UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXII **CICLO DEL DOTTORATO DI RICERCA IN BIOMEDICINA MOLECOLARE**

VALIDATION OF PREDICTIVE AND PROGNOSTIC BIOMARKERS AS A GUIDE FOR A PERSONALIZED APPROACH IN SOLID TUMOURS

Settore scientifico-disciplinare: biomedicina molecolare

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CHAPTER 1

MONITORING NSCLC WITH EGFR MUTATION ANALYSIS ON CIRCULATING FREE DNA

INTRODUCTION

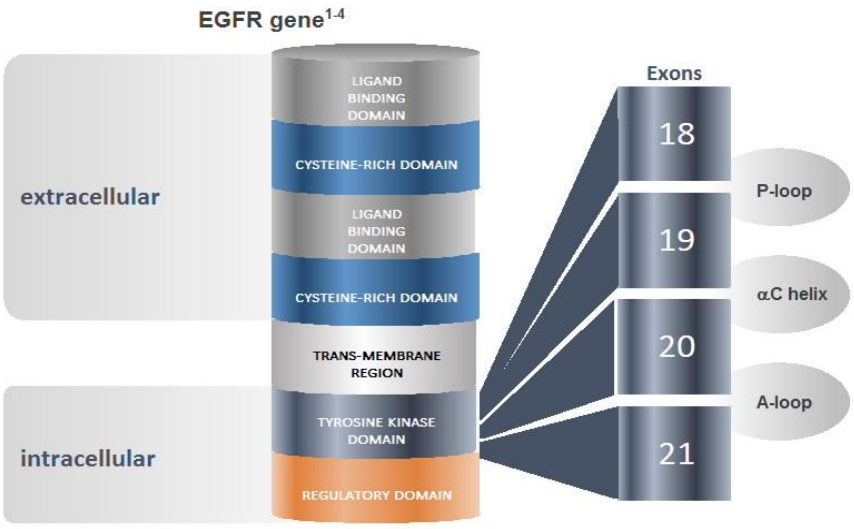
Lung cancer is one of the most diagnosed malignancy and the leading cause of cancer death worldwide. Most lung cancers (85%) are classified as Non Small Cell Lung Cancer (NSCLC) and most NSCLC patients are at an advanced stage when diagnosed [1]. Over half of NSCLC diagnosis are defined histologically as adenocarcinomas, other variants are squamous and large cell lung cancer (LCC). Tobacco smoking remains the main cause of lung cancer but several other factors have been described as lung cancer risk factors, including exposure to asbestos, arsenic, radon and non-tobacco-related polycyclic aromatic hydrocarbons.

Surgery is the elective treatment in the early stages of the disease, resections are indicated only with curative intent, where tumor removal can be achieved with a surrounding healthy margin of tissue, histologically confirmed. Since most NSCLC patients are diagnosed at an advanced stage, surgery is no longer possible and it is hard to get sufficient tissues for molecular testing [2]. Advanced NSCLC treatment choice depends on various factors: histology (squamous VS non-squamous histology, non-oncogene-addicted disease); clinical conditions (age, comorbidities, performance status); PD-L1 (programmed cell death-ligand 1) expression and finally the presence of driver mutations for oncogene-addicted disease: mainly EGFR (Epidermal Growth Factor Receptor) or ALK (Anaplastic lymphoma kinase) translocation. The study of the molecular characteristics of lung tumors has highlighted a specific role of some genes that represent important therapeutic targets, including EGFR [3].

EGFR Structure and function

EGFR (ERBB1) is a transmembrane tyrosine kinase receptor, it belongs to the ERBB family. Other ERBB receptors are HER2 (ERBB2), HER3 (ERBB3), and HER4 (ERBB4). Activation of EGFR induces trans-phosphorylation of the ERBB dimer partner and regulation of transcription, protein synthesis, proliferation and cell survival via various intracellular pathways like RAS/RAF/MEK/ERK, PI3K/AKT/TOR, STAT transcription factor and Src kinase.

The locus encoding EGFR is located on the short arm of chromosome 7 (7p11.2) and is encoded by 30 exons. EGFR activating mutations in NSCLC tend to cluster at exons 18 through 21, which encode the tyrosine kinase domain and the adenosine triphosphate (ATP) substrate-binding cleft of the EGFR.



Distribution of mutation types[4,*]

Mutation type	Percent
Exon 18 G719A/C	1.03%
Exon 19 deletion[+]	46%
Exon 20 T790M de novo Other exon 20 mutations	4.1% 2.3%
Exon 21 point mutation L858R[†]	37.5%
L861Q	1.12%

Fig.1 EGFR domains and activating mutation distribution.*Literature review as reported in COSMIC (Catalogue of somatic mutations in cancer) database; may vary depending on study and population factors. [+]Sensitizing mutations that confer sensitivity to first and second generation TKIs. *1.Shigematsu H, et al.J Natl Cancer Inst 2005;97(5):339-346. 2. Lynch TJ, et al. N Engl J Med. 2004;350(21):2139. 3 Paez JG, et al. 2004;304(5676):1497-1500. 4. Siegelin MD, et al Lab Invest 2014;94(2):129-137.*

The presence of EGFR activating mutations results in constant signaling by EGFR and therefore activation of downstream pathways. As shown in figure 2, the exons 18-21 encode for the tyrosine-kinase region of the receptor and most frequent mutations (90% of all activating mutations) are deletion on exon 19 (E746-A750, LREA deletion) and substitution of leucine to arginine on exon 21 (L858R).

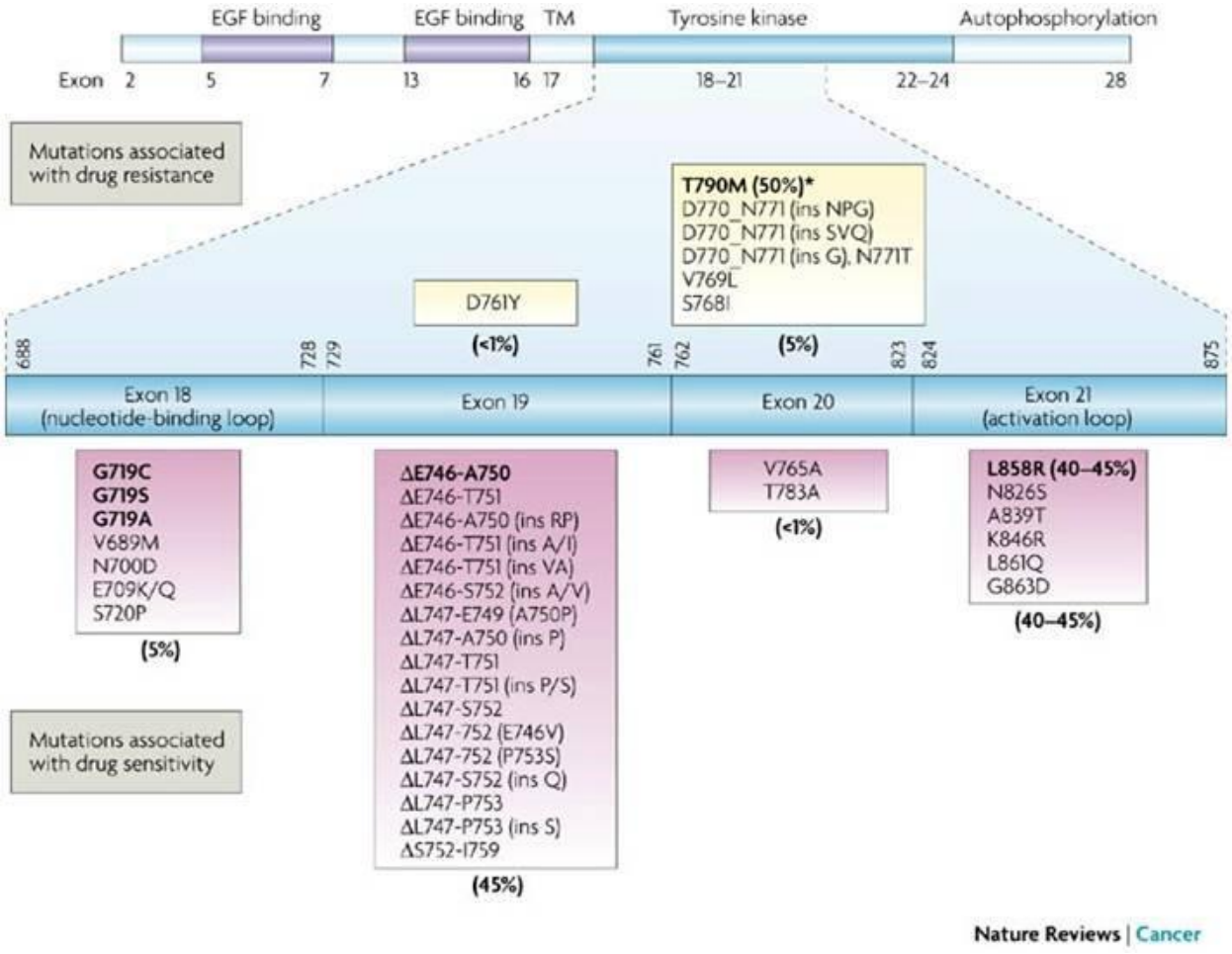


fig.2 Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nature reviews Cancer 2007;7:169-81 [4]

In NSCLC, in particular in 20% of adenocarcinomas of caucasian patients and in 40% of Asian patients, the presence of activating mutations represents the most important predictive factor for the

adoption of molecular target therapies with specific EGFR tyrosine kinase inhibitors (EGFR-TKI) [5]. On the other hand, the point mutation in exon 20 (T790M) indicates resistance to EGFR-TKIs and poor prognosis [6].

EGFR Tyrosine Kinase Inhibitors

At least 8 randomized phase III studies have shown, in patients suffering from advanced NSCLC with EGFR mutation, superiority in patients on oral treatment with an EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib (250 mg/day), erlotinib (150 mg/day) or afatinib (40 mg/day) in the first line of treatment compared to standard platinum-based chemotherapy, in terms of both RR and PFS[6-10]. Patients that do not carry a mutation on the EGFR gene don't show any clinically relevant activity with Erlotinib (Tarceva), Gefitinib (Iressa) or Afatinib (Giotrif). Gefitinib response rate is higher in tumors expressing most common activating mutations (L858R and exon 19 deletion), but scarce on those tumors carrying less common mutations, available data show that G719X, L861Q e S7681 are sensitizing mutations. NSCLC with less common mutations of EGFR in exon 18 (G719X) and in exon 21 (L861Q) are particularly sensitive to treatment with afatinib in clinical and non-clinical settings. Afatinib is an irreversible, strong and selective inhibitor of the ErbB family approved for second-line therapy. Afatinib bind is covalent and irreversibly blocks the signal from all the homo and heterodimers formed by the members of the ErbB EGFR (ErbB1), HER2 (ErbB2), ErbB3 and ErbB4 family.

Progression disease occurs on average after 9 to 13 months of EGFR-TKI treatment. T790M is a resistance mutation that is found in nearly 50% of patients progressing under first-line TKI treatment. Osimertinib (Tagrisso) is a third-generation EGFR-TKI that targets specifically the T790M mutated cells, approved by EMA in January 2019 in the first or second line of therapy. Its

superiority over platinum-based treatment in terms of PFS, Overall response rate (OR), and duration of response (DOR) has been demonstrated in the AURA 3 trial [7].

Liquid Biopsy

Several studies have been carried out to assess the cases in which the EGFR gene mutation was present both in the blood sample and in the tissue finding, but the diagnostic accuracy has always revealed very wide range variation. The sensitivity for the analysis of circulating DNA ranges from 17% [8] to 100% [9,10], while the specificity fluctuates between 71.4% [11] and 100% [12]. The great variability that characterizes the different studies can be explained by using different cfDNA laboratory extraction and sequencing procedures. Also, the dimensions of the tissue sample influence the sensitivity and specificity data [13]. Two large meta-analysis [14,15] were conducted to compare cfDNA with tumor tissue in terms of diagnostic accuracy for EGFR mutations. Both found that cfDNA has a high diagnostic accuracy to identify EGFR mutations in NSCLC patients. The sensitivity of cfDNA has been shown to be 67.4% and 62%, Qiu et al. and Liu et al. respectively, and the specificity of 93.5% and 95.9% respectively [13].

Techniques approaching the identification of molecular alterations in cfDNA differ in many aspects. Real-time or quantitative PCR (rt-PCR) and most of its variants have targeted (narrow) approaches, while Next-generation sequencing (NGS) platforms have a broad, untargeted approach. The former detects mutations in small defined regions of DNA, while the latter investigate larger regions of multiple genes in a single run and usually focus on a panel of genes relevant to cancer therapeutics [16]. Diagnostic accuracy, turnaround time and costs, as well as the amount of total cfDNA extracted, are all important features to be considered in the choice of the most appropriate platform for clinical practice and the variability in the rigorousness of the validation

of each assay has led to confusion as to how to best utilize this new technology for clinical care in lung cancer [17]. Real-time or quantitative PCR (rt-PCR) Real-time or quantitative PCR (qPCR) differs from classic PCR because the intensity of a fluorescent light emitted by the probes is read every cycle, which allows for an estimate of the quantity of the loaded sample based on the number of cycles needed to obtain a threshold fluorescent signal [18]. Digital droplet PCR (ddPCR) quantification, based on the emulsion of fractionated droplets, is considered more precise than the one of rt-PCR. Also BEAMing methods, similar to ddPCR but with the binding of the DNA to the magnetic beads before the emulsion, outperformed rt-PCR in terms of sensitivity. NGS technology (Illumina platforms or Ion Torrent) certainly enables efficient use of limited DNA and the identification of a wider range of mutations, including the rarest [19].

Nonetheless, availability and cost-effectiveness are important limiting factors, a treatment strategy that takes into account the patient's clinical status, the clinical relevance of the test results and the local feasibility of the different assays must be taken into account when planning diagnostic procedures to avoid potential delays in identifying mechanism of therapy resistance [20]. rt-PCR is a validated and solid technique for targeting specific mutations such as EGFR in NSCLC, and results are available in less than two weeks, the time limit over which a plasma analysis is preferable than a tissue biopsy .

Considering these data, the cfDNA is an attractive approach with a high specificity/sensibility to investigate mutations of the EGFR for treatment purposes. As the clinical need to quickly identify the proper patients for the proper treatment especially in NSCLC is crucial, the aim of the present study was to provide the importance of clinical use in a daily practice of liquid biopsy (LB) in NSCLC treatment.

MATERIALS AND METHODS

Patients

This study included 30 patients, treated at the Oncology Unit of Azienda Sanitaria Universitaria Integrata (ASUI) of Trieste (Italy) in the period between December 2016 and May 2019. The last follow-up update was on 19th August 2019. Patients with NSCLC, all of whom tested positive for mutational analysis of the EGFR gene at histological/cytological first evaluation (baseline), with signed informed consent, were included in the analysis. Majority of patients were diagnosed at a locally advanced/advanced stage, except for three patients (stages Ib to IIb); two of them progressed later on. Patients characteristics are detailed in table 1. A subset of 3 patients underwent surgical resection only, followed by instrumental follow-up; 4 patients received adjuvant chemotherapy after surgery (platinum based or Vinorelbine); 21 patients had unresectable disease and only received chemotherapy, two patients did not have sufficient data for follow-up and where therefore excluded from analysis. Patients' characteristics are detailed in table 1. A second blood sample (T1) was proposed for patients who had been on EGFR-TKI therapy for at least 4 months and/or, according to clinician opinion and/or unclear CT results, had shown early signs of clinical disease progression (n=19) in order to evaluate potential changes of the mutational status of EGFR during treatment. The LB has been performed at the same time of the CT scan in order to correlate the EGFR status with the disease with response/resistance to treatment.

Diagnosis and molecular test of EGFR status

Data of mutational status on surgical/cytological biopsy were collected retrospectively. In order to perform the mutational comparison between the basal cell/tissue and the cfDNA analysis performed on liquid biopsy, the blood sampling (approximately 10 ml in EDTA tubes) has been taken at the

same time of diagnosis or before starting the target-treatment (T0). Patients that underwent surgical removal of tumor (n=7) performed LB at the time of progression as no adjuvant treatment nor more mutational analysis was needed after surgery.

A second blood sample (T1) was proposed for patients who had been on therapy for at least 4 months and/or, according to clinician opinion and/or unclear CT results, had shown early signs of clinical disease progression (n=19) in order to evaluate potential changes of the mutational status of EGFR during treatment. The LB has been performed at the same time as the CT scan in order to correlate the EGFR status with the disease with response/resistance to treatment.

Extraction, purification and concentration of circulating free DNA (cfDNA) was performed from 3-5 ml of plasma with “Helix Circulating Nucleic Acid kit” (Diatech Pharmacogenetics) within 60 minutes from blood withdrawal. The detection analysis has been performed with 200µl by real-time PCR using the “Easy EGFR kit” or the "EasyPGX® ready EGFR" kit (Diatech Pharmacogenetics). The kit selectively amplifies the mutated DNA in samples containing a mixture of mutated and wild-type DNA; detection is performed by fluorescent probes marked with FAM and HEX. The kit consists of 7 assays for the detection of mutations and a control assay for the evaluation of DNA content in the sample. Each assay allows simultaneous detection of the target by a probe labeled FAM and an endogenous control gene by a probe labeled HEX, with a sensitivity of up to 0,5%. Amplification of the internal control gene allows for verification of the correct execution of the amplification procedure and the possible presence of inhibitors, which may cause false negative results. The principal mutations are detected are: codon 719 (without discrimination), T790M, S768I, mutations or insertions on exon 20 (without discrimination), L858R, L861Q, mutations on exon 19 (without discrimination).

Statistical analysis

The clinical-pathological characteristics of the study population were described by means of ± standard deviation (SD) or median and range (minimum-maximum) for continuous variables, while with absolute and percentage frequencies for categorical variables. The association between presence or absence of mutation and the categorical variables of interest (type of mutation, type of withdrawal and clinical response of the patient) was evaluated by Fisher's exact test. The proportions test was used to compare the difference in the percentage of mutated patients at baseline and the first plasma sample. The comparison of the mutational status between T0 and T1 was performed with the Mc-Nemar test for paired data and the degree of agreement was evaluated by Kappa index of Cohen [19]. For the patients in oncological therapy with targeted drug and with T1 sample available (n=18), progression free survival (PFS) and overall survival (OS) were evaluated: PFS was defined as the time from the beginning of the therapy to the date of the first progression and OS was defined as time between the end of therapy and time of death or date of last visit for living patients. PFS and OS were estimated using the Kaplan-Meier method and the differences between the groups were tested using the Log-rank test. All statistical analysis were performed using the statistical software R (the R Foundation for Statistical Computing; Version 3.5.0) and the software STATA 14.2 (StataCorp, College Station, TX).P-value values less than 0.05 were considered statistically significant.

RESULTS

All 30 patients included in the study had been diagnosed with NSCLC, stage I-IV, the vast majority of them being diagnosed already as metastatic; median age was 69.6, majority of patients is non-smoker (19, 63,3%) or former smoker (4, 13,33%) clinical characteristics are reported in Table 1.

		N	%
Gender	Female	23	76.6
	Male	7	23.3
Age	>=60 years	25	83.3
	<60 years	5	16.7
Age, years (Mean±SD)	69.58 ± 10.63		
Smoke	Smoker	3	10.0
	Non smoker	19	63.3

	Former smoker	4	13.3
	NA	4	13.3
Stage of disease	IA2	1	3.3
	IB	1	3.3
	IIB	1	3.3
	IIIA	1	3.3
	IIIB	2	6.6
	IIIC	1	3.3
	IV	20	66.6
	NA	3	10.0

Histopathology	Adenocarcinoma	30	100

Table 1: Baseline characteristics of NSCLC patients. SD= standard deviation, NA = not available

Baseline EGFR mutational analysis were performed using cytological samples in 7 patients (23.3%), on tissue biopsy in 16 patients (53.3%) and on sample after surgery on 7 patients (23.3%). Type of mutation detected is not linked to the origin of the sample, in fact different mutations are equally distributed between surgery, cytology and biopsy (table 2).

Sample origin	Deletion 19	Exon 21 (L858R)	Ins 20
cytology	4	3	0
biopsy	7	8	1
surgical sample	3	3	1
Total	14	14	2

Table 2 mutations distribution according to sample origin

In 18 out of 30 patients (60%), the mutation detected at baseline is also been confirmed on the cfDNA at time point T0, while in 12 patients (40%), the mutation at baseline was not detected on the corresponding cfDNA (Table 3), this difference is statistically significant ($p<0.01$, binomial proportion test). The sensitivity of mutation-status detection between baseline tumour and T0 samples for patients evaluable for both samples was 60.0% (95% CI: 41.0%–77.0%).

EGFR Mutation Status	Tissue Samples	T0
Deletion 19	14 (46.7%)	12 (40.0%)
Exon 21 (L858R)	14 (46.7%)	5 (16.7%)
Addition Exon 20	2 (6.7%)	1 (3.3%)
Mutation-Negative	0 (0.0%)	12 (40.0%)
Total	30	30

Table 3: EGFR mutations status according to tissue samples and T0

Comparing the analysis performed on cell/tissue versus cfDNA, we noted the absence of mutation is associated with the type of mutation itself (see Figure 3). Discordance between the absence of detection of the mutation was greater in patients carrying the exon 21 mutation (64.3%) than with the deletion of exon 19 (14.3%) or mutation in exon 20 (50.0%) (p=0.02, Fisher Exact test)

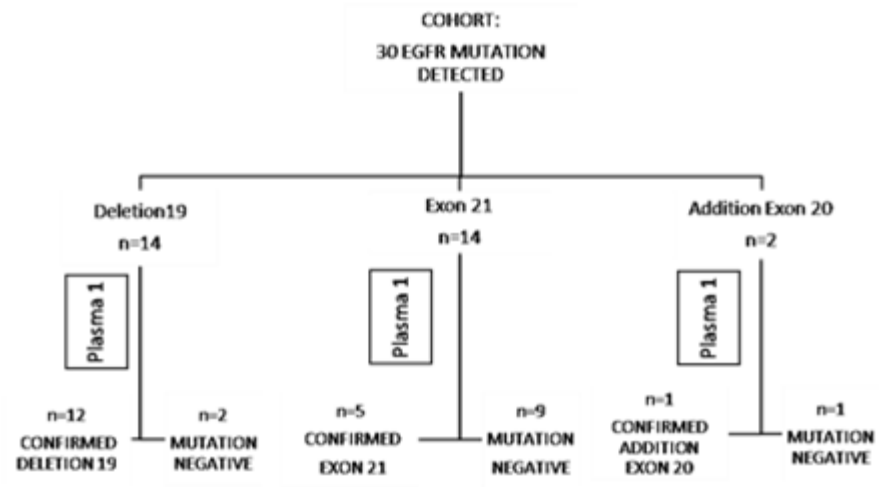


Figure 3: EGFR mutation status summary for tissue sample versus T0 circulating free DNA (patients evaluable for both samples, n=30)

Patients with a mutation detected at baseline with FNA or biopsy detected the same mutation on cfDNA (71% and 69% respectively); on the other hand only in 29% of the patients carrying a mutation detected from samples obtained from surgery has been detected the same mutation on cfDNA. Thus, overall, a significant association is observed between samples (FNA and biopsy versus surgery) on which the mutation at baseline is performed and the mutational state at the T0 ($p = 0.08$, Fisher Exact test).

Moreover, a mutational analysis on cfDNA was performed on 19 patients (63.3%) during treatment with anti EGFR-TKI. A second blood withdrawal (T1) was taken approximately 6 months after the beginning of therapy (5.84 months, standard deviation 2.02).

In 9 out of 19 patients, the same mutation was detected in T0 and in T1 while in 4 patients we found a change from a mutation to a wild type during the therapy. No changing from wild type to mutation has been detected during the two time points and in 6 patients no mutation has been detected, neither in T0 nor in T1.

All of the patients that had a confirmed mutation on T1 (9, 100%) are in a progression state (p = 0.003, Fisher Exact Test). The percentage decreases both for patients not mutated on both samples (2, 33.3%) and for mutation-negative patients (1, 25%) (Table 4).

	Progression Disease	Stable disease
Mutation-positive in both T0 and T1	9	0
Mutation-Negative in both samples	2	4
Mutation-positive in T0 and mutation-negative in T1	1	3

Table 4: EGFR Mutational status by patients treatment response

In the survival analysis were only included patients that received EGFR-TKI therapy (22, 73.3%): 10 patients received gefitinib, 8 afatinib and 4 erlotinib. Moreover two of the 22 patients had progressive disease prior to EGFR-TKI treatment and received first line therapy were therefore excluded from survival analysis.

Disease was instrumentally evaluated according to clinical schedule to assess response to therapy. 12-month Progression Free Survival (PFS, 95% CI) was 64.90% (40.0-81.9). Median 12 months PFS was 74.2% (CI 95%: 484%-100.0%) at first plasma evaluation (T0) (Figure 4a) and 33.3% (CI 95%: 13.2%-84.0%) at T1(Figure 4b).

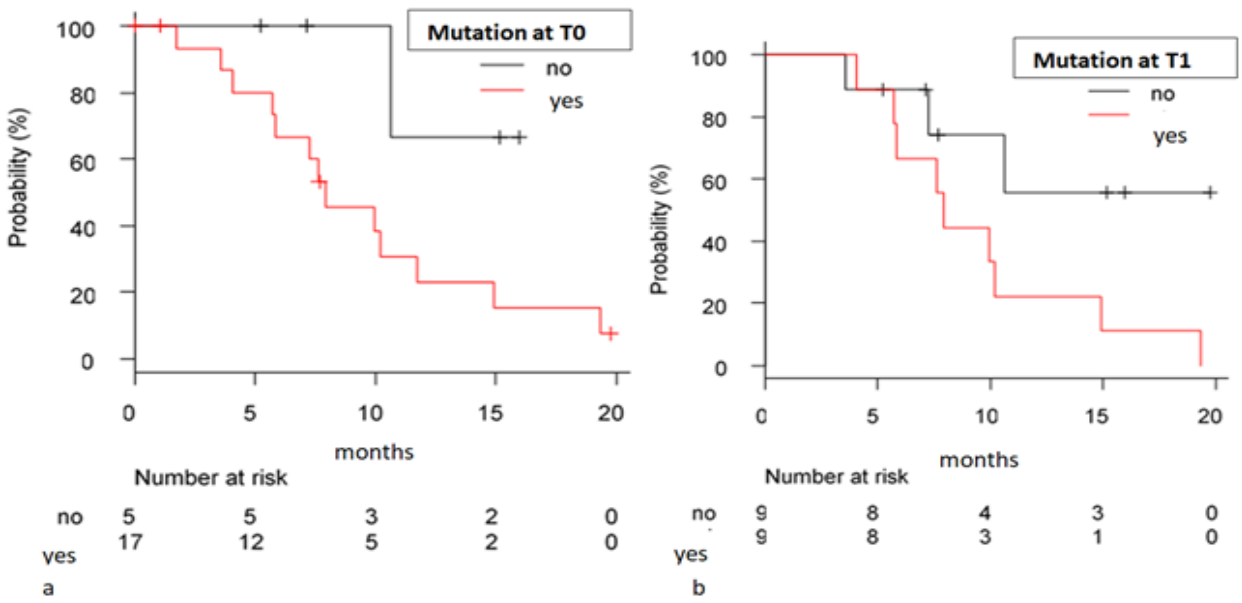


Figure 4 Kaplan-Meier progression free survival (PFS) curves for patients treated with target therapy detected in T0 (a) and T1 samples (b)

We compared the PFS curves between three groups: wild type on both T0 and T1 (n=5, median PFS has not yet been reached -more than half of patients were still living), mutation on both plasma samples (n=9, median PFS=7.97 months), mutated on T0 but non-mutated on T1 (n=4, median PFS= 7.27 months) (Figure 5). Patients without mutation at both time points showed the longest PFS at 12 month (66.7%, 95% CI: 27.2% -100%;); whereas patients that maintained a mutation during treatment showed the shorter PFS (22.2%, 95% CI: 6.5% -75.4%). Patients with a discordance in the mutational status detected on cfDNA on the two time points showed an intermediate situation in terms of PFS at 12 months (50.0%; 18.8% -100%).

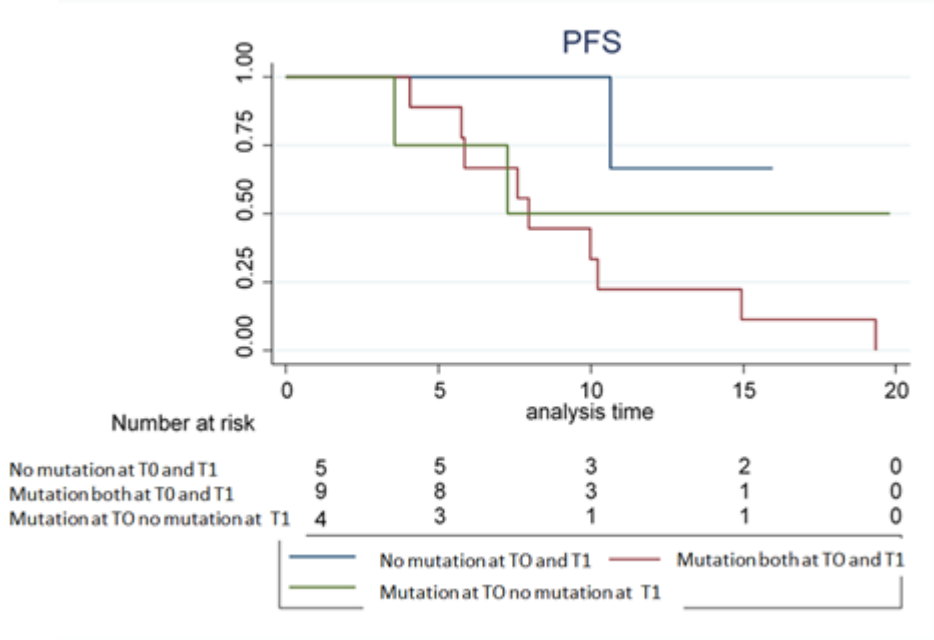


Figure 5: Kaplan-Meier progression free survival (PFS) curves for patients treated with target oncological therapy respect to EGFR mutational status detected in T0 and T1 samples

The median OS at 12 months was 18.21 months (95% C.I., 7.97-NA) with 12 events occurred as reported in other studies on EGFR-mutated NSCLC patients treated with target therapy [21]. Patients whose basal mutation was confirmed by liquid biopsy at T0 had a 12 months OS of 53.8% [26.7%-74.7%] with a median OS of 13.2 months (Figure 6a). On the other hand all of the patients with a discordance between cyto/histological and plasma mutation (EGFR mutation not detected on T0) were alive at the 12th month of follow-up (OS 100%; 100-100) (p=0.02, Log-Rank test)(Figure 6b).

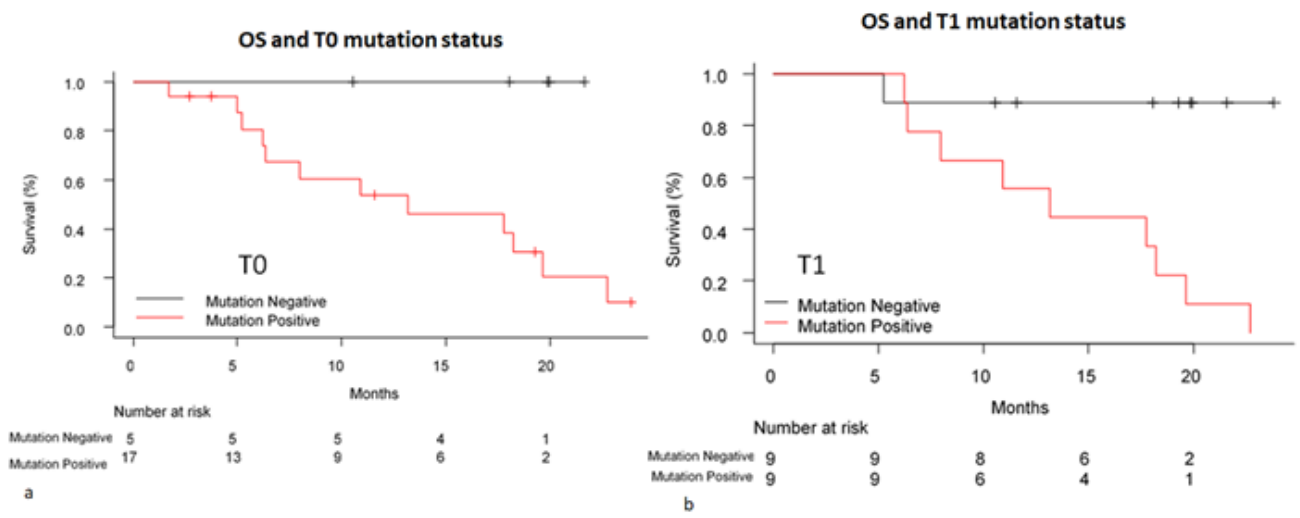


Figure 6 Kaplan-Meier overall survival (OS) curves for patients treated with target therapy detected in T0(a) and T1 samples (b)

The overall survival at 12 months was then evaluated between basal and T1 mutation. The OS was 88.9% (95% CI; 0.433-0.984) for patients that did not reveal the mutation at T1, and was significantly lower (55.6%, 95% CI; 0.204-0.805) in patients mutated both at basal and T1 (p=0.004). Lastly, the OS was evaluated in relation to mutation status between T0 and T1 (Figure 7). All of the patients (100%) that resulted negative both at T0 and T1 were alive at 12 months (95% CI; 1.00-1.00), patients that had a positive T0 but negative T1 registered an intermediate OS (75.0%, 95% CI; 12.8%-96.1%) and patients that tested positive both at T0 and T1 had a 12 month OS of 55.6% (95% CI, 20.4%-96.1%). Differences between groups were statistically significant (p=0.01).

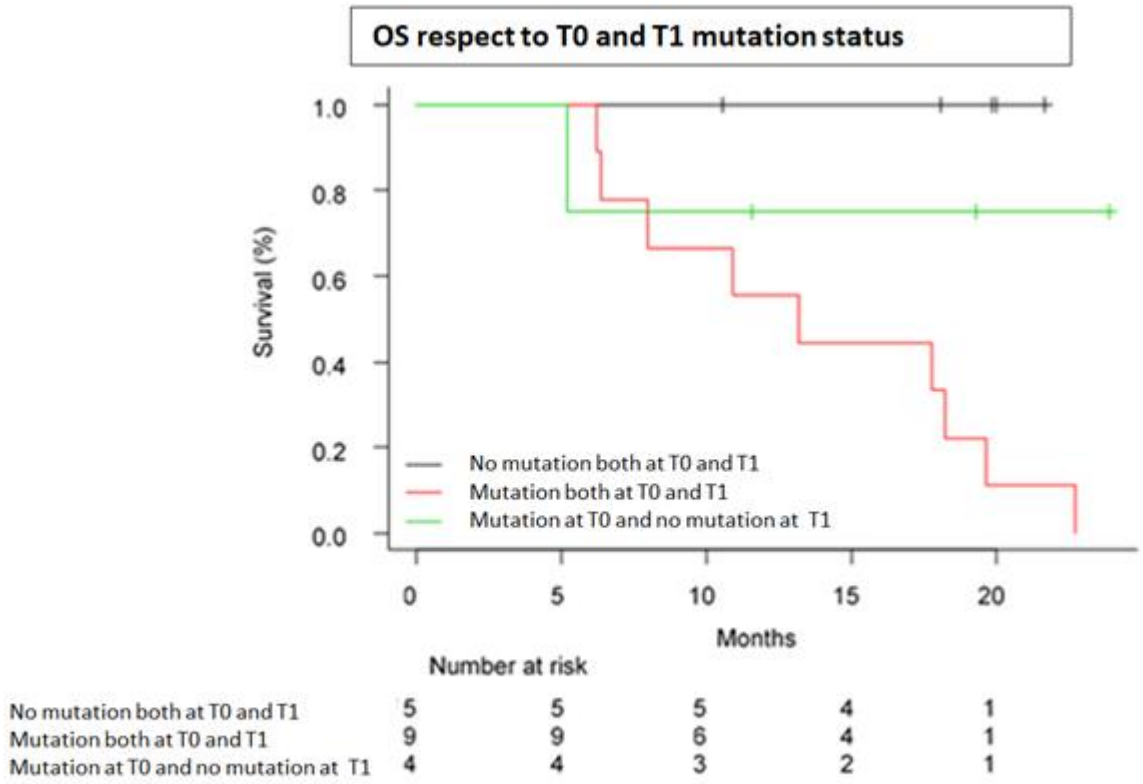


Figure 7 Kaplan-Meier Overall survival (OS) curves for patients treated with target therapy respect to EGFR mutational status detected at T0 and T1

DISCUSSION

Onset of drug resistance in NSCLC EGFR mutated patients undergoing first line tyrosine kinase inhibitors treatment usually occurs between 9 to 12 months of therapy [22], which leads to the need to follow as closely as possible the evolving of the disease. The patients selection focused on advanced or locally-advanced NSCLC stages and confirmed presence of EGFR mutation on tissue at baseline. Patients had to consent to one or more blood withdrawal in addition to blood test routine, this limitations led to an accrual number of only 30 patients. Nevertheless the cohort

reflects accurately NSCLC EGFR mutated patients as far as clinical and histopathological characteristics, smoke habit, gender and median OS and PFS.

The distribution of the mutations' frequency and the sensitivity, understood as the detection rate of the liquid biopsy for the mutational structure, were comparable to literature data (0.61; 0.50-0.71, 95% CI) [14,15]. There was a reduced relationship between the mutation present at baseline on the surgically excised tissue and whose identified in the plasma. An hypothesis that could possibly explain this difference lies in the therapeutic choice, namely the surgery itself. The absence of mutation at the cfDNA plasma analysis, suggests the disease has been eradicated by surgery; therefore decreasing the number of tumor cells responsible for releasing the tumor DNA into the circulation at the point that they are undetectable. Furthermore, patients not eligible for surgery have generally higher stage disease (metastatic stage in 18 out of 23 patients); this is an indication of a more aggressive disease and higher tumor burden that can justify the higher concordance between basal and liquid mutation detection in respect to analysis performed on surgical samples. Non detection of mutation on cfDNA is associated also with type of mutation. Exon 19 deletion is confirmed at T0 on 12 over 14 baseline-samples; mutation on exon 21 is confirmed only on 5 out of 14 baseline-samples; mutation on exon 20 is confirmed in 1 over 2 cases. This data show that patients carrying exon 21 mutation have a higher probability of mutation-negative status on plasma than patients carrying exon 19 or 20 mutation (64.3% versus 14.3% and 50% respectively). In the meta-analysis by Mao et al. however, no differences emerge in the subgroups of analysis regarding the concordance between mutations of exons 19 and 21. Differences in type of mutation distribution may lay in the methodology used; majority of studies report data using next generation sequencing (NGS) a method that still needs to be validated but is being implemented because it may allows a more efficient and automated handling of the samples.

The evaluation of the second plasma sample allows the evaluation of the response to treatment and the possible insurgence of any mutations responsible for resistance to therapy. All patients for which EGFR mutation was identified in plasma assessment both at time 1 and time 2 are in a state of disease progression (PD), while the percentage of progressive disease falls both for patients who have preserved a non-mutated profile (33.3% of PD) and for those who had a first mutated profile but changed to non-mutated in the second sample (25% of PD). In 2 patients, it was identified the positivity to the T790M mutation of exon 20, a factor involved in the mechanisms of resistance to anti-EGFR generation I and II drugs and that makes the patient eligible for treatment with an anti EGFR-TKI of third generation, osimertinib. Negativization of mutation indicates a response to treatment which also involves an improvement in survival, however the cohort numbers are limited in order to be able to demonstrate this trend with strength.

In terms of PFS, patients who change their mutational profile (on LB) from mutated to non-mutated had a worse prognosis than the patients whose mutation is not detected in T0 and T1 but a better prognosis in respect to those that maintain the mutation over time. Due to the small samples size of the analyzed cohort, the results were not statistically significant; however, patients with both T0 and T1 EGFR mutations showed a trend for worse progression-free survival compared to patients without EGFR mutations detected on ctDNA ($p=0.14$, Log-Rank Test). Similarly, the detection of a plasma mutation held a negative prognostic value in terms of OS. Indeed patients whose mutation is detected at both plasma timepoint registered the lowest OS (55.6%, 95% CI, 20.4%-96.1%). All of the patients that maintained a status of WT on liquid biopsy are alive at time of analysis. This preliminary study showed that the non-detection of EGFR mutation by LB during target treatment foster a longer survival, and that the earlier the negativization of the mutation, the better the prognosis.

Therefore, even with the limitation due to the small number of patients included in this preliminary analysis, the negativization of the EGFR mutation evaluated by liquid biopsy at a second plasma sampling seems to coincide with a prognostic improvement.

CONCLUSION and FUTURE PERSPECTIVE

The results obtained and the high concordance between cyto/histological and liquid evaluation (60%, 95% CI: 41.0%–77.0%) described in this study allow us to state that liquid biopsy (LB) today is a valid technique to use in clinical routine. At the present time, international guidelines allow prescription of first line EGFR-TKI therapy based only on LB in those patients for whom a surgical procedure or biopsy is infeasible, or the amount of tumor tissue is scarce [20].

The status and modification of EGFR mutations and the occurrence of new mutations related to drug resistance during therapy with anti-EGFR TKI drugs determined on cfDNA showed a strong correlation with PFS and OS. Indeed, the negativization of the plasma mutation was consistent with a longer PFS and so with a better prognosis. If this data would be confirmed on a larger scale; monitoring EGFR mutation via liquid biopsy in advanced stages NSCLC patients, could give the clinician a prediction on treatment response, disease progression and survival itself, fostering a modified scheduled monitoring based on plasma mutational status. Therefore, we endorse the use of cfDNA EGFR liquid biopsy as a validated instrument of clinical significance.

There is a growing interest and development in the use of molecular and genetic analyses within the diagnostic and therapeutic path. The healthcare system must be prepared to integrate modern DNA study techniques and digital PCR to guide everyday clinical practice.

Following our encouraging preliminary results, a schedule of blood sampling is being implemented for all NSCLC patients undergoing EGFR TKI therapy. The monitoring will also hopefully allow

the early identification of resistance-mutation onset. Switching to a more appropriate therapy will spare the patients useless and potentially invalidating adverse reactions, bettering the compliance and, ultimately, validating the cost-benefit ratio of therapeutic choice.

CHAPTER 2

PREDICTIVE AND PROGNOSTIC ROLE OF Δ KI67 IN LUMINAL BREAST CANCER

ABSTRACT

A key tool for monitoring breast cancer patients under neoadjuvant treatment is the identification of reliable predictive marker. Ki67 has been identified as a prognostic and predictive marker in ER-positive breast cancer.

90 ER-positive, HER2 negative locally advanced breast cancer patients received letrozole (2,5 mg daily) and cyclophosphamide (50 mg daily) with/without sorafenib (400 mg/bid daily) for six months before undergoing surgery. Ki67 expression and tumor size measured with caliber were determined at baseline, after 30 days of treatment and at the end of treatment. Patients were assigned to a clinical response category according to RECIST criteria, both at 30 days and before surgery and further classified in high responder and low-responder according to the median variation of Ki67 values between biopsy and 30 days and between biopsy and surgery time. The predictive role of Ki67 and its changes with regards to clinical response and survival was analyzed.

No differences in terms of survival outcomes emerged between the arms of treatment while we observed a higher percentage of women with progression or stable disease in arm with the combination containing sorafenib (20.5% vs 7.1%, p=0.06). Clinical complete responders experienced a greater overall variation in Ki67 when compared to partial responders and patients with progressive/stable disease (66.7% vs 30.7%, p=0.009). High responders showed a better outcome than low responders in terms of both disease-free survival (PFS, p=0.009) and of overall survival (OS, p=0.002).

ΔKi67 score evaluated between basal and residual tumor at definitive surgery showed to be highly predictive of clinical complete response, and a potential parameter to be used for predicting DFS and OS in luminal BC treated with neoadjuvant endocrine-based therapy.[23]

INTRODUCTION

Neoadjuvant systemic therapy (NST) has become a mainstream approach in breast cancer treatment. Aside from helping the achievement of disease local control with breast conserving surgery, NST allows prompt evaluation of tumor response and guides therapy adjustments accordingly. Furthermore, NST allows to test new therapeutic compounds and to monitor the impact of treatment on biological, molecular and pathological characteristics of the tumor, thus providing invaluable information on the mechanisms of action of anticancer drugs [24-27]. Monitoring the treatment response allows to assess if the cytotoxic treatment is effective in increasing the Disease Free Survival (DFS) and the Overall Survival (OS) to provide information on the mechanisms of action of the anticancer drugs and to identify intermediate endpoints of treatment response [28].

Pathological complete response (pCR), Ki67 tumor expression value and the changes induced by treatment as well as SUV variation on PET-TC have been identified as potential surrogate endpoints of treatment efficacy, i.e. observational variables that can replace the true outcome of interest in clinical studies and routine [28]. Moreover, recent studies have indicated that Ki67 and pCR in NST are independent predictor of DFS and OS [29-31].

NST in breast cancer was originally limited to locally advanced inoperable disease but has been extended first to operable disease and later to earlier-stage tumors [32-33]. Therapeutic strategy strongly depends on molecular classification; ER status is the most successful predictive biomarker

for endocrine therapy. A number of clinical trials in the recent years evaluated the efficacy of Aromatase Inhibitors (AIs) over Tamoxifen. In the neoadjuvant setting; AIs have shown to be more effective than tamoxifen, with a response rate between 40 to 60% [34-36]. Among the AIs, letrozole (Let) showed an overall response rate of about 80% [37]; additionally, 12 months letrozole based-therapy resulted more effective in overall response and complete response than 4 or 8 months therapy [38].

In an integrated approach setting, hormonal therapy may cause a reduction in cell proliferation and this may be counter-productive to chemotherapy, which has an effect on high proliferating cells. This "issue" can be bypassed by administration of chemotherapy in a metronomic regimen (LDM) [39]. The combination of letrozole and metronomic cyclophosphamide (Cyc) has already been reported by Bottini *et al*. in a phase II study safely conducted on elderly breast cancer patients [37]. In view of the action of LDM on the endothelial vasculature, Bazzola et al. hypothesized a synergism with other anti-angiogenic drugs and designed a phase II study to address this question, comparing the combination of letrozole and cyclophosphamide with Let-Cyc plus sorafenib, a serin-threonine kinase, RAF-1 inhibitor with anti-angiogenic activity, in breast cancer patients [40]. The triplet combination was well-tolerated and effective in reducing tumor size, Ki67 and VEGF-A [40].

As these promising results warranted further studies, we conducted a study evaluating the combination of letrozole, metronomic administration of cyclophosphamide and sorafenib with a focus on clinical response, on tumor proliferation and how they may affect the DFS and OS.

MATERIALS AND METHODS

Study design and patients selection

Following analysis were performed tissue of patients who participated in a phase III trial titled "Primary Systemic treatment with metronomic administration of Letrozole + Cyclophosphamide +/- Sorafenib in patients with hormone sensitive operable or locally advanced breast cancer, the FEN study". FEN is the acronymous to the three drugs of the combinatorial therapy: Femara® (letrozole), Endoxan® (Cyclophosphamide) and Nexavar® (Sorafenib). 90 postmenopausal women (mean age 66.6 ± 8.6) with ER positive, HER2 negative breast cancer were included in this prospective, open-label, single-center, randomized Phase III study. Eligible patients had T2-4, N0-N2, M0 breast cancer, uni-dimensionally measurable by objective examination according to RECIST criteria (Response Evaluation Criteria in Solid Tumors), and performance status 0-2 according to ECOG (Eastern Cooperative Oncology Group). Women were randomly assigned 1:1 to receive Let 2,5 mg daily and metronomic oral Cyc 50 mg daily with (arm B; n=45) or without (arm A, n=45) sorafenib 400 mg/bid daily for six months before undergoing surgery.

Written consent was provided by each participant. The study was approved by the Val Padana Ethics Committee (Eudract Number 2007-006208-39). The study was prematurely closed due to an unexpected high number of progressions in Arm B (with sorafenib); from the ethical point of view it was decided to interrupt the recruitment.

Proliferation Index

Proliferation index was tested with the KI67 expression; Ki67 was evaluated by immunohistochemistry at three different time-points. Tissue was obtained from patients from an incisional biopsy performed at presentation, from tru-cut biopsy performed after 30 days of

treatment and at definitive surgery. Immunohistochemistry was performed on paraffin-embedded tumor samples; Ki67 staining was performed using standard protocols as described in a previous article [41]. Briefly, an antigen retrieval step was performed by heating a tissue section in a citrate buffer. The primary antibody applied was mouse monoclonal Mib-1 (Dako, Glostrup, Denmark), dilution 1:30, 1 h incubation at RT; biotinylated horse anti-mouse IgG and avidin–biotin–peroxidase complex were applied as a staining method (Vectastatin ABCkit; Vector Laboratories, Inc, Burlingame, CA). A solution containing hydrogen peroxide (0.06% v/v) and diamino-benzidine4 HCL (DAB; 0.05 v/v) was used as chromogen.

Percentage variation of proliferation index have been calculated as follows: ΔKi67(%) Short variation (baseline-30 days) = (Ki67 baseline − Ki67 30 days/ Ki67 baseline) × 100; ΔKi67(%) Intermediate variation (30 days-surgery) = (Ki67 30 days − Ki67 surgery/ Ki67 30 giorni) × 100; ΔKi67(%) Long variation (baseline-surgery) = (Ki67 baseline − Ki67 surgery/ Ki67 baseline) × 100.

Response assessment

Primary tumor size was measured with a caliber by a clinician at three time points: enrollment, after 30 days and at the end of treatment (before surgery). Early clinical response (eCR) - between baseline and 30 days - and pre-surgical clinical response - between baseline and surgery - was assessed according to RECIST criteria (version 1.1) [42]. Lesions were scored as follows: Complete Response (CR, disappearance of all target lesions), Partial Response (PR, decrease of ≥30% in the sum of the longest diameter of target lesions), Stable Disease (SD, does not meet the criteria for CR, PR or Progression Disease) and Progression Disease (PD, an increase in tumor size of ≥20% in the sum of the longest diameter of target lesions). Progression-free survival (PFS) and overall survival

(OS) were respectively defined as the time from the date of surgery to the date of appearance of metastasis or death and as the time from the date of surgery to the date of death by any cause [43].

Statistical analysis

Characteristics of the study population are described using means ± standard deviation or median and range (minimum-maximum values) for continuous variables, depending on the distribution's shape. Data were tested for normal distribution using the Shapiro-Wilk test. Categorical variables were summarized with absolute frequencies and percentages; cross-tabulations were generated to compare frequency distributions and Chi-square or Fisher Exact test, when appropriate, were used to assess possible associations.

Analyses were performed to test for differences among Ki67 median values at three different time points (baseline, 60 days, and at surgery) using Friedman test for paired data and the post-hoc analysis performed by the Wilcoxon test applying the Bonferroni adjustment for multiple comparisons. Differences among percentage variations of Ki67 at three different time points (short, intermediate and long) were evaluated by Kruskall-Wallis test for independent variables and post-hoc analysis with the Mann-Withney test applying the Bonferroni adjustment for multiple comparisons. The association between clinical response (total, partial, non-responding) with respect to continuous variables (Ki67, Ki67 variation) was assessed by Kruskall-Wallis test. DFS and OS were estimated using the Kaplan-Meyer method and differences between the curves were tested for significance by the Log-rank test. All statistical analyzes were performed using the R (the R Foundation for Statistical Computing; Version 3.0.3, library “survival”). A p-value less than 0.05 was considered statistically significant.

RESULTS

From 2009 to 2013, 90 women were enrolled onto the trial; 45 were randomly assigned to receive only LET-CYC (arm A) and 45 were assigned to receive LET-CYC plus Sorafenib (arm B). The trial was interrupted due to the occurrence of post-treatment progression but all patients completed the planned 6 months of therapy. Patients'characteristics enrolled into the trial are detailed in Table 1.

Variables	All Cohort (n=90) n(%)	Arm A (n=45)	Arm B (n=45)	p-value
Sex				
Female	90 (100)	45 (100)	45 (100)	1.00
Age				
≥ 60	70 (77.8)	8 (17.8)	12 (26.7)	0.31
<60	20 (22.2)	37 (82.2)	33 (73.3)	
Histology before surgery				
IDC	71 (78.9)	34 (75.6)	37 (82.2)	0.86
ILC	14 (15.6)	8 (17.8)	6 (13.3)	
IDC+ILC	2 (2.2)	1 (2.2)	1 (2.2)	
Others	3 (3.3)	2 (4.4)	1 (2.2)	

Grading before surgery [a]				
G1	2 (2.4)	0 (0.0)	2 (4.7)	0.28
G2	46 (54.1)	25 (59.5)	21 (48.8)	
G3	37 (43.5)	17 (40.5)	20 (46.5)	
Ki-67 before surgery				
≤ 20%	62 (68.9)	32 (71.1)	30 (66.7)	0.65
> 20 %	28 (31.1)	13 (28.9)	15 (33.3)	
Molecular Profile before surgery				
Luminal A	51 (56.7)	26 (57.8)	25 (55.6)	0.83
Luminal B Her2-neg	39 (43.3)	19 (42.2)	20 (44.4)	
Type Surgery [a]				
Conservative	64 (72.7)	30 (68.2)	34 (77.3)	0.34
Mastectomy	24 (27.3)	14 (31.8)	10 (22.7)	

Clinical Response at end of neoadjuvant treatement [a]				
CR	48 (55.8)	22 (52.4)	26 (59.0)	0.06
PR	26 (30.2)	17 (40.5)	9 (20.5)	
SD/PD	12 (14.0)	3 (7.1)	9 (20.5)	
Histology after surgery [a]				
IDC	65 (73.7)	33 (75.60)	32 (72.7)	0.99
ILC	16 (18.2)	8 (18.2)	8 (18.2)	
IDC+ILC	2 (2.3)	1 (2.3)	1 (2.3)	
IN SITU	2 (2.3)	1(2.3)	1 (2.3)	
Others	3 (3.4)	1 (2.3)	2 (4.6)	
pT [a] *after surgery*				
Tis	2 (2.3)	1 (2.3)	1 (2.3)	0.99
< 1 cm	14 (16.3)	7 (16.3)	7 (16.3)	
1-2 cm	45 (52.3)	22 (51.2)	23 (53.3)	
≥ 2 cm	25 (29.1)	13 (30.2)	12 (27.9)	

pN [a] *after surgery*				
N0	44 (50.0)	20 (45.5)	24 (54.6)	0.39
N+	44 (50.0)	24 (54.6)	20 (45.5)	
Ki67 after surgery [a]				
≤ 20%	80 (96.4)	40 (95.2)	40 (97.6)	0.57
> 20 %	3 (3.6)	2 (4.8)	1 (2.4)	
Grading after surgery [a]				
G1	3 (2.4)	1 (2.3)	2 (4.6)	0.45
G2	53 (54.1)	29 (67.4)	24 (54.5)	
G3	31 (43.5)	13 (30.2)	18(40.9)	
Molecular Profile after surgery [a]				
Luminal A	28 (33.3)	14 (33.3)	14 (33.3)	0.84
Luminal B Her2-neg	53 (63.1)	26 (61.9)	27 (64.3)	
Luminal B Her2-pos	3 (3.6)	2 (4.8)	1 (2.4)	

Adjuvant Chemotherapy (after surgery)				
Yes	32 (35.6)	14 (31.1)	18 (40.0)	0.51
Adjuvant Hormonotherapy (after surgery)[a]				
Yes	81 (95.3)	39 (90.1)	42 (100.0)	0.12

Table 1 Patients'characteristics. [a]Numbers do not add up to the total due to missing values. CR= complete response, PR= partial response, SD/PD= stable disease/progressive disease, IDC= invasive ductal carcinoma, ILC= invasive lobular carcinoma

Treatment response

Data on early clinical response (after 30 days of therapy) was available for 77 of the 90 patients: only one patient registered a complete response (1,3%), 22 patients had partial response (28,6%) and 54 had either clinical stable disease or clinical progression (70,1%). The arm of treatment did not significantly influence early clinical response (Pr = 0.71, Fisher Exact test).

At the end of treatment assessment, clinical response data was available for 86 patients, 4 patients were missing either basal or post-treatment assessment. None of the 86 patients showed a complete pathological response, however 55.8% had a complete clinical response (n= 48), 30.2% had partial clinical response (n=26) and 14.0% had stable disease or clinical progression (n=12, Table 1). A greater number of patients in arm B experienced disease stability or progression (p = 0.06, Chi-Squared Test, Table 1). Even if sorafenib-treated women had a median age greater than the control arm (69.2 vs 63.8, p=0.003), age was not associated with early or late clinical response (p=0.40,

One-Way Anova, p=0.15, student t-test respectively). Tumor classification at diagnosis (Luminal a or Luminal B type) was not associated with pre-surgical clinical response (p=0.94).

Early and Pre-surgical clinical response

Treatment response changes significantly between early (30 days) and pre-surgical evaluations (p<0.001, Stuart Maxwell Test for paired data). Among the 75 patients for whom data are available, 25 women (33,3%) who had eSD/ePD and 15 with ePR (20%) registered a complete response at the end of treatment. 17 patients (22,7%) had an improved response (from eSD/PD to PR), 17 patients (22,7%) maintained clinical response between 30 days and end of treatment evaluation, whereas only one woman (arm B, 1,3%) worsened her response, going from early partial response to stable/progressive disease.

Treatment response rate at 30 days and end of treatment was statistically correlated with the treatment arm (p<0.001, Stuart Maxwell Test for paired data). Clinical response classification between 30 days and end of treatment was not changed for 16,7% of patients in arm A and 28,2% of patients in arm B. A greater percentage of patients in arm A experienced an improvement of clinical response (83,3% VS 69,2%) even if this difference didn't reach statistical significance (p=0.27, Fisher Exact Test).

Change in clinical response during and after treatment was evaluated in relation to the Ki67 variation. Our population was divided in three groups: no change between early and pre-surgical response, change to complete response and change to partial response; the patient who progressed was excluded from the statistical evaluation. A greater variation of Ki67 between basal and 30 days was observed in the patients who achieved a complete response from PR/PD-SD at 30 days (p=0,625), however the comparison between the three groups is not statistically significant (p=0.11,

Kruskall Wallis Test). The comparison between the two groups with an experience of tumor change (to complete response or to partial response) resulted in a difference at the limit of statistical significance (p = 0.05, Mann Withney test).

Survival analysis

Survival analysis was performed on 79 women (11 excluded: 4 due to lack of information on the clinical response and 7 due to lack of information on the follow up). Median follow up was 55.6 months and 8 deaths by any cause occurred.

There were not any significant differences between arms of treatment in terms of DFS (p=0.84) and of OS (p=0.74).

Clinical response and survival

There are no significant differences in terms of DFS and OS with regard to early clinical response but survival analysis according to pre-surgical clinical response was performed and revealed significant differences between groups (p=0.015 log rank test, figure 1). Survival at 60 months (5 years) was significantly greater in women with clinical complete response than in partial responders and patients with stable or PD: 98% vs 66% of women with PR and 65% of women with stable or progressive disease. Five deaths occurred during the evaluation period and 12 women had progressive disease, 3 of which died, for a total number of events of 17.

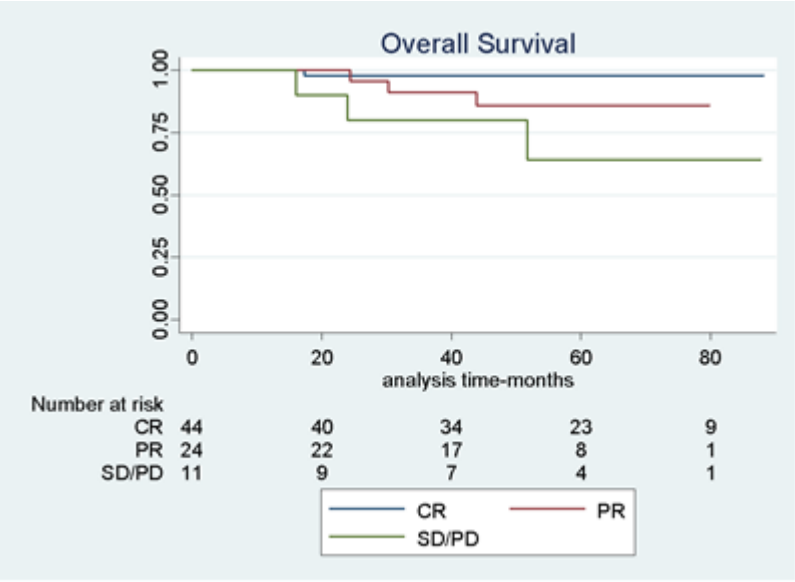


Fig 1 Overall survival (OS) and Disease Free Survival (DFS) according to clinical response (RECIST criteria). CR= complete response, PR= partial response, SD/PD= stable disease/progressive disease

DFS was also evaluated in relation to clinical response. 21 events occurred: 19 patients progressed and 2 died without prior recurrence. Although no significant differences were registered between clinical response groups (p=0.10), patients with SD or PD experienced recurrence early if compared to patients with CR (Figure 1). Indeed DFS at the 2-year time point was 95% in the CR group and 60% in the SD/PD group.

Proliferation index: correlation with clinical response and survival

Proliferation index values decrease significantly in both arms of treatment ($p<0.001$) but no statistically significant differences were observed between the two arms ($p = 0.39$, linear mixed effects model for repeated measurements (figure 2). Overall, the Ki67 values comparison before and after therapy shows that the number of patients with high proliferative index (values of Ki67 > 20%) decreases significantly (28 pre VS 3 post), while the number of patients with ki67 ≤20% increases from 61 to 80 ($p < 0.001$ Chi square test). Ki67 expression was available at baseline for

90 patients, at 30 days time-point for 49 patients and at surgery for 83 patients. Ki67 values decreased significantly (p<0.001 Friedman test) between Baseline [median value 16.5 (2-70)] and 30 days [median value 6 (0.9-50)] and between baseline and surgery [median value 5 (0-30)] (Figure 3). Reduction between 30 days and surgery was not statistically significant (p=0.3 Wilcoxon test adjusted for multiple comparisons).

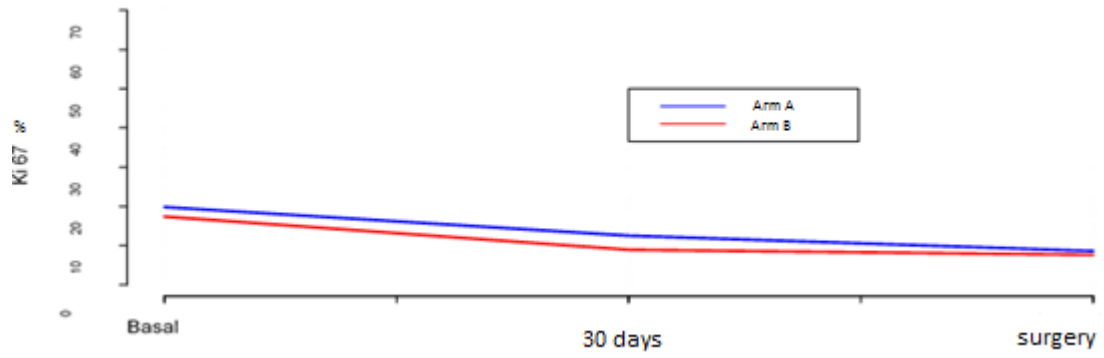


Fig 2 Plot of the Ki67 mean values at different times according to arm of treatment. Arm A received Letrozole + Cyclophosphamide, Arm B receive Letrozole, cyclophosphamide and Sorafenib.

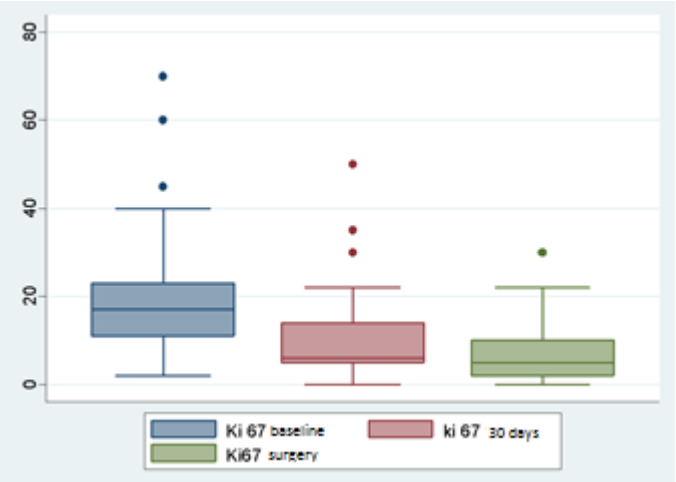


Fig 3 Ki67 values at different time point

Ki67 values and percentages variations were evaluated in relation to early and pre-surgical CR. Early response was not associated with proliferation index variation, irrespective of the time of evaluation (baseline, 30 days and surgery). Conversely, Ki67 values at surgery are significantly lower in clinical responders (CR) in comparison to SD/PD patients (p=0.008). Therefore, percentage variation of Ki67 between baseline and surgery was evaluated in relation to late clinical response. Patients who had a complete clinical response registered a considerably greater Ki67 variation if compared to patients who had SD or PD (66.7% vs 28.0%, p=0.003, Mann Withney test) but no differences were noted between PR and SD/PD (p=0.09) nor between CR versus PR (p=0.11).

To evaluate the impact of ki67 changes on survival, we chose the median percentage of variation of ki67 between time-points as a cut-off to define high versus low responders. For the early variations we chose ΔKi67 50%, for pre-surgical variations we chose ΔKi67 60%. At first early variations were explored and the population was divided into early low responders ΔKi67 < cut-off value, n=24) and early high responders (ΔKi67≥cut-off value, n=25). Results showed no statistical difference in terms of DFS and OS (p=0.76, log-rank test both; data not shown). Thereafter, we focused on pre-surgical clinical response and ΔKi67(%) Long variation (baseline-surgery). 36 patients were classified as low responders ΔKi67 < cut-off value) and 44 as high responders (ΔKi67≥cut-off value). At 5 years, DFS was significantly longer in the group with the highest variation of Ki67: 92% (95% CI:77%-97%) in the high responders group versus 60% (95% CI: 41%-75%, p=0.002 Log Rank test, figure 4) in the low responders. Similarly in terms of OS, high response group had a better prognosis in comparison to the low response group (p=0.009 Log-Rank test, figure 5), and OS at 5 year was 92% (95% CI: 77%-97%) in the high response group and 60% (95% CI: 41%-75%) in the low response group.

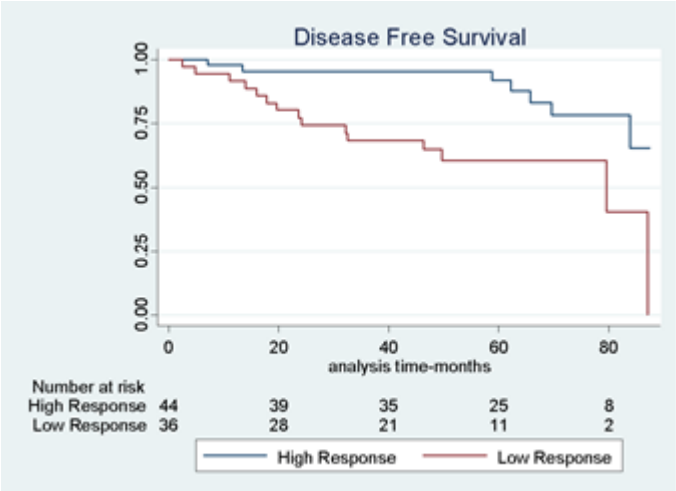


Fig 4 Disease free survival (DFS) according to ΔKi67 between baseline and surgery. Low response (ΔKi67 <60%) N=36, High response (ΔKi67 ≥ 60%) N=44.

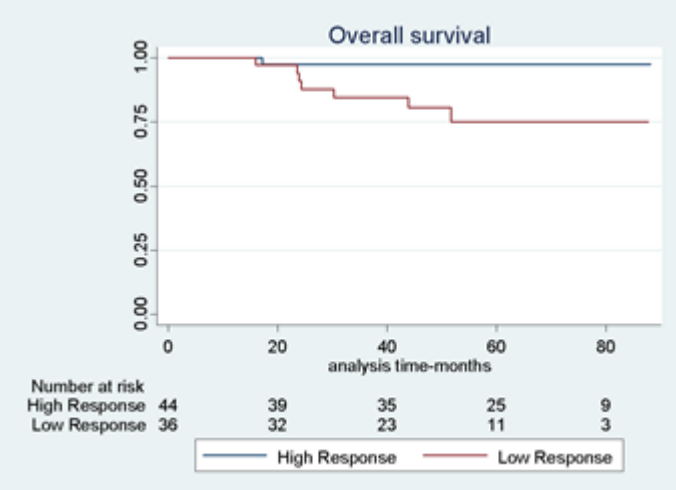


Fig 5 Overall survival (OS) according to ΔKi67 between baseline and surgery. Low response (ΔKi67 <60%) N=36, High response (ΔKi67≥ 60%) N=44

DISCUSSION

Monitoring treatment response has become a key factor in managing cancer patients and this is even more true in the neoadjuvant setting [27]. While endocrine therapy represents the most tailored NST for women with ER-positive breast cancer, previous studies have suggested that response rate and survival could be improved in post-menopausal, luminal breast cancer patients with the concomitant administration of metronomic chemotherapy (CYC) [37,39,40]. In our study, we explored the efficacy of the LET-CYC combination with or without sorafenib, a serine-threonine kinase inhibitor that has shown anti-angiogenic activity due to the interaction with VEGFR-2 and PDGFR-β. None of the patients enrolled in the study achieved a pathological complete response (pCR). Usual characteristics associated with increased pCR rate are age < 40 years, high expression of Ki67, ER-negative, triple-negative subtype, HER2 positive disease, ductal histology, high nuclear grade tumors [44] and these features are not well represented in our study population. This fact could perhaps explain the low pCR rate. Pathologic complete response is not often achievable with neoadjuvant systemic therapy (NST) especially in luminal breast cancer, but it is reported that even a reduction in tumor size affects the clinical response. In our trial, more than half of the enrolled patients had a complete clinical response (55,8%). The experimental combination of Sorafenib plus LET-CYC did not demonstrate superiority in comparison to LET-CYC alone; however, a higher number of non-responders (SD or PD) was identified in the sorafenib-treated group at the end of treatment, leading to the premature closing of enrollment.

Early assessment of clinical response (eCR, after 30 days of therapy) showed no sufficient correlation with survival endpoints (DFS and OS, data not shown), nor with proliferation index and its percentage variation. Nevertheless, eCR was a useful intermediate tool to determine disease status and treatment efficacy. Statistically, significant differences were noted between the arms of

treatment ($p<0.001$, Stuart Maxwell Test for paired data). Overall, 53,3% of the evaluable patients with ePR, eSD or ePD achieved a complete clinical response after treatment, 22,7 % improved their response between early and pre-surgical assessment, 22,7% maintained it and only one patient (arm B, 1,3%) worsened the response. The improvement of clinical response was more evident in arm A if compared to arm B ($p=0.27$, Fisher Exact Test).

The right Ki67 cut-off is currently still being debated, with values ranging from the 12% to 25% [45]. The accepted threshold value according to the latest indications from St Gallen expert panel is 20% [29]: beyond this value the tumor is considered proliferative and consequently more aggressive. Proliferation index was assessed at three time-points allowing for monitoring throughout the whole treatment period.

Neoadjuvant treatment significantly lowered the quote of proliferating cells affecting Ki67 measurements as only 3 patients had Ki67 greater than 20% at the end of treatment. In more detail, we found the most significant variation of Ki67 between the first 30 days of treatment and till the end of treatment ($p<0.001$ Friedman test), whereas there was not a significant differences between measurements at 30 days and pre-surgery ($p=0.3$ Wilcoxon test adjusted for multiple comparisons). The establishment of a Ki67 decrease trend in the first treatment period preludes to a clinical improvement over the entire period (change from ePR/ePD/eSD to CR, $p=0,625$). Therefore, it seems that a decrease with greater slope in the first 30 days may represent a valid predictive indicator of treatment response, even in the presence of a non-complete eCR. Patients with luminal breast cancer with a relatively low to mid risk disease, as in our study, benefit from this early response prediction, as it increases the chances of a conservative surgery.

Percentage decrease of proliferation index is strongly associated with clinical response: the greater the Ki67 variation the greater the probability of clinical complete response after treatment. In fact

patients who performed worse in terms of clinical response (PD/SD patients) recorded the lower percentage variation of Ki67. These results suggest that strict monitoring of the proliferation index could help the clinician with first-hand information on therapy efficacy [46]. Furthermore, achieving a clinical complete response also fosters longer OS, as demonstrated by survival rate: after 5 years 98% of women with complete response are still alive. Even if this data is not statistically significant, patients with CR tend to relapse or progress later than SD/PD patients: DFS at 5 years is 95% for CR vs 60% in SD/PD. Given the correlations between proliferation index, clinical response to treatment and survival, in our study we calculated the ΔKi67 between baseline and end of treatment and used the median value to discriminate between high and low-responder patients. High responders recorded a significantly longer DFS (p=0.009 Log-Rank test, figure3), meaning that a decrease of Ki67 between baseline and end of treatment greater than 60% lengthens recurrence time. ΔKi67 showed consistency not only as a predictive factor but also as a prognostic marker for HR-positive patients, as confirmed in literature [47]. High Ki67 value on residual tumor after treatment, rather than at baseline, has a negative prognostic value, as patients record a higher distant metastasis recurrence rate and poorer DFS and OS [48]. In our study the decrease of the proliferation index is linked to a better prognosis (p=0.002 Long-rank test, figure 2), as previously reported by von Minckwitz et al [49], with a 5-year survival rate 32% greater in high responders than in low responders. Proliferation index on residual tumor holds inarguably a prognostic importance but our results suggest that a greater attention should be given to the percentage of reduction of Ki67, rather than focusing merely on a fixed value. In the era of personalized medicine, this would certainly be a more comprehensive and patient-oriented approach.

Therefore, even in the presence of a pathological residue, a reduction in the proliferative index indicates a less aggressive tumor, a more stable response over time with a longer survival.

CONCLUSION

Neoadjuvant hormone-based treatment has shown a clinical and biological activity with a 55,8% complete clinical response overall in our study. Clinical complete response correlates with a lower risk of disease progression and a greater overall survival, and 5-year survival was improved in complete responders. Cellular proliferation measured by Ki67 levels can be safely used as an predictive clinical marker. Moreover ΔKi67 between baseline and surgery time demonstrated both a predictive and prognostic value as it allows to discriminate between responders and non-responders and correlates with a better outcome in terms of both DFS and OS.

CHAPTER 3

METABOLIC RESPONSE USING 18 FDG PET SUV VALUES AS NOVEL THERAPEUTIC GUIDE TOOLS IN PATIENTS WITH HORMONE-RESPONSIVE BREAST CANCER

INTRODUCTION

Monitoring response to treatments is a crucial aspect of disease management, particularly during neoadjuvant phase. Cytostatic treatments interfere with set pathways of the transformed cell and therefore particular attention is to be used to the biological asset of the tumor, that influences response and outcome.

The use of 18F FDG Positron Emission Tomography (PET) for initial staging of breast cancer has been debated in the past and levels of recommendations vary greatly between different guidelines [50]. Bernsdorf et al. found a substantial impact on initial staging and on clinical management in patients with BC, even in early-stages and with tumor dimensions $\geq$ 2 cm [51]. Endocrine and cytostatic treatments both interfere with uncontrolled proliferation of tumor cell, therefore combined therapies effects on cell metabolism of the chemotherapy can indirectly be monitored collecting Standardized Uptake Value (SUV) of 18F FDG PET: decreasing of SUV values would indicate pathologic response in breast cancer [52,53] thus confirming the validity of therapy, whilst an increase in this values could determine an higher risk of progression or therapy inefficacy, due to a greater biological aggressiveness of the tumor [54]. New strategies and technologies allow the researchers and the clinicians to strive for a better and more complete understanding of breast cancer complex evolution, an integrated and focused approach to the early disease could become the future of breast cancer disease management.

MATERIALS AND METHODS

Patients

This biological substudy dataset included 88 patients from the FEN trial, whose characteristics are described in chapter 2.

SUV values, metabolic and clinical response

PET imaging was performed at enrollment, after 30 days of therapy and at the end of treatment, before surgery. SUV values, tumor dimension (mm) and date of analysis were registered for each timepoint. Metabolic response was established as short response (ΔSUV1 %), intermediate response (ΔSUV2 %) and late response (ΔSUV3 %) and defined as follows: ΔSUV1 (%) = [(SUVbaseline - SUV30 days) /SUV baseline]x100; ΔSUV2 (%)= [(SUV30 days - SUVpre-surgery) /SUV30 days]x100 and ΔSUV3 (%)= [(SUVbaseline - SUVpre-surgery) /SUV baseline]x100.

Primary tumor size was measured with a caliper by a clinician at the same three time points: enrollment, after 30 days and at the end of treatment (before surgery). Lesions were scored according to RECIST Criteria: Complete Response (CR, disappearance of all target lesions), Partial Response (PR, decrease of ≥30% in the sum of the longest diameter of target lesions), Stable Disease (SD, does not meet the criteria for CR, PR or Progression Disease) and Progression Disease (PD, an increase in tumor size of ≥20% in the sum of the longest diameter of target lesions).

Statistical Analysis

Continuous variables were tested for normality with the Shapiro-Wilk test and are reported as median and range (min-max). Qualitative (categorical) variables are reported as absolute frequencies and/or percentages and compared with the Chi-Squared test or Fisher exact test whenever appropriate. Comparisons of SUV's values at three different time points (baseline, 30

days, and end of therapy) were performed using Friedman test for paired data and the pairwise post-hoc analysis by the Wilcoxon test (p-value adjusted with Holm method for multiple comparisons). Differences among percentage variations of SUV at the three different time points (short, intermediate and late response) were evaluated by Kruskal-Wallis test and post-hoc analysis with the Mann-Whitney test applying the p-value's Holm adjustment for multiple comparisons. The association between clinical response (complete response, partial, stable/progression) and with respect to continuous variables such as (SUV and, metabolic response) was assessed by Kruskal-Wallis test. SUV's variations over time of SUV's values respect to in accordance with the arm of treatment arm were evaluated with non-linear mixed-effects models (NLME) for repeated measures. Since age at diagnosis was normally distributed, association among age and clinical response was assessed through One-Way Anova

Statistical analyses were performed using R software version 3.5.0 (2018) The R Foundation for Statistical Computing, and a p value <0.05 indicates statistical significance.

RESULTS

SUV values were available for 88 patients. PET was performed at diagnosis (n=88), after 30 days of therapy (n=88) and before surgery (n=77) and values showed statistical differences ($p<0.001$). SUV decreased from diagnosis and 30 days time point ($p<0.001$) and from diagnosis and to end of treatment/pre-surgery ($p<0.001$). The reduction between 30 days and to pre-surgery was also statistically significant ($p <0.001$ Wilcoxon test adjusted for multiple comparisons). Results are summarized in Table 1.

Time-point of SUV evaluation	SUV Value Median (Range)	p-value
Enrollment (N=88)	3.55 (0.00-25.00)	<0.001
30 days (N=88)	2.30 (0.00-12.50)	
Pre-surgery (N=77)	0.00 (0-13.10)	

Table 1 Standardized uptake values (SUV) at different time-points: enrollment, after 30 days of therapy and at the end of therapy (pre-surgery)

While SUV values decreases significantly over time in both arms of treatment, there is no significant differences between arms (p=0.43).

Short, intermediate and late metabolic response were based on percentage variation between time periods, as described before. There is a statistically significant difference between time points groups, in particular both the intermediate and late response being greater than the short response (100% vs 43.75%, p <0.001) as well as the late response versus short response (p <0.001). No statistically significant differences were observed between intermediate and late response are observed (p = 0.09).

Clinical response according to Recist criteria was assessable for 86 over 88 patients as data of 2 patients were not complete. None of the patients registered a pathological complete response. 55.81% (n=48) achieved complete response (CR), 30.23% (n=26) were classified as partial responders (PR) and 13.95% (n=12) had stable or progressive disease (SD/PD). A greater percentage of progressive disease has been registered in arm B (20%) in comparison to arm A (7%). Median age at diagnosis was 67 and there was no correlation between this variable and clinical response (p=0.40).

Values of SUV uptake have been analyzed according to clinical response classification. The first evaluation, at enrollment time, revealed statistically significant differences between groups (p=0.02). Complete responders scored lower values; in particular CR SUV values differ from those of the SD/PD group (p=0.013, Mann Whitney test), while no statistically significant differences are observed between CR and PR patients (p=0.12) and between PR and SD/PD (p=0.21). After 30 days of therapy differences between the three groups are more evident (p=0.004). Post-hoc comparisons between CR and SD/PD and PR groups are statistically significant (p=0.01 and p=0.007 respectively); comparison between the PR versus SD/PD groups comparison did not show any statistically significant difference (p=0.30). At the end of therapy, at last time-point check before surgery, differences in uptake values differed significantly between CR, PR and SD/PD (p=0.012, Kruskal Wallis Test) (Figure 1). In particular post hoc analysis revealed a significant discrepancy in values between complete responders and women with stable or progressive disease (p=0.02) and between CR and PR (p=0.02) whereas differences between PR and SD/PD were not significant (p=0.34).

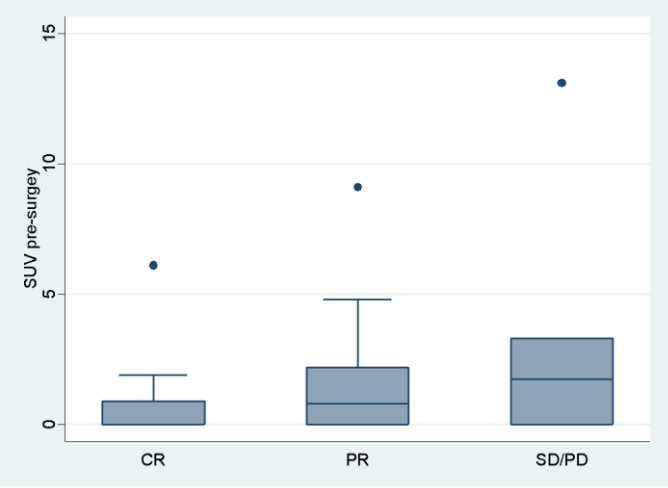


Figure 1 Standardized uptake values (SUV) according to clinical response at end of treatment. CR = complete response, PR = partial response, SD/PD stable disease/progression disease

Finally, metabolic response defined as percentage variation of SUV in the different time points was evaluated in relation to clinical response (table 2). Neither short response (ΔSUV1) nor intermediate (ΔSUV2), nor late metabolic response (ΔSUV 3) was predictive of a clinical response (p=0.42 , p=0.34 and p=0.11 respectively).

Metabolic response (%)	CR	PR	PD/SD	p-value
Short response Median (Min-Max)	45.55% (-189.47;100)	40.34% (0;100)	34.52% (4.76;100)	0.42
Intermediate response Median (Min-Max)	100% (0;100)	61.90% (0;100)	51.44% (-84.51;100)	0.34
Late response Median (Min-Max)	100% (5.26;100)	80.95% (26.47;100)	86.08% (-0.60;100)	0.11

Table 2 Metabolic response according to clinical response. CR = complete response, PR = partial response, SD/PD stable disease/progression disease

DISCUSSION

Breast cancer patients undergoing neoadjuvant systemic therapy can benefit from an early prediction of clinical response using SUV trends during treatment. The preoperative SUVmax of primary breast cancer can be considered a prognostic marker of recurrence [52] and several studies have also assessed the predictive potential of 18 FDG-PET and SUV variation in terms of pathological complete response.

Interim FDG-PET parameters are becoming more and more useful as a trustworthy technique that allows to evaluate solid tumor response to therapy and predict in part its future behaviour. In our study, the decrease of SUV values in response to therapy is a clear indicator of therapy efficacy

itself ($p < 0.001$ Wilcoxon test adjusted for multiple comparisons). The interim uptake values analysis is consistent with therapy response according to RECIST: in fact standardized uptake values (SUV) were significantly lower in clinical responders in comparison to patients who registered only a partial response or a clinical progression .

The longitudinal approach (PET-scan during therapy) is helpful in stratifying and characterizing patients accurately. Letrozole is a slow acting cytostatic drug and this aspect can influence the trend of SUV-percentage decrease of SUV, which in this case that become more evident after 30 days of therapy and reaches its highest at final control. Therefore metabolic response analysis should be tailored based on the drugs characteristics and administration regimen protocol administered, in order to be able to see the appropriate cytostatic effect.

CONCLUSION

The main limitation of this substudy is the low number of samples available for analysis, further validation are warranted on a larger number of patients.

Nonetheless it seems clear that SUV trends reflects the metabolic response to therapy. Clinical responders had a greater decrease of SUV over therapy period respect to partial and non responders. Therefore monitoring metabolic response could become a powerful predictive tool in early management of breast cancer.

CHAPTER 4

RNA DISRUPTION ASSAY AND DRUG RESPONSE IN EARLY BREAST CANCER

INTRODUCTION

Degradation of nonfunctional RNAs occurs in regulated stages in the cell in order to prevent aberration in protein synthesis and the others biological functions downstream. This process usually involves RNAses and other cofactors as polymerases, ubiquitynilases, helicases and support proteins, according to the specific RNA involved [55].

Cellular stressors, cytotoxic or proapoptotic factors have shown to induce an increase in ribosomal RNA degradation in a number of eukaryotic cell lines [56,57]. Therefore the activation of rRNA degradation in relation to cytotoxic chemotherapy should be investigated.

Ovarian and breast tumour cell lines were exposed to increasing doses of cytotoxic chemotherapy drugs, total RNA was isolated and northern blotting was performed to detect the origins of the degradation bands [55]. The percentage of apoptotic cells and effect of treatment on cell cycle progression was determined by flow cytometry, after propidium iodide and annexin V staining. Results show that all chemotherapy agents induced RNA disruption in the cell lines and that RNA disruption bands origin from the 28s rRNA. Disrupted RNA consistently reflected the above differential drug sensitivities, by displaying higher RNA Disruption Index (RDI) values and RNA disruption bands in drug-sensitive cells. Cytofluorimetric analysis indicated that exposure to cytotoxic drugs such as docetaxel resulted in concurrent induction of apoptosis and RNA disruption, as cells were found to be in early apoptosis, this data confirmed by cycle cell analysis because sub G_1 peak (associated with apoptotic bodies) increased according to time of exposure.

In a substudy of CAN-NCIC-MA.22 trial (NCT00066443), Parissenti et al. underlined the correlation between low tumour RNA integrity (RIN) and pathological clinical response (pCR), confirming the *in vitro* hypothesis [58]. In this study tumour biopsies were analyzed pre, mid-term and post neoadjuvant chemotherapy in 50 breast cancer patients. Levels of estrogen receptors (ER), progesterone receptors (PR), HER2 and topoisomerase 2 were assessed immunohistochemically and RIN was measured with capillary electrophoresis. They observed that mid-term lowest RIN was associated with high dose of chemotherapy and eventually with pCR, and that lower post-treatment RIN values were to be identified in PR+ tumours. This lead to the hypothesis that a better prognosis is to be associated with higher levels of disrupted RNA [59] .

Starting from this assumption it has been developed the RNA disruption assay (RDA) that quantifies the levels of RNA disruption and correlate the results with pCR and disease free survival (DFS). RDI was determined for each sample using a proprietary algorithm and levels of disrupted RNAs were stratified in three zones based on pCR [60]. RDI increasing was directly proportional to the increment of intensity of abnormal RNA bands and to concurrent decreasing of intensity of 18S and 28S bands. The mean RDI wasn't significantly different between pCR non-responders and pCR responders before treatment, but mean RDI value in mid-treatment were more than 2 fold higher in pCR responders than in pCR non-responders (p=0,005), this effect perpetuate also after treatment. Patients with low RDI didn't experience a pCR and had inferior DFS irrespective of histological subtype [61].

Based on the above observations RNA disruption seems to be commonly associated with chemotherapy-induced tumour cell death, quantifying RNA disruption early after initiation of therapy could be a novel biomarker of response, such that non-responding patients could be moved forward to downstream treatments, avoiding toxic effect of a fairly useless therapy [60]. However there's a need for validation of the clinical value of RDA and assessment of the relationship

between RDI and both mid-term or post treatment pCR and DFS. We investigated over a possible link between chemotherapy-induced RNA disruption and survival/progression.

Materials and Methods

Following analysis were performed on 40 biopsies of patients who participated in the FEN study, characteristics are detailed in chapter two. Biopsies were taken at baseline and 15 days after the beginning of the neoadjuvant therapy. Larger biopsies were cut into multiple pieces and the RNA isolated from the individual pieces. The RNA for each sample or subdivided sample was then assessed using the RNA Disruption Assay®. The maximum RDI value for each patient at day 15 was used for all analyses. The RDI values have been recently revised using version 8.1 of the RDA algorithm and clinical correlations have been updated with 5 year recurrence data.

Results

RDI values according to arm of treatment are detailed in table 2. Majority of patients had no evidence of disease at time of evaluation but 9 patients registered a recurrence (5 in arm A and 4 in arm B). Median RDI values was similar in both arms of treatment with an overall median RDI value of 2.4 (0.5-12.4).

RDI Value	Number of patients	Range of RDI values	Medium RDI values	PD	NED
Arm A	25	0.9-12.4	2.6	5	20
Arm B	15	0.5-6.4	2.2	4	11
total	40	0.5-12.4	2.4	9	31

Table 2 Maximum RDI values obtained from patients separated by drug treatment group. Arm A : letrozole+ Cyclophosphamide; arm B: Letrozole + Cyclophosphamide + sorafenib; PD: progression disease; NED: non evidence disease)

Maximum RDI value didn't appear to be dependent on drug regimen and varied from 0.5 to 12.4, suggesting that some patients were responding to treatment. Maximum cut-off RDI value of 2.4 was used to generate Kaplan Meier curves, because most of patients with RDI below this number had a recurrence (Fig 1).

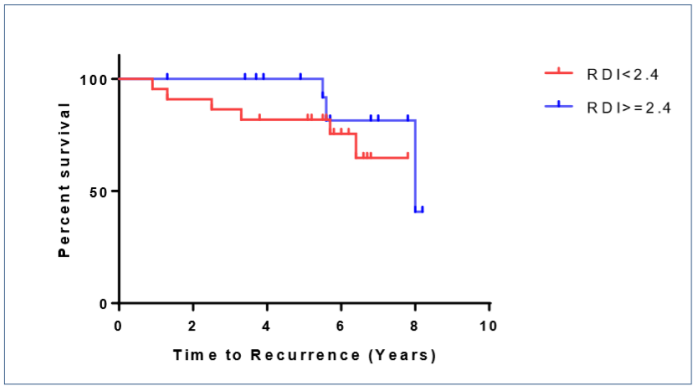
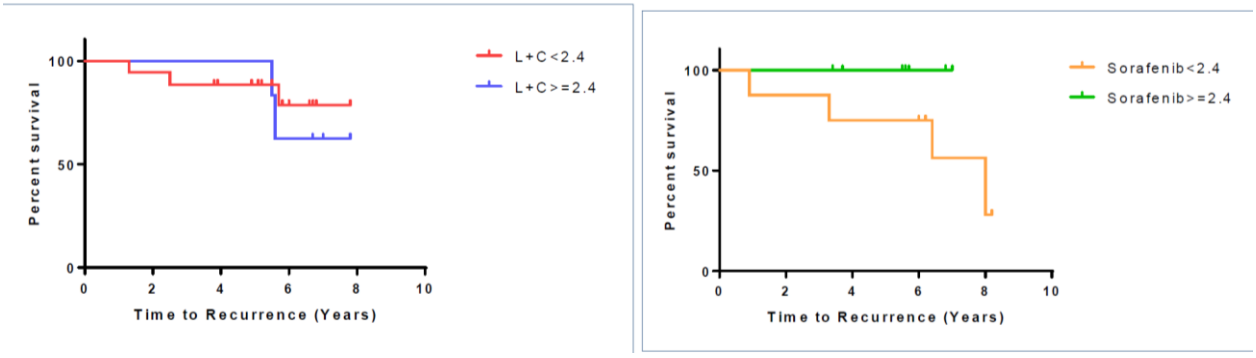


Fig. 1 time to recurrence (years) according to RDI values

Despite the low number of patients it appears that that patients with maximum RDI value equal or greater than cut-off have improved survival.

Survival curves were generated also separating patients for treatment group, confirming the tendency previously seen. (Fig 2A and 2b).



A B

Fig 2 Kaplan Meier curves using cut-off of maximum RDI ≥ 2.4 in patients treated with letrozole and cyclophosphamide (A) and in patients treated with letrozole, cyclophosphamide and sorafenib (B)

Discussion

Even though there aren't statistically significant differences between the two arms, an RNA Disruption Index greater than 2.4 has a positive prognostic value and this effect is more evident in the Sorafenib treated group. The main limitation is the low number of samples available for analysis; the RDI values may have possibly been affected by an internal resistance between the treatments (aromatase inhibitors reduce the quote of proliferating cells) or by the low cytostatic effect of letrozole at the 15 days post-therapy-timepoint. Aromatases maximum suppression is reached after 48-78 hours, but steady state between absorption/elimination establish during 2-6 weeks. It has been reported that Ki67 values are lowered even after 15 days of aromatase inhibitors treatment [62] and that such changes are predictive, but this period of time could not be sufficient to reach the cytostatic effect needed for the Rna Disruption Assay.

CHAPTER 5

ROLE OF IL-6 IN RESISTANCE TO NEOADJUVANT CHEMOTHERAPY IN BREAST CANCER

Cytokines are pleiotropic factors that regulate a number of pathway and their role in cancer development and tumor progression has yet to be defined. High circulating IL-6 family cytokine levels have been correlated with poor prognosis and tumor burden [63]. The aim of this study was investigating Il-6 levels and its potential role in determining patients who will benefit from endocrine-based treatment.

In our study serum of 32 breast cancer patients undergoing neoadjuvant letrozole based treatment with (N=16) or without (N=16) Sorafenib, before and after treatment, were analyzed via Multiplex Panel technology. Plasma serum of 32 patients at baseline were screened for 38 cytokines values via Bio-plex assay (panel 17-plex and panel 21-plex). Luminex platform (Biorad) is a bead-based color-coded multiplexed immunoassay system in a microplate format. The system can simultaneously detect many targets in a single sample (from plasma, fresh tissue or serum). Ligands specific to the target are coupled with the beads; after incubation a mixture of biotinylated antibodies is attached to the beads, followed by another time of incubation and addition of the fluorochrome (streptavidin). Intensity of fluorescence is converted into target concentration (pg/ml). 38 analytes (cytokines and growth factors) were simultaneously measured before and after treatment according to the arm of treatment. Clinical response at the end of the treatment has been assessed following Response Evaluation Criteria in Solid Tumor scale (RECIST). One patient was excluded from the analysis due to lack of informations.

The percentage of responders and non-responders were similar between two arms of treatment (p=0.88, Chi square test). First goal was to look for resistance/response markers, establishing for every cytokine any relevant difference between arms of treatment and then between responders and non-responders in arm B.

Cytokines analysed were divided into groups according to family (interleukin, chemokine, interferon, growth factors, Tumor necrosis factors) and according to principal function or pathway: Cancer stem cell markers, promotion of proliferation, anti/proinflammatory and prognostic value.

Even with a low number of cases analysed (10 B responders VS 5 B non responders) among others IL-6, IL-8, Stem cell growth factor beta (SCGF-b) and CD25 (IL-2Ra) had statistically significant (Wilcoxon non parametric test) different values between non responders and responders of sorafenib treated patients (arm B) (Table 1).

IL-6 in particular, a cytokine with anti-inflammatory activity, cancer stem cell marker and that has been previously linked with prognostic negative influence, had different expression values between A and B arm, therefore suggesting a resistance marker significance.

CSC MARKERS	RESPONDERS	PARTIAL/NON RESPONDERS	p-value
Hu IL-6	0.91 [0.01-3.92]	2.63 [0.52-161.82]	**0.12***

Table 1. Expression of Interleukin 6 (IL6) in Clinical responders and in partial/non responders of patients treated with letrozole, cyclophosphamide and sorafenib..

Overall, independently from treatment arm, at baseline, non-responders women showed a median level IL-6 of 3.92 (range [0.52-161.82] significantly higher than responders' level 1.9 [0.01-8.12](p=0.03, Mann-Whitney test). The Il-6 values comparison between pre and post treatment in all cohort of patients, revealed that treatment induced an increase of IL-6 (p=0.27, Wilcoxon test): in particular in non-responders, IL-6 values post treatment were higher than responders (medians

respectively 3.49 vs 1.39, p=0.07, Mann-Whitney test). However the evaluation of the percentage variation between baseline and post-treatment values (delta) showed a trend of decrease in non-responders (-25.9%) and of increase in responders (+11.8%).

In conclusion, Sorafenib did not affect the clinical response. High baseline levels of Il-6 promote it as a potential predictive marker of resistance but its role remains controversial and certainly deserves future insights.

CHAPTER 6

CLINICAL IMPACT AND PROGNOSTIC ROLE OF DISCORDANCE IN MUTATIONAL STATUS IN COLORECTAL CANCER

Introduction

Colorectal carcinoma is one of the most common cancers worldwide. A considerable proportion of patients may present with metastatic disease either at upfront presentation (synchronous with the primary) or following diagnosis and treatment of the primary tumor (metachronous) [64]. Generally, their optimal management includes surgical resection of the primary tumor and metastatic site [64]. However, most patients further progress and are candidate to chemotherapy plus targeted drugs. [65]. In this setting, it is well known that biomolecular characterization of disease is mandatory to better define the optimal combination of drugs. In particular K- and N-RAS wild type tumors are best candidate to first-line chemotherapy plus monoclonal antibodies targeting the epidermal growth factor receptor [66]. For this reason, several questions on the identification of the most appropriate tissue to analyze in synchronous or metachronous colorectal cancer are raised. In fact, although KRAS mutations are considered an early event in the colorectal tumorigenesis [67] and therefore a concordance in bio-molecular characteristics is assumed from primary and metastatic site, a discordance of RAS mutational status should be detected in a small percentage of patients [68]. Tumor heterogeneity, differences in technical methodologies and late acquisition or loss of KRAS mutations may explain this event [68]. However only few studies describe the impact of discordance in mutational status and clinical outcomes of synchronous or metachronous colorectal patients [69-71]. Therefore, the aim of this report is to investigate the presence of a correlation between concordance or discordance in mutational status from primary tumor and metastatic lesion in a cohort of patients treated for synchronous or metachronous metastatic colorectal cancer and their clinical outcomes.

Material and methods

Patients

The analysis was performed on paraffin-embedded formalin fixed tumour specimens of colorectal cancer (CRC) patients operated in our institution. All the samples were stored in the archive of the Institute of Pathological Anatomy of Trieste. The institutional review board approved the study, and patients provided informed written consent. K-RAS, N-RAS, B-RAF and PIK3CA mutations were investigated in both primary and metastatic lesion. Mutational status was determined by pathologists at our institution and clinicopathological information was collected from patients‘ charts.

Molecular Analysis

DNA was extracted from paraffin-embedded formalin fixed tumour of each tumour specimen. QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was performed for the extraction of DNA. After, DNA was amplified through multiplex real-time polymerase chain reaction (PCR) PCR followed by sequencing (Sequenom MassARRAY System; Sequenom, San Diego, CA, USA). Data were evaluated using MassARRAY Typer Analyser software 4.0, which allows to identify mutated alleles by comparing the ratio of the wild-type peak of all suspected mutants and to generate a specific report. KRAS, NRAS, BRAF and PIK3CA genes were identified for mutational status.

Statistical analysis

Numerical variables were expressed as median and range. Qualitative data were expressed as frequencies and organized into contingency tables; the concordance between mutational status of primary tumour and metastatic lesion was investigated by McNemar's test for paired data. Time dependent variables were calculated according to the Kaplan–Meier method. Differences in subgroup were investigated by long-rank test. The disease-free survival (DFS) was defined as the

time from the date of diagnosis to the progression of disease; the overall survival (OS) was defined as the time from the date of diagnosis to the death from any cause; patients who were still alive were censored. For the entire statistical analysis, the significance levels were established at $p<0.05$. All data were analyzed with STATA software (Statacorp version 14.2).

Results

Patients characteristics

From January 2015 to July 2018, we identified 178 patients with stage IV colorectal cancer (synchronous or metachronous) 21 of them were included in this analysis. Inclusion criteria were: diagnosis of stage IV CRC or a lower stage CRC that progressed in the period from January 2015 to July 2018, having mutational analysis of both primary and metastatic site. Baseline characteristics of patients are summarized in Table 1. The metastasis was synchronous in 11 patients (52%) and metachronous in 10 (48%). Metachronous adenocarcinomas were identified 8 months to 8 years after excision of the primary lesion. Primary tumour was colon in 15 (71%) patients and rectum or sigma rectum in 7 (33%) patients. Main site of metastasis was liver in 10 (48%) subjects, others were lung, peritoneum, bladder, brain and ovary.

Mutation analysis included K-RAS, N-RAS, BRAF and, when available PIK3CA. A patient was considered mutated if at least one of the three main mutation occurred. A mutation in primary tumour was found in 17 (81%) patients while a mutation in metastasis was found 18 (86%) patients; details are reported in table 2. The McNemar test revealed no significant discordance between primary and metastatic disease ($p=0.9$) (table 3). Three cases (14%) had a different mutational status between primary tumour and metastasis, two cases had a wild type primary tumour and a mutation on metastasis while the last patient had a mutation in primary tumor and a wild type status in the metastasis.

SEX	N°	(%)
Male	11	52
female	10	48
PRIMARY SITE		
Colon	15	68
rectum	7	32
T		
1	1	5
2	0	0
3	13	65
4	6	30
N		
0	10	50
1	5	25
2	5	25
ONSET METASTASIS		
synchronous	11	52
Metachronous	10	48
SITE OF METASTASIS		
Liver	10	48
Lung	4	19
other	7	33

Table 1 Baseline characteristics of patients

MUTATION AT PRIMARY SITE	N°	(%)		MUTATION AT METASTASIS	N°	(%)
KRAS	11	50.0		KRAS	10	45,5
BRAF	5	22.7		BRAF	5	22,7
NRAS	1	4.5		NRAS	2	9,1
BRAF+NRAS	1	4.5		BRAF+NRAS	1	4,5
WT	4	18.2		WT	4	18,2

Table 2 Mutational asset at primary and metastatic site

		MUTATION AT METASTATIC SITE		
		YES	NO	Total
MUTATION AT BASELINE	YES	17	1	18
	NO	1	3	4
	Total	18	2	22

Table 3 Concordance between primary and metastatic site mutation, a patients is considered mutated if at least one of the three main mutation occurred.

Efficacy analysis according to the concordance in mutational status

After a median follow-up of 7.6 months (range 4.9-32.6), a total of 16 patients developed a recurrence of disease with a median DFS for the all patients of 15.2 months (10.4-28 95% IC). A median DFS of 20.5 months (95% CI 9.9-29.6) was found in patients with concordance in mutational status versus 10.4 months (95% CI 6.1-not reached) in patients with discordance (p=0.01) (Figure 1). After a median follow-up of 26 months (range 5-43.1), a total of 8 patients died with a median OS for the entire population of 35.9 months (29.6-not reached 95%IC). In particular, median OS was 35.9 months (95% CI 26.3-not reached) in patients with concordance in mutational status between primary and metastasis versus 25.6 months (95% CI 6.6-not reached) in patients with discordance (p=0.038) (Figure 2).

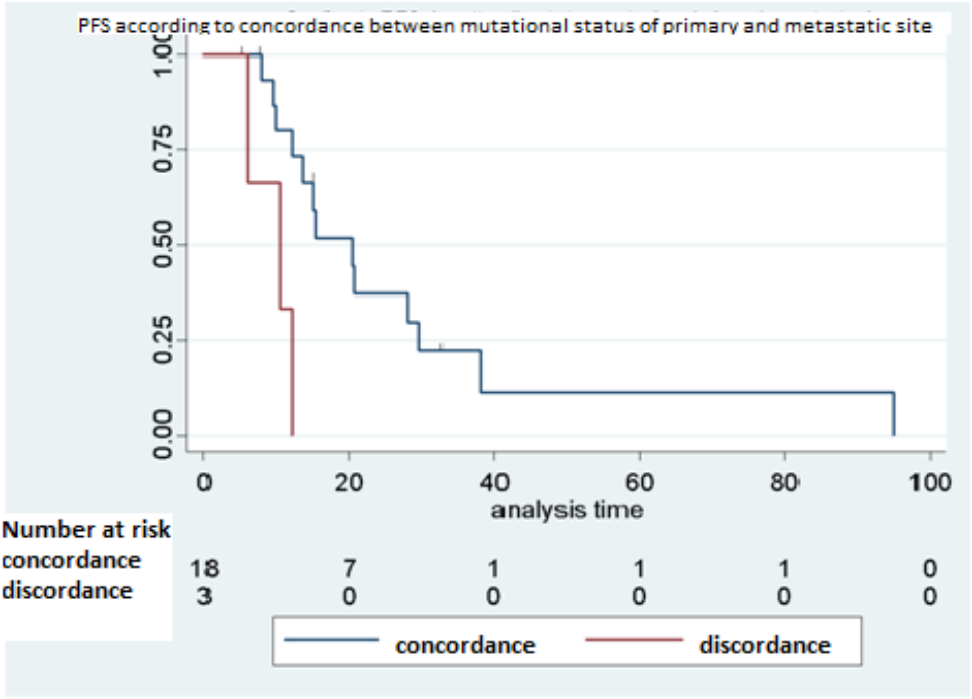


Figure 1 PFS according to concordance/discordance of mutational status between primary and metastatic site. Patients with concordance in mutational status had a median PFS of 20.5 months (95% CI 9.9-29.6) versus 10.4 months (95% CI 6.1-not reached) of patients with discordance (p=0.01)

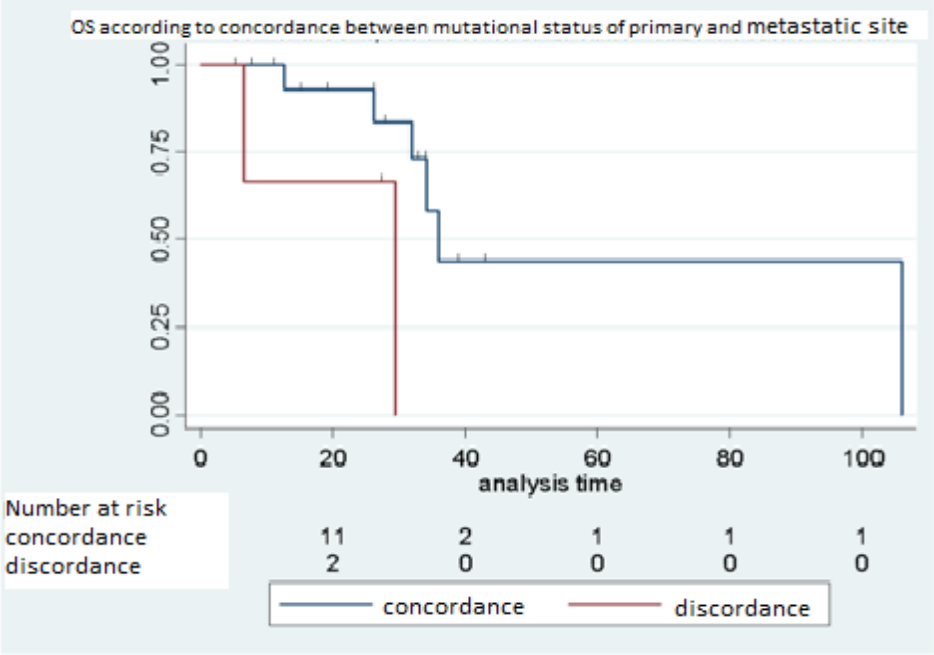


Figure 2 OS according to concordance/discordance of mutational status between primary and metastatic site. Median OS was 35.9 months (95% CI 26.3-not reached) in patients with concordance in mutational status between primary and metastasis versus 25.6 months (95% CI 6.6-not reached) in patients with discordance (p=0.038)

Discussion

In this study, we investigated the role of concordance between mutational status of primary tumor and mutational status of metastasis with the clinical outcomes of a cohort of patients treated for a synchronous or metachronous metastatic colorectal cancer. While the possibility of a discordance of mutational status between primary tumors and metastases has been largely investigated [68], the relationship between the concordance and clinical outcomes has been rarely analyzed. Although with the limitation of a small sample size, our results showed a prognostic role in term of DFS and OS of patients with discordance in mutational status.

Discordance of RAS mutational status between primary tumors and metastases from colorectal cancer is a rare phenomenon that account less the 10% of cases (ranged from 3-12%) [68]. Generally, a highest discordance has been showed between primary tumor and non-liver metastasis [68]. In addition, discordance seems to involve KRAS mutated patients and in a lower measure KRAS wild type tumors. In fact, it has been established that discordance between primary tumor and metastases account less than 15% of KRAS mutated cases and it accounts around the 5% of KRAS wild type patients. In line with all these data, our study reported a concordance near the 90% and the most of patients with discordance had wild type primary tumor and a mutation on the metastatic site.

In 2015, Siyar Ekinc et al [72] retrospectively investigated 31 patients with colorectal cancer who underwent metastasectomy of their liver and/or lung metastases showed a discordance in the 22% (7/31) of the patients; however, no progression free survival (PFS) difference was detected between patients with determined discordance and patients with undetermined discordance (10.6 vs 14.7 months, p=0.719) [73]. Conversely with the study by Siyar Ekinc et al, we found that the discordance between primary tumor and metastasis could be a negative prognostic factor in term of DFS and OS. In fact, patients with discordance survived on average 10 months less than patients

with concordance and PFS was even halved in discordant cases. However, it is very difficult the compare two small retrospective studies and definitive data are uncertain. Another important challenge that emerges is the criteria to follow for the early identification of patients who need a closest evaluation of the mutational concordance between primary site and metastasis. Testing the mutational status on both the primitive tumor and the metastasis doesn't seem to be cost-effective nor clinically useful. However we have observed that in early progression (less than 12 months) the double evaluation on primary and metastatic site could help better understanding the prognosis and, in the new era of target therapy, this patients could possibly benefit from a proper treatment. Actually, patients with quadruple wild-type metastatic colorectal cancer are being recruited for a phase II trial (NCT03457896) evaluating the efficacy of combinatorial target therapy, based on HER2 status and prior chemotherapy received (neratinib plus trastuzumab or neratinib plus cetuximab). The role of PIK3CA mutation still needs to be defined but is growing interest among the scientific community. Activating mutation in PIK3CA gene have been associated with resistance to anti EGFR therapy [74] and some treatment targeting the PI3K axis have been investigated in preclinical studies [75], as well as in phase I/II trials in CRC (NCT02861300).

In conclusion, notwithstanding the limitation of a small number of evaluated patients and the retrospective nature of the data, our study seems to define a prognostic role on the tumor discordance between primary mutational status and metastatic mutational status of patients with synchronous or metachronous metastatic colorectal cancer, therefore prospective large-scale trials are warranted to further evaluate this issue.

CHAPTER 7

CONCLUSIVE REMARKS AND FUTURE PERSPECTIVE

In recent years the discovery of cancer biomarkers has become a major focus of cancer research. The widespread use of tumor markers in managing cancer and its related therapies has motivated researchers to identify suitable markers for different types of cancer. Biomarkers are useful for diagnosis, monitoring disease progression, predicting disease recurrence and therapeutic treatment efficacy. With the advent of new and improved genomic and proteomic technologies such as DNA and tissue microarray, Next generation sequencing, ddPCR, protein assays etc coupled with advanced bioinformatic tools, it is possible to develop biomarkers that are able to reliably and accurately predict outcomes during cancer management and treatment. In years to come, a tissue or plasma based test for every phase of cancer may drive clinical decision making, supplementing or replacing currently existing invasive techniques.

In this scenario the Chapter 1 of my thesis was focused on the plasma genotyping as a novel diagnostic approach in thoracic oncology. NSCLC is a rapidly evolving malignancy, thus being able to test the molecular changes occurring during the cancer progression and driving the therapeutic choice (e.g. mutation in EGFR gene related to resistance/sensibility to target therapy) is clinically relevant. The analysis of cfDNA is even more appealing considering the non-invasive format: it spares the patient a tissue biopsy or, even better, offers a viable alternative to those patients where tissue biopsy is not feasible. The growing interest in the diagnostic possibility that liquid biopsy offers, has lead to the development of a huge number of assays and platforms.

Currently, I would suggest two potential scenarios in which cfDNA on plasma analysis has the greatest clinical relevance: the molecular diagnosis and the monitoring of progression during targeted therapy. However, the longitudinal approach I embraced in the study I have reported has given us a comprehensive view of the disease in its entirety; it helped to discriminate between bulky

and non-bulky disease, longer and shorter progression and, ultimately, to identify the patients most likely to experience early progression or resistance mutation.

The detection of genomic alterations in cfDNA, however, requires accurate yet rapid DNA extraction and isolation to avoid cell rupture (2 hours for EDTA tubes and 3 days for preservative tubes). The use of plasma has been recommended compared to serum [20], polymerase chain reaction (PCR) amplification based methods were the first to be used in this area and are now recognized as validated and established methods with a high specificity of detection despite a slightly lower but still acceptable sensitivity compared to beams or NGS methodology [17]. Undoubtedly, the overall burden of the disease influences the final result. Since the ability of any plasma genotyping test to detect a given mutation is directly related to the degree of cfDNA released into the circulation, it has been shown that the cfDNA shed of a metastatic tumor is superior to that of an early disease, particularly with bone and liver metastasis [76]. Plasma biopsy is therefore a robust logical option for the cohort of patients examined that focuses exclusively on advanced or locally advanced cases, which would not otherwise be suitable for TKIs therapy. The data reported in chapter 1 support the use of this technique as a predictive tool: the early identification of T790M mutation at diagnosis, even on cfDNA alone, spares the patient 9 to 13 months of unnecessary first generation treatment and inevitable rapid progression. It is also hoped that in the future technologies as ddPCR will be available to manage a wider range of EGFR exon mutation [77], giving a wide and clear landscape of the disease and at the same time helping the clinicians to chose the right treatment for the right person and the right time. The upcoming challenges we are facing in the closed future include the possibility of predicting certain sites of metastasis, and the early detection of mutation involved in the drug resistance.

The second part of my research focused on luminal breast cancer investigating the parameters that could influence the efficacy of neoadjuvant treatment. It has been previously

reported that an early response to neoadjuvant chemotherapy correlates both with the pathological response to surgery and with a longer overall survival [78], and the same feedback was found in non-responders to the primary regimen that respond to an alternative protocol [79]. Response-guided neoadjuvant therapy may lead to an extension of neoadjuvant therapy for respondents or a change in protocol for non-responders, both strategies have led to an improvement of DFS and OS rates. In addition, hormone receptor-positive tumours seem to benefit the most from this approach [80].

The need for reliable predictive markers becomes more evident during neoadjuvant treatment due to the lack of robust data on the optimal duration and combination of hormone-therapy and chemotherapy in HR+ HER2- tumors. Data from recent studies are promising, although less impressive than in the metastatic setting. In this context, the use of genomic-transcriptomic technology (such as ONCOTYPE, PAM50) and the identification of new biomarkers (ESR1, PI3Kca, PDGF-R) on tissue or with liquid biopsy could help to select patients inclined to respond to endocrine combined therapy and able to obtain pCR [81].

I approached this clinical issue from different points of view trying to identify those markers that could have clinical relevance and/or impact. The predictive and prognostic value of Ki67 is the subject of much debate and, despite numerous positive evidences, it is not yet universally accepted as a reliable parameter. Instead of focusing only on the value of the Ki67 itself and its predictive role, I have decided to test the potential informative value provided by the variation of Ki67 over time. In our study the ΔKi67 percentage variation is lower in those patients who did not achieve a complete clinical response (according to RECIST), and those same patients had an overall shorter PFS and OS [23].

Another controversial tool for initial staging of breast cancer is 18F FDG Positron Emission Tomography (PET). Its use is usually limited, or preferred, in the restaging phase [50]. However,

our analysis has shown that SUV trends reflect the metabolic response to therapy. Patients with clinical complete response had a greater decrease in SUVs during therapy period than partial responders and patients with stable or progressive disease. Therefore, metabolic response monitoring could become a powerful predictive tool in breast cancer even at an early stage.

In the same setting I have tested also a new bimolecular test potentiality involved in the early prediction of treatment response. The Rna Disruption Assay is an innovative tool allowing to measure the cytostatic effect of therapy. Since RNA alteration appear to occur with different types of drugs, this technology could potentially be applied to a variety of clinical settings. The early data seem to be promising and following these encouraging results I have found, an interventional prospective study "RNA Disruption Assay (RDA)-Breast Cancer Response Evaluation for Individualized Therapy-BREVITY" (NCT03524430) has been started. The aim of the study is to provide validation of RDA results as a response assessment tool. This is a single arm interventional study, patients with invasive breast cancer of any subtype or grade scheduled to receive neoadjuvant treatment according to clinical choice. The RDA will be performed on core needle biopsies taken after 35 +/-4 after starting of the administered treatment and, if there are no changes in the treatment administered, at 55 +/- 5 days after the start of the first neoadjuvant treatment. If therapy is changed, the second core biopsy is performed two to three weeks after the start of the new therapy, according to the therapy schedule. The outcome measures will be the pathological complete response (pCR) measured at surgery and disease free survival with a 5 year follow-up (at the moment accrual is suspended due to the Coronavirus emergency).

In chapter 5 of my thesis I focused on the possible role of cytokines and growth factors in response or resistance to therapy. Multiplexed analysis allows simultaneous quantitative measurement of circulating factors, but due to the pleiotropic nature of cytokines it can be challenging to find statistically significant ranges. Although the trend we have identified in IL-6

values before and after treatment is encouraging, there is a need for extensive analysis, possibly examining variations during cytotoxic therapy in groups of similar effector molecules.

Finally, in chapter 6, I investigated the presence of a correlation between concordance or discordance in the mutational status of primary and metastatic site with the clinical outcomes of a cohort of colorectal patients. Discordance of mutational status between primary and metastatic site is a rare event in colorectal cancer but, despite the limited number of patients included in this study, it seems to be related to a poor prognosis in synchronous or metachronous metastatic colorectal cancer.

In order for the oncologists to be able to monitor over time mutational status on plasma, proliferation index and SUV changes, or to identify an inconsistency between the mutational status of primary and metastatic sites, an effort is needed to collect all relevant clinical information and make it clinically available. Based on this urgent need, I have started to collaborate on the The MOzART (Understanding the MOlecular Aberrations related to Resistance/Responsiveness to Novel Drugs in Metastatic Solid Tumors), one of the main project of the “Dipartimento di Eccellenza- DS;” of University of Trieste, supported by MIUR. The program is a real-life exploratory study aimed at patients with metastatic solid tumors (mST) in different treatment lines with tyrosine kinase inhibitors, or monoclonal antibodies associated or not with chemotherapy and / or hormonal drugs. The MOzART is based on the collaboration between University of Trieste and Sloan-Kettering Memorial Cancer Center of New York (USA). The program will allow the molecular screening of metastatic solid tumors, and therefore the identification of potential biomarkers that may have a predictive value of response or resistance to ongoing treatment with TKIs or mAbs using genomics or transcriptomics data, which would allow the identification of so-called "outliers" or exceptional responders or rapid progressors. I am involved in the evaluation of the prognostic relevance of genetic alterations detected in tissue biopsies (de novo on metastatic or

on primitive archived samples) and liquid on plasma (cfDNA) performed routinely in clinical practice, as it will open to the possibility to build new therapeutic hypotheses based on the findings generated by the bioinformatic integration of all the obtained data.

The future of cancer management is expected to be profoundly dependent upon the use of biomarkers that will guide physicians at every step of disease management. Cancer biomarkers can be used for the accurate evaluation and management of the disease in different stages. They can be useful for predicting several outcomes during the course of disease including early detection, outcome prediction and detection of disease recurrence. Most importantly, with the clinical appearance of many new therapeutic agents, appropriate markers can be used to determine which tumors will respond to which treatments in order to predict the likelihood of drug resistance

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