conducted on a sample for patients 5, 7, 9 and 12; two samples for patients 18 and 51 and three samples for patient 10.

Table 7. Mutational comparison between CTCs, ctDNA and corresponding breast tumor tissue

Pt	CTCs		ctDNA	Tissue concordance		BC tissue mutations	
	N°. CTC	Mutations (OB-cfRAv2)	Mutations (OB-cfRAv2)	CTCs	ctDNA	OBcfRAv2	custom panel*
5	3	*TP53* c.1100+30A>T	*TP53* c.1100+30A>T *PIK3CA* p.E542K	*Yes*	*Partial*	*TP53* c.1100+30A>T	*ERBB2 p.Ile654Val* *ERBB2 p.Ile655Val* *ERBB2 p.Pro1170Ala*
7	3	*TP53* p.Arg213=	*TP53* p. =	*Yes*	*Yes*	*TP53* p.Arg213=	*PIK3CAp.Asn345Lys* *ERBB2* p.Ile655Val
9	3	Not found	Not found	*Yes*	*Yes*	Not found	*PIK3R1 p.Met326Ile* *ERBB2 p.Pro1170Ala*
10	2 2 4	Not found	*PIK3CA* p.E542K	*No*	*No*	*TP53* p.Arg248Gln	
12	5	*TP53* c.1100+30A>T	*TP53* c.1100+30A>T	*Yes*	*Yes*	*TP53* c.1100+30A>T	*ERBB2* p.Pro1170Ala
18	3 3	Not found	Not found	*No*	*No*	*TP53* p.Cys275Leufs	*ERBB2* p.Pro1170Ala
24	2 5	*TP53* c.1100+30A>T *TP53* c.1100+30A>T	*TP53* p.Arg248Trp *TP53* p.Gly302Trp *TP53* p. =	*partial*	*No*	*TP53* p.Arg248Trp *TP53* c.1100+30A>T	*ERBB2* p.Pro1170Ala
51	3 6	*PIK3CAp.Hys1047Arg* *PIK3CAp.Hys1047Arg*	*PIK3CAp.Hys1047Arg*	*yes*	*Yes*	*PIK3CA* p.Hys1047Arg	

Fig. 12 represents Circulating Tumor Cells (CTCs) recovered from BC patients on DEPArray™ and directly analysed with the optimised method.

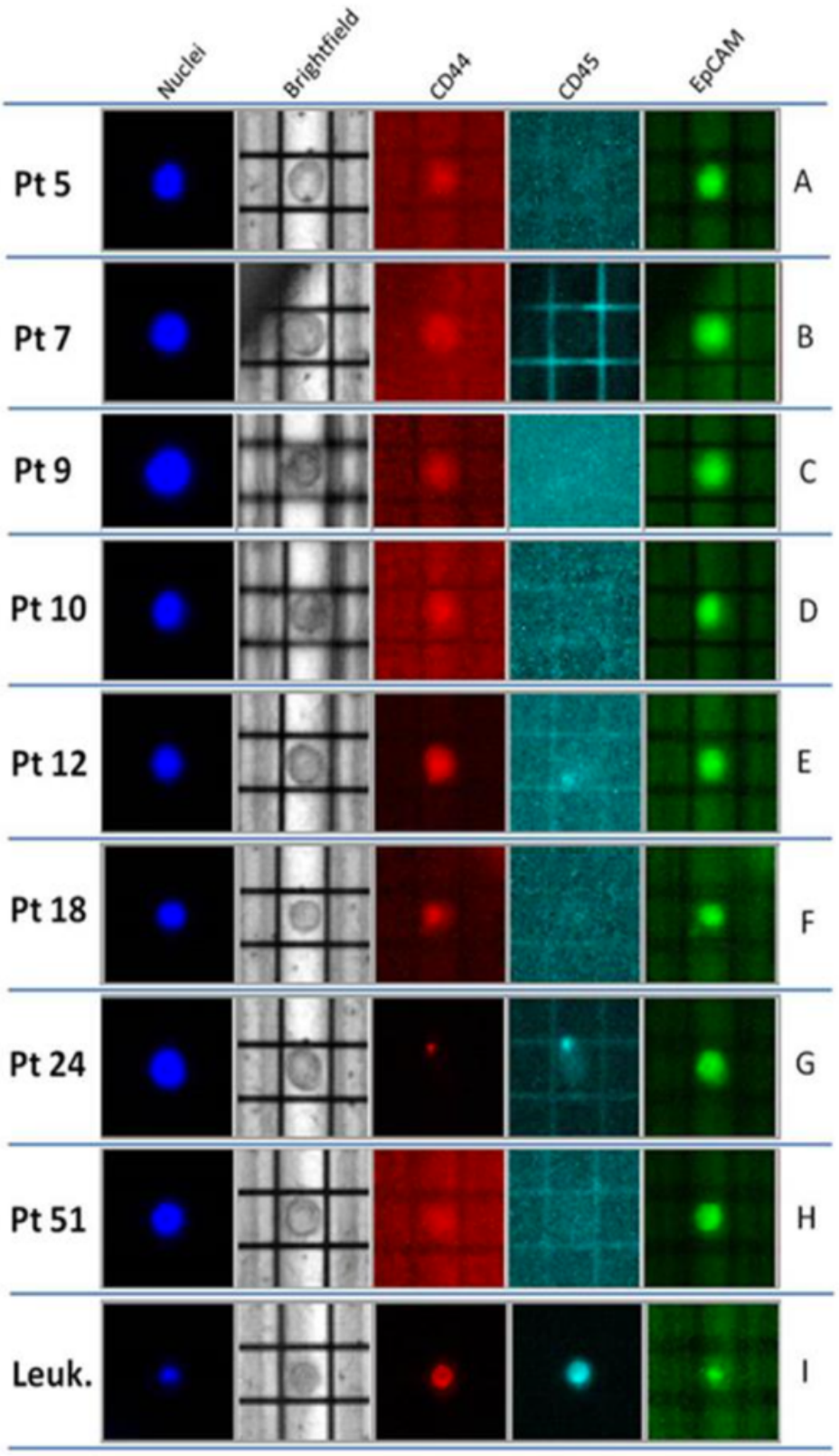

Fig. 12 Representative CTCs, obtained from BC patients by DEPArray™ method and actually analyzed in NGS

The results show that it was possible to detect mutations for 6 of the 8 patients analyzed. In particular, an intronic variant in the TP53 gene (c.1100 + 30A> T, chr17: 7573897) was determined in the CTCs of patients 5, 12 and 24, while patient 7 had a synonymous variant in the TP53 gene (p.Arg213 =). It should be noted that all CTCs samples from patient 51 were characterized by the PIK3CA mutation (p. Hys1047Arg).

The comparison between the mutations highlighted in the CTCs and the corresponding primary tumor (analyzed with the custom panel described in materials and methods) shows agreement in the mutations found in 6 out of 8 patients. In details, 6 out of 8 cases (62.5%; patients 5, 7, 9, 12, and 51) were completely concordant, while patient 24 showed partial agreement, since the tissue analysis showed an additional mutation by TP53 (p.Arg248Trp). For 2 patients (patients 10 and 18) a complete discrepancy was instead determined: no mutation was found in the CTCs while the tissue analysis revealed a variant of TP53 pArg248Gln and p.Cys275Leufs. This discrepancy may reflect the intrinsic nature of the CTCs, which can both have the same mutational profile of the primary tumor but also diverge completely. The comparison with the mutations found in the ctDNA shows agreement in 5 out of 8 cases. It is interesting to report that the mutations found in PIK3CA (p.E542K) identified in the ctDNA of patients 5 and 10 were negative in the corresponding CTCs. This last finding is also inconsistent with the mutational profile of the tissue.

Finally, it is possible to point the presence of other mutations in tissues that can only be revealed by the larger custom panel compared to the Oncomine Breast V2 panel.

The mutational analyses of the samples obtained from the other patients are still in progress.

5.2 BaReLiB

This study is still ongoing, and the final results are expected for the end of 2022. In this thesis are presented the evidences obtained so far.

5.2.1 CTCs recovery from mice blood

In this project, the PhD research activity was focused on the recovery of CTCs from mouse blood, that was developed with an approach similar to the procedure applied in the CITUCEL trial to enrich CTCs from patients' samples. CTCs recovery was optimized performing spike–in experiment using GFP-modified MDA-MB-231 cells: the methodology is based on immunonegative selection of human CTCs using the same e approach already described, which takes the advantage of the use of the DEPArray™ System. Briefly, this method has been adapted to recover human CTCs from blood mouse samples, using specific antibodies cocktails (Mouse Cell depletion kit, Miltenyi Biotech) after mice RBC lysis. Immunonegative selection was carried out on LS columns and enriched cells (GFP positive) were further labelled with HOERSCH, anti-human CD44 and anti-mouse CD45, in order to better discriminate human cell from mouse ones.

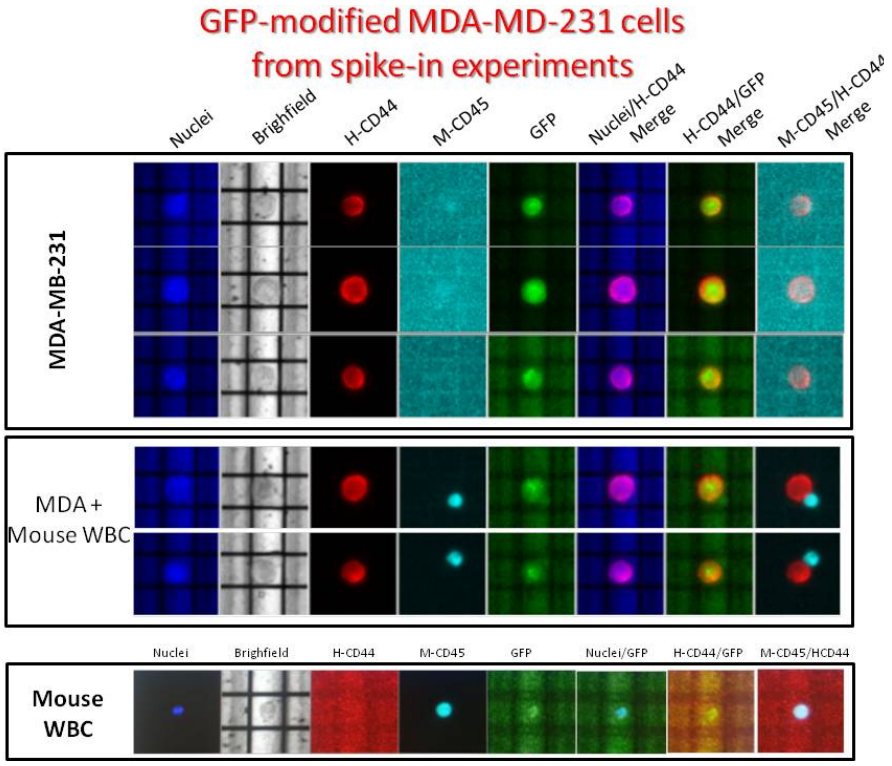

Fig. 13 Spike-in experiments on MDA-MB-231 cell line

Finally, cells were fixed using Inside Stain Kit (Miltenyi Biotec).

In vitro experiments demonstrated that with this system it is possible to recover human cancer cells from mouse peripheral blood. Cells were analysed and selected with DEPArray™ (Fig. 13) and DNA content has been successfully amplified by pCR from single or pooled cells, suggesting that this method is reliable.

5.2.2. CTCs recovery from xenotransplanted mice blood

In vivo experiments have been performed injecting the GFP-modified MDA-MB-231 cells into NOD-SCID mice. Compared to the analysis of spiked-in cells in mouse blood, the identification of CTCs from xenografted mouse blood has been not so straightforward.

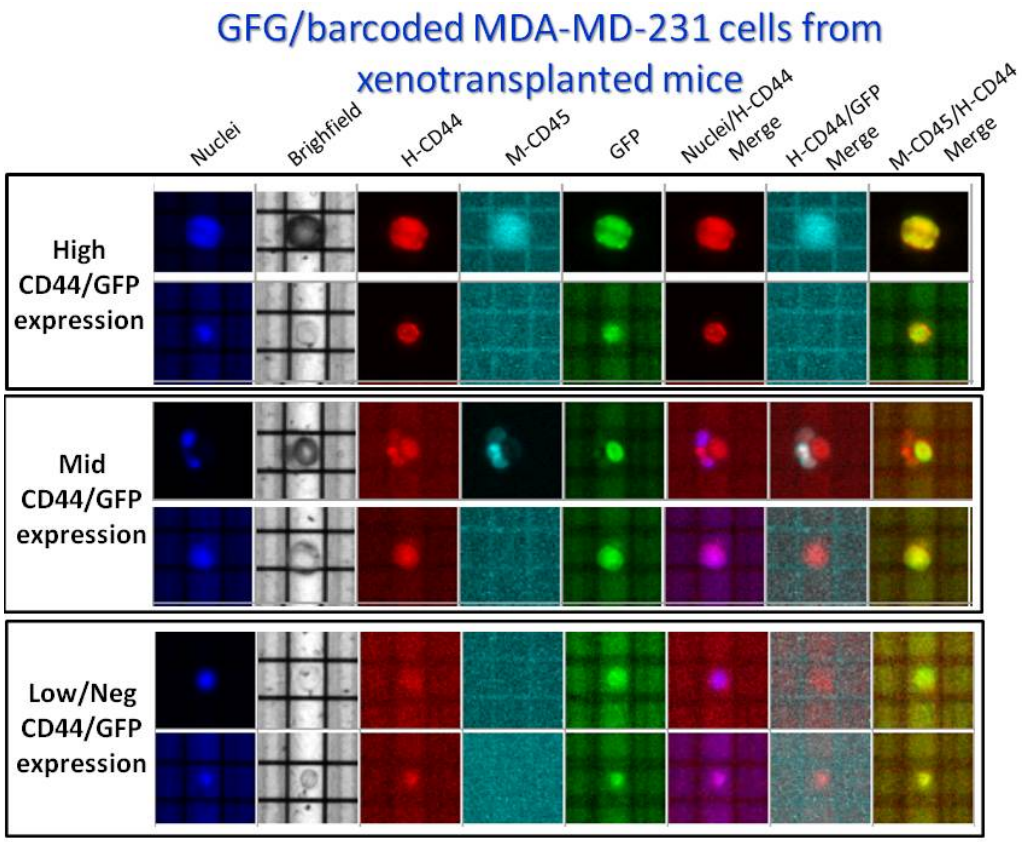

Fig. 14 DEPArray™ Analysis from xenotransplanted mice blood. Cells were labelled with anti-human CD44 and anti-mouse CD45. CTC were identified and selected as $CD45^-$ and $CD44^+$ and/or GFP^+

Infact, CTC showed a different morphology and structure, as it is possible to appreciate in Fig. 12: they did not maintain round shape, assuming often ellipsoid contours, and were found in the majority of cases associated in cluster with mouse WBC. Moreover, GFP expression was very low compared to original modified MDA-MB-231 cells (Fig. 14). Therefore, the collection and the consequent isolation was much more difficult and not univocal. The DEPArray™ selection was done in order to obtain as much as possible CTCs candidates to be downstream NGS analysed, therefore collecting pools including cells or cells aggregates not univocally characterised as human derived CTCs.

5.2.3. Barcode recovery from cell culture

After the development of tumor masses, tissue and liquid biopsies were collected and analysed using NGS, by Malatesta's laboratory. Exploiting in-house developed software, clonal composition of independent tumors has been successfully reconstructed showing a good maintenance of clonal heterogeneity in the tumor masses (Fig. 15) but this was almost lost in bloodstream, where only few barcodes were detected at ctDNA level (Fig. 16) and CTCs (10-20 tumor cells detected in 100µl of mouse blood).

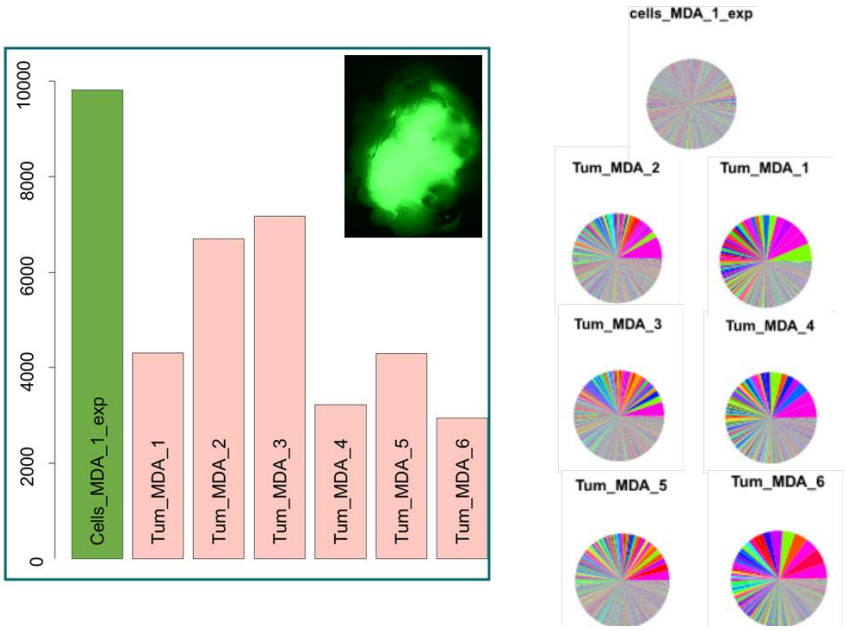

Fig. 15 Clonal composition of initial cell culture and derived *in vivo* expanded tumors

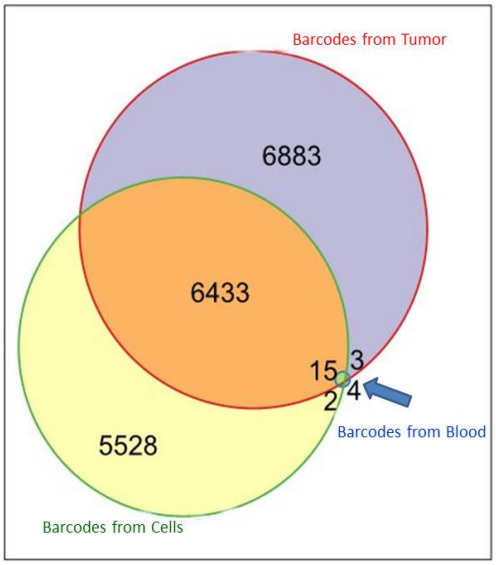

Fig. 16 Comparison of the retrieved barcodes between initial cell culture, derived tumor and ctDNA

Those evidences may suggest that the ability to migrate in the bloodstream is owned only by few clones. However, additional sequencing analysis showed that even if the number of clones is limited, they are not consistent between the replicated, indicating that circulating clones are not present as multiples copies but are few “single” molecules

The results obtained with modified MDA-MB-231 cell on xenografted mouse blood showed that cell lines could not be a good model to mimic human BC formation and CBM dissemination, since the tumor formed was well represented but the barcodes found in the circulation were very few.

However, those preliminary experiments with cell lines allowed to set-up the different experimental conditions and verify the study workflow.

5.2.4. Barcode recovery from patients

After the first part conducted using cell lines, the So far, 21 mouse xerographs have been obtained from 3 patients (for one patient was possible to use primary and tissues obtained at progression to prepare primary culture). In 9 of these 21 xerographs, enriched CTCs were analysed in with DEPArray™ system, while the other CTCs were only enriched, in order to verify whether or not the cells were lost during the entire

enrichment/recovery procedure. In these experiments the identification of CTCs was even furthermore complicated compared to cell lines obtained from xenografted experiments, as they appeared more aggregated to WBC clusters. Data showed an average of 21 cells retrieved from the primary culture of first patient, 10 from the sample of the same patient but in which their cells were obtained from tissue collected at relapse of the disease and 11 from the second patient.

Preliminary comparative results obtained from NGS barcodes analysis showed that ctDNA well represents the primary tumor. Infact, in the comparison with barcode retrieved from tumor masses and CBM showed that barcodes found in ctDNA are well representative, while very few were found in CTC pools. Finally, in regards of Ex analysis, the results obtained so far have shown less reliability and more experiment will be necessary.

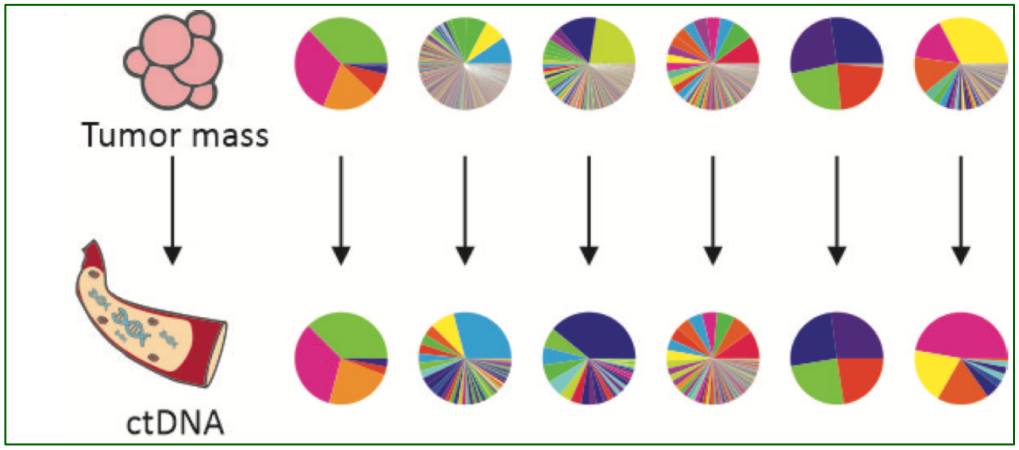

Fig. 17 Comparison between barcodes from tumor masses and from ctDNA

The work is still in progress and more data are needed to confirm the obtained results and to determine which of the CBM is the best in representing tumor heterogeneity.

5.3. Ixazomib

Starting from a case of a patient with MM and BC treated at the Breast Unit of Ospedale Policlinico San Martino, we have planned an *in vitro* study to assess the possible therapeutic effect of Ixazomib and Bendamustine in BC.

The results of this part of the research activity were deeply delayed due to the COVID-19 emergency. Therefore, only very preliminary data are presented.

To assess the antitumor effect of Ixazomib on BC cells, experiments were performed *in vitro* using different cell lines, representative of different BC subtypes: MDA-MB-231 (TNBC cell line), MCF-7 (HR+, PR+ and HER2-) and SKBR3 (HR-, PR- and HER2+).

First, it was necessary to set-up of the optimal concentration, for each BC cell lines, in order to obtain a confluence of about 60-70%.

Toxicity experiments were performed using 10 mM stock Ixazomib solution in DMSO (single aliquot 0.5mL to be used for experiment stored at -80°C); Bendamustine was obtained already diluted at clinical dose (10mM) from HMS Antiblastic Unit.

The effects of Izaxomib and Bendamustine alone were roughly tested on the different cell lines.

Then preliminary experiments were performed using with 3000 cells/well 96-well plate of MDA-MB-231, 4000 cells/well MCF-7 and 5000 cells/well of SKBR3. Cells were treated with 100µM Ixazomib and 10µM Bendamustine final concentration for 24 hours and 48 hours after overnight seeding. Control experiments were done incubating cells with medium alone. Initial tests showed that Ixazomib has high activity on all cell lines (MDA-MB-231>MCF-7>SKBR3), probably due to the high initial concentration used. On the contrary Bendamustine showed basically no effect on all the cell lines. Therefore, only a few experiments have been done with Bendamustine, as even at very high concentrations of this drug

we did not obtain valid results; as a consequence, the experimental activity was first focused on finding the optimal concentration of Ixazomib alone.

The best results were obtained seeding into a 96-well plate 10000 cells/well MCF-7, 9000 cells/well MDA-MB-231 and 10000 cells/well of SKBR3 for the treatment of 24 hours and 7500 cells/well MCF-7, 6500 cells/well MDA-MB-231 and 8000 cells/well of SKR3 for the treatment of 48 hours.

Overall, the only few experiments carried out showed that the effect of this drug on our BC cell lines is present both on MCF-7 and on SKBR3, while it is even much greater on MDA-MB-231. This behaviour is detectable and coherent in both 24- and 48-hour treatments.

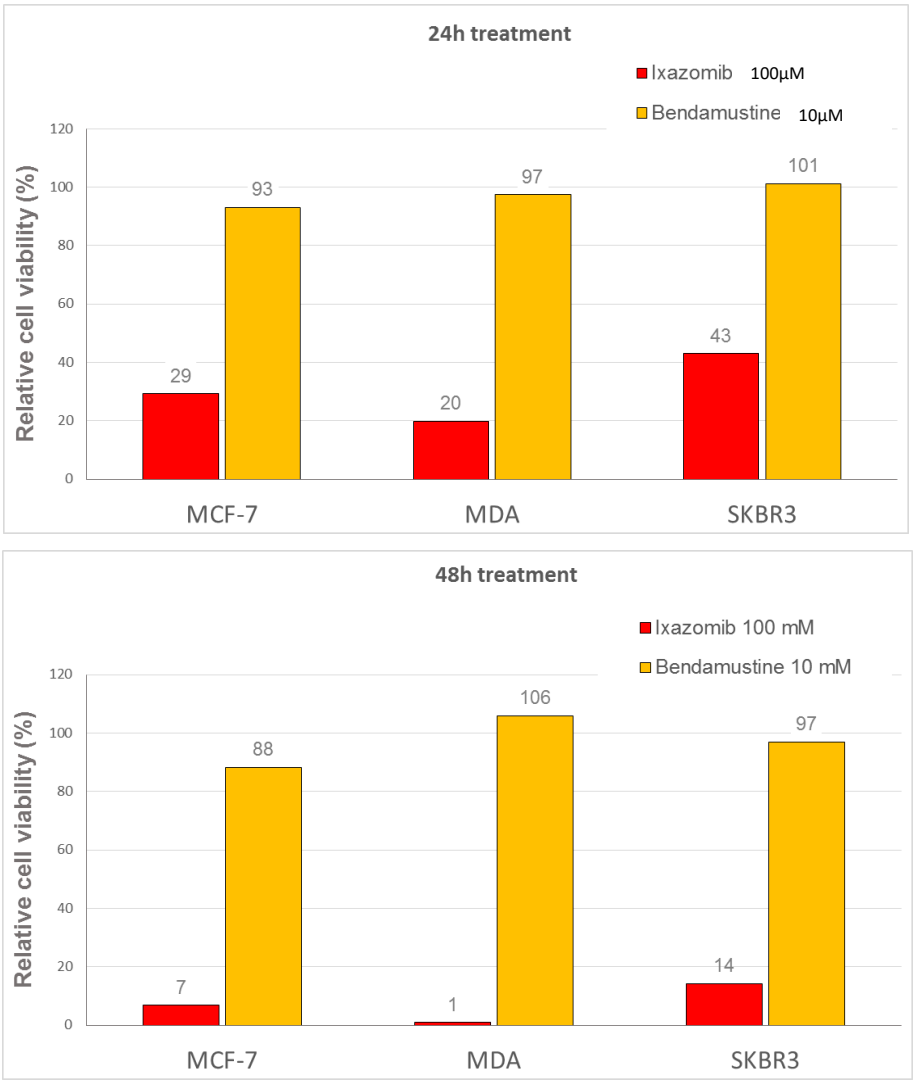

Fig. 18 Treatment with 100µL of Ixazomib and with 10µL of Bendamustine for 24 and 48

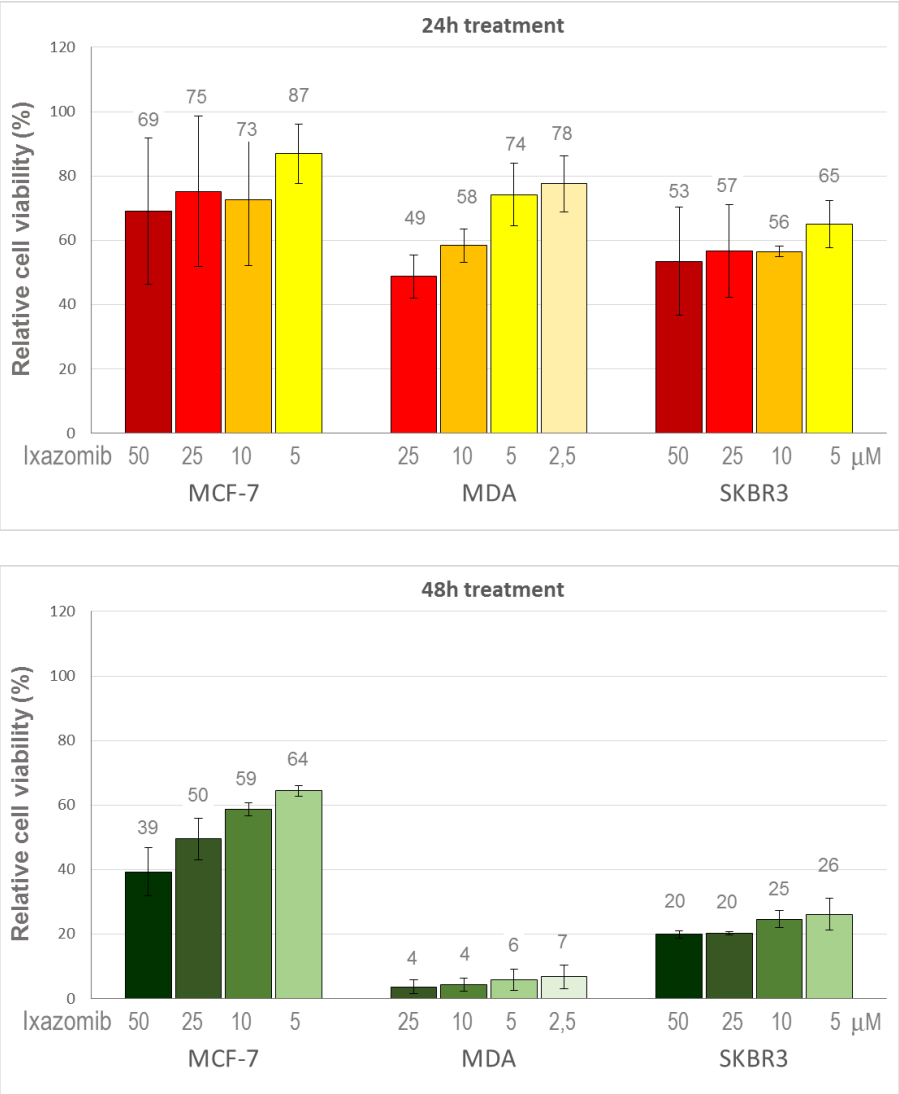

Fig. 19 Treatment with Ixazomib at different concentrations for 24-48 hours

However, these results are different from other study in this contest, in which the experiments, executed in the same BC cell line, shown that Ixazomib was more efficient on MCF-7 at lower concentration[48].

More experiments are therefore needed to confirm the results.

Once the more efficient and powerful concentration will be determined for Ixazomib, combination experiments will be performed for 24 or 48 hours on cell lines, with the aim to evaluate which of these is the most sensitive cell line to the treatment and to evaluate if an additive or synergic effect of these drugs could be achieved.

6. DISCUSSION

In this study the evaluation of CTCs and ctDNA in different disease settings were performed using cutting-edge technologies and innovative approaches. In particular, the analysis of the CTCs used an independent EpCAM enrichment process and the use of DEPArray™ technology that allows resolution down to the single cell level.

Furthermore, the analyses of CTCs and ctDNA presents a double aspect: the quantitative one, which can provide useful prognostic information, and the qualitative one, aimed at molecular characterization, which allows the determination of predictive response mutations or monitoring of the cloning evolution of the neoplasm.

The results, although still partial, seem encouraging and fit well into the current translational research based on liquid biopsy and are in line with the most recent literature. In fact, it has been shown that the ctDNA detected in plasma samples after neoadjuvant chemotherapy and surgery is correlated with a more rapid relapse of the disease in triple negative patients.

On the other hand, CTCs have been frequently identified in both patients with early and advanced disease; with this regard there is a direct correlation between the quantity and the prognosis of the disease.

Considering the results obtained in the CITUCEL trial, presence of cells at baseline was verified between the various therapeutic settings and correlates with the disease burden and becomes significant between neoadjuvant/adjuvant and metastatic patients.

The cells that most seem to differentiate the settings are the EpCAM positive which have been determined in greater numbers in metastatic patients than other settings in which most of the CTCs detected were EpCAM negative. Focusing on the number of cells found in pre- and post-therapy blood samples, no significant difference was determined between the number of cells found, otherwise it is interesting to underline that the number of CTCs determined at the baseline in patients treated with

neoadjuvant therapy correlates with the response to treatment, suggesting that this value may have predictive significance. Greater strength to these considerations is offered by the analysis of ctDNA, which is present in much higher quantities in the blood of patients in the advanced setting. As for the number of CTCs, there was no significant difference in the comparison between the amount of pre- and post-adjuvant and neoadjuvant ctDNA amount. Furthermore, the baseline ctDNA concentration does not appear to correlate with the complete pathological response in the neoadjuvant setting, differently to what was found for CTCs. However, it should be noted that the data of the CTCs must be validated by the mutational analysis as well as the concentration values of the ctDNA may not derive exclusively from tumor DNA.

The mutational analysis, still ongoing, was carried out on 8 patients but already offers promising results. In fact, it has been observed that in some cases there is complete agreement between the analysis of ctDNA and/or CTCs and the corresponding tumor, while in others there is a discrepancy between the identified mutations, suggesting that CTCs and ctDNA could provide complementary information both on the biology of the tumor and on its evolution.

Once completed, this analysis could provide additional information on the mutational profile of patients and will allow us to determine which biomarker in circulation could best reflect the histopathology of the tumor and its progression.

In this perspective to ensure that CTCs became an important prognostic and predictive marker of tumor, their unambiguous and standardized evaluation is necessary. In fact, the reliability of CTCs characterization is still a source of conflict in this field; the next step of the CITUCEL project will be the optimization of the detection antibodies cocktails and their consequently use in CTCs patients' samples detection.

Moreover, one additional point is related to the need of improving molecular analysis method for a more comprehensive and accurate detection of occurring molecular alteration. Our group already developed a

new molecular approach to analyse CTCs mutational profile. Until now, the results obtained are encouraging for a possible translational application of the proposed approach.

Based on our results, even if very limited, it is evident that CTCs and ctDNA could provide different - and maybe complementary - information. Therefore, it could be really important to establish which of those could be more representative of the tumor type and tumor progression.

With the hope to establish this open issue, the study BareLiB has been designed. The initial data confirmed that the system is able to label clones and to retrieve them maintaining their heterogeneity.

Moreover, our optimized experiments allow to recover human CBM from xenotransplanted mice: using tumor induced by TNBC cell line our preliminary data support that the circulating tumor derived biological material is very limited (data presented at ACCC 2019, Corfu' – Grecia).

On the contrary, initial experiment using patients' derived cells showed that ctDNA seems to well represent the heterogeneity of primary tumor, but this result was not obtained analysing CTCs or EX.

Overall, the identification of the most sensitive liquid biopsy method could allow the detection of specific biomarkers necessary to address the therapeutic choices and to monitor the response to therapy, contributing to improve the clinical treatments, the prognosis and the quality of life of BC patients. The results of this study could lead to the development of new diagnostic approaches specific for this neoplasia.

Finally, once optimized, the in vitro study on Ixazomib and Bendamustine will provide new data useful to obtain preliminary evidences of the efficacy of the use of these drugs in BC treatment, with the possibility to change the management of BC cancer therapy.

7. CONCLUSIONS

The different and multidisciplinary studies conducted in this PhD thesis have the purpose of obtaining a reliable biomarker derived from liquid biopsy, that allows not only to have an early diagnostic test but also to monitor the therapy and associate their presence with prognosis and resistance.

The data obtained within the CITUCEL trial, using molecular analyses and innovative platforms such as NGS and DEPArray^{TM}, have demonstrated the feasibility of CTCs and ctDNA analyzes in various clinical settings.

The analysis of the CTCs showed numerical and phenotypic/morphological differences between early and advanced setting and a correlation of the basal count with the pCR. The ctDNA analysis supports and confirms this difference information between the various settings. Furthermore, promising data on the mutational profile of the tissue and the related "circuloma" has been obtained.

Once completed, the study could provide new data to better define the mechanisms underlying tumor progression and resistance to therapies.

On the other hand, the ongoing trial BaReLiB will provide insight regarding which CBM will better represent the heterogeneity of BC. So far, the results support the fact that ctDNA well reproduce tumor clonal complexity suggesting that it could be implemented in clinic for treatment choice and monitoring.

Finally, the results of the pilot study on Ixazomib could provide new insight of a potential novel treatment strategy in BC and could be a starting point for a larger and more complex study.

8. REFERENCES

1. AIOM Breast Cancer Guidelines (2019).

2. Bray F, Ferlay J, Soerjomataram I, Siegel R.L., Torre L.A., Jemal A. Estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. 68, 394–424 (2018)

3. Klein, C.A. Parallel progression of primary tumours and metastases. Nat. Rev. Cancer, 9, 302–312 (2009)

4. Coumans, F.A., Siesling, S., Terstappen, L.W. Detection of cancer before distant metastasis. BMC Cancer (2013)

5. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N. Detection of circulating tumor DNA in early- and late-stage human malignancies. Science translational medicine 6: 224ra24. (2014)

6. Krishnamurthy N, Spencer E, Torkamani A, Nicholson L. Liquid biopsies for cancer: coming to a patient near you. J Clin Med; 6:3. (2017)

6b. Radovich M, et al “Association of Circulatimg Tumor DNA and Circulating Tumor Cells After Neoadjiuvant Chemotherapy with with disease recurrence in patients with Triple_Negative Breast Cancer: Preplanned Secondary Analysis of the BRE12-158 Randomized Clinical Trial; JAMA Oncol. (9):1410-1415 (2020)

7. Kaiser J. “Liquid Biopsy” for Cancer Promises Early Detection. Washington, DC: American Association for the Advancement of Science; (2018)

8. Wan JC, Massie C, Garcia-Corbacho J. Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nat Rev Cancer. 17: 223-238

(2017)

9. Torga G, Pienta KJ. Patient-Paired Sample Congruence Between Commercial Liquid Biopsy Tests. JAMA Oncol; 4: 868-70 (2018)

10. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, et al. Detection and localization of surgically respectable cancers with a multi-analyte blood test. Science 359: 926-30 Clin. 2018, 68, 394–424, doi:10.3322/caac.21492. (2018)

11. Byrd Dr AJCC cancer staging annual 8th ed. (2016)

12. Mego M, Karaba M, Minarik, G, Benca, J.; Silvia, J.; Sedlackova, T.; Manasova, D.; Kalavska, K.; Pindak, D.; Cristofanilli, M. Circulating Tumor Cells With Epithelial-to-mesenchymal Transition Phenotypes Associated With Inferior Outcomes in Primary Breast Cancer. Anticancer Res, 39, 1829–1837 (2019).

13. Seyed Mohammad Kazem Aghamir, Ramin Heshmat, Mehdi Ebrahimi and Fatemeh Khatami (2019). Liquid Biopsy: The Unique Test for Chasing the Genetics of Solid Tumors. SAGE Journals (2020).

14. Fabisiewicz A, Szostakowska-Rodzos M, Zaczek AJ, Grzybowska EA. Circulating Tumor Cells in Early and Advanced Breast Cancer; Biology and Prognostic Value. International Journal of Molecular Sciences (2020)

15. Vetter M, Landin J, Szczerba B.M, Castro-Giner F, Gkountela S, Donato C, Krol I, Scherrer R, Balmelli C, Malinovska A. Denosumab treatment is associated with the absence of circulating tumor cells in patients with breast cancer. Breast Cancer Res (2018)

16. Wang Q, Zhao L, Han L, Tuo X, Ma S, Wang Y, Feng X, Liang D, Sun C, Wang Q. The Discordance of Gene Mutations between Circulating Tumor Cells and Primary/Metastatic Tumor. Mol. Ther. Oncolytic (2019)
17. Gkountela S, Castro-Giner F, Szczerba B.M, Vetter M, Landin J, Scherrer R, Krol I, Scheidmann M.C, Beisel C, Stirnimann C.U. Circulating Tumor Cell Clustering Shapes DNA Methylation to Enable Metastasis Seeding. Cell, 176, 98–112.e114 (2019)
18. Bidard F.C, Michiels S, Riethdorf S, Mueller V, Esserman L.J, Lucci A, Naume B, Horiguchi J, Gisbert-Criado R, Sleijfer S. Circulating Tumor Cells in Breast Cancer Patients Treated by Neoadjuvant Chemotherapy: A Meta-analysis. J. Natl. Cancer Inst. *110*, 560–567 *(*2018).
19. Felden Jv, Craig AJ, Villanueva A. Role of circulating tumor DNA to help decision-making in hepatocellular carcinoma. Oncoscience 5: 7-8. (2018)
20. Bronkhorst AJ, Ungerer V and Holdenrieder S. The emerging role of cell-free DNA as a molecular marker for cancer manage- ment. Biomol Detect Quantif 17: 100087, 30. (2019)
21. Cheng FT, Lapke N, Wu CC, Lu YJ, Chen SJ, Yu PN, Liu YT and Tan KT. Liquid biopsy detects relapse five months earlier than regular clinical follow-up and guides targeted treatment in breast cancer. Case Rep Oncol Med 2019: 6545298, (2019).
22. Russnes HG, Lingjærde OC, Børresen-Dale AL and Caldas C. Breast cancer molecular stratification. Am J Pathol 187: 2152-2162, (2017)
23. Rodriguez BJ, Córdoba GD, Aranda AG, Álvarez M, Vicioso L, Pérez CL, Hernando C, Bermejo B, Parreño AJ, Lluch A. Detection of TP53 and PIK3CA

mutations in circulating tumor DNA using next-generation sequencing in the screening process for early breast cancer diagnosis. J Clin Med 8: 1183, 2019.

24. Chung JH, Pavlick D, Hartmaier R, Schrock AB, Young L, Forcier B, Ye P, Levin MK, Goldberg M, Burris H. Hybrid capture-based genomic profiling of circulating tumor DNA from patients with estrogen receptor-positive metastatic breast cancer. Ann Oncol 28: 2866-2873, (2017).

25. Stover DG, Parsons HA, Ha G, Freeman SS, Barry WT, Guo H, Choudhury AD, Gydush G, Reed SC, Rhoades J. Association of cell-free DNA tumor fraction and somatic copy number alterations with survival in metastatic Triple-Negative breast cancer. J Clin Oncol 36: 543-553, (2018).

26. F. André, E. Ciruelos, G. Rubovszky, M. Campone, S. Loibl, H.S. Rugo, H. Iwata, P. Conte, I.A. Mayer, B. Kaufman, T. Yamashita, Y.-S. Lu, K. Inoue, M. Takahashi, Z. Pápai, A.-S. Longin, D. Mills, C. Wilke, S. Hirawat, and D. Juric. Alpelisib for PIK3CA-Mutated, Hormone Receptor–Positive Advanced Breast Cancer. NEJ (2019)

27. Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol. 10:1470–76 (2008)

28. Vader P, Breakefield XO, Wood MJ. Extracellular vesicles: emerging targets for cancer therapy. Trends Mol Med. 20:385–93 (2014)

29. Théry, C., Ostrowski, M. & Segura, E. Membrane vesicles as conveyors of immune responses. Nat. Rev. Immunol. 9, 581–593 (2009)

30. Greening DW, Gopal SK, Mathias RA, Liu L, Sheng J, Zhu HJ, Simpson RJ. Emerging roles of exosomes during epithelial-mesenchymal transition and cancer progression. Semin Cell Dev Biol. 40:60–71. (2015)
31. Vaupel P, Briest S, Höckel M. Hypoxia in breast cancer: pathogenesis, characterization and biological/therapeutic implications. Wien Med Wochenschr. 152:334–42 (2002)
32. Ciravolo V, Huber V, Ghedini GC, Venturelli E, Bianchi F, Campiglio M, Morelli D, Villa A, Della Mina P, Menard S, Filipazzi P, Rivoltini L, Tagliabue E, Pupa SM. Potential role of HER2-overexpressing exosomes in countering trastuzumab-based therapy. J Cell Physiol. 227:658– 67 (2012)
33. Lehmann BD, Jovanovic B, Chen X, Estrada MV, Johnson KN, Shyr Y. Refinement of Triple-Negative Breast Cancer Molecular Subtypes: Implications for Neoadjuvant Chemotherapy Selection. PLoS One 11(6): e0157368 (2016)
34. Ciechanover A. The ubiquitin-proteasome proteolytic pathway. Cell (1994)
35. Kupperman E, Lee EC, Cao Y, Bannerman B, Fitzgerald M, Berger A, Yu J, Yang Y, Hales P, Bruzzese F, Liu J, Blank J, Garcia K, Tsu C, Dick L, Fleming P, Yu L, Manfredi M, Rolfe M, Bolen J. Evaluation of the proteasome inhibitor MLN9708 in preclinical models of human cancer. Cancer Res (2010)
36. Offidani M, Corvatta L, Caraffa P, Gentili S, Maracci L, Leoni P. An evidence-based review of ixazomib citrate and its potential in the treatment of newly diagnosed multiple myeloma. Dovepress (2014)

37. Wang H, Yu Y, Jiang Z, Cao WM, Wang Z, Dou J, Zhao Y, Cui Y, Zhang H. Next-generation proteasome inhibitor MLN9708 sensitizes breast cancer cells to doxorubicin- induced apoptosis. Scientific Report (2016)

38. Chauhan D, Tian Z, Zhou B, Kuhn D, Orlowski R, Raje N, Richardson P, Anderson KC. In vitro and in vivo selective antitumor activity of a novel orally bioavailable proteasome inhibitor MLN9708 against multiple myeloma cells. Clin Cancer Res (2011)

39. Inoue M, Honma Y, Takeshi Urano T, Suzumiya J. Japanese apricot extract (MK615) potentiates Bendamustine-induced apoptosis via impairment of the DNA damage response in lymphoma cells. Oncology letters (2017)

40. Giuseppa De Luca, Barbara Cardinali, Lucia Del Mastro, Sonia Lastraioli, Franca Carli, Manlio Ferrarini, George A. Calin, Anna Garuti, Carlotta Mazzitelli, Simona Zupo and Mariella Dono. Optimization of a WGA-Free Molecular Tagging-Based NGS Protocol for CTCs Mutational Profiling. International Journal of Molecular Sciences (2020)

40.b Reduzzi C, Motta R, Bertolini G, et al. Development of a protocol for single-cell analysis of circulating tumor cells in patients with solid tumors. Adv Exp Med Biol 2017;994:83–103 (2017)

41. Hyun KA, Koo GB, Han H. Epithelial-to-mesenchymal transition leads to loss of EpCAM and different physical properties in circulating tumor cells from metastatic breast cancer. Oncotarget, Vol. 7, No. 17 (2018)

42. Zhu G, Song P, Zhou H. Role of epithelial-mesenchymal transition markers E-cadherin, N-cadherin, β-catenin and ZEB2 in laryngeal squamous cell

carcinoma. Oncology Letters 15: 3472-3481, (2018)

43. Nath S and Mukherjee P. Muc1: a multifaceted oncoprotein with a key role in cancer progression. Trends Mol Med (2014)

44. Zhou I, Zhu YF, Chen XY, Han B. Black rice-derived anthocyanins inhibit HER-2-positive breast cancer epithelial-mesenchymal transition-mediated metastasis in vitro by suppressing FAK signaling. International Journal of Molecular Medicine 40: 1649-1656, (2017)

45. www.pnas.org/cgi/doi/10.1073/pnas.1111053108

46. Grzegrzolka J, Biala M, Wojtyra P. Expression of EMT Markers SLUG and TWIST in Breast Cancer. Anticancer Research 35: 3961-3968 (2015)

47. Subimerb C, Pinlaor S, Lulitanond V. Circulating CD14+CD16+ monocyte levels predict tissue invasive character of cholangiocarcinoma. Clinical and Experimental Immunologydoi: 10.1111/j.1365-2249.2010.04200 (2010)

48. Hao Wang H, Yang Yu Y, Zheng Jiang Z, Cao WM, Wang Z, Dou J, Zhao Y, Cui Y, Zhang H. Next-generation proteasome inhibitor MLN9708 sensitizes breast cancer cells to doxorubicin- induced apoptosis. Scientific Report (2016)

9. PUBLICATION

Giuseppa De Luca, Barbara Cardinali, Lucia Del Mastro, Sonia Lastraioli, Franca Carli, Manlio Ferrarini, George A. Calin, Anna Garuti, Carlotta Mazzitelli, Simona Zupo, Mariella Dono. Optimization of a WGA-Free Molecular Tagging-Based NGS Protocol for CTCs Mutational Profiling. International Journal of Molecular Sciences (2020)