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ABOUT COVER

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AIM AND SCOPE

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MINIREVIEWS

Cell-free DNA integrity for the monitoring of breast cancer: Future perspectives?

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Author contributions: Scaggiante B developed the original idea, researched the literature and edited the manuscript; Sobhani N contributed to the development of the idea, researched the literature and edited the manuscript's content and language; Generali D contributed to developing the oncological aspects and edited the manuscript; Zanconati F contributed to developing the idea as a pathologist; Bortul M contributed to the editing the manuscript as a breast cancer surgeon.

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Abstract

Breast cancer (BC) is the most common cancer and the second cause of death in women worldwide. Therapeutic options are increasing, but the response to treatments is not always efficient and the risk of recurrence covers decades. In this perspective, the need to have a proper follow-up for the therapeutic responses and for anticipating recurrence it is urgent in the clinical setting. Liquid biopsy provides the basic principle for a non-invasive method for the routinely monitoring of BC. However, due to the heterogeneity of tumors during onset and progression, the search for tumor DNA mutations of targeted genes in plasma/serum is a limiting factor. A possible approach overtaking this problem comes from the measurement of cell-free DNA integrity, which is an independent factor from the mutational status and theoretically is representative of all tumors. This review summarizes the state-of-the-art of cell-free DNA integrity researches in BC, the controversies and the future perspective.

Key words: cfDNA integrity; Liquid biopsy; Breast cancer; ALU sequences; LINE-1 sequences

Core tip: Despite the potentiality of cell-free DNA integrity as a useful tool for the monitoring of Breast Cancer (BC), evinced in some clinical studies, the scientific community has not reached agreeable conclusions to translate the results from the bench-to-the-bedside yet. The main controversy regards

the targets' choice and the size of circulating cell-free tumor DNA fragments. This work underlines the utility of cell-free DNA Integrity evaluation for BC follow-up and at the same time highlights the common concepts explaining the different results in line of future directions.

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INTRODUCTION

Breast cancer (BC) is still the most common cancer and the second cause of cancer-related death in women worldwide[1]. A timely knowledge of its occurrence, responsiveness to therapies and recurrence is becoming of paramount importance for clinicians to adopt specific and more efficient approaches with regards to any single patient's health assistance. In clinical routine, the evaluation of serum markers as CEA or CA15-3 is still used for BC follow-up, but with a low specificity and sensibility[2-5]. Up to now, one of the most promising frontiers in this field is the liquid biopsy. Recently, the meta-analysis on the clinical utility of circulating tumor cells (CTC) in early BC or in metastatic BC (MBC) provides a solid rationale for their use in oncological settings[6-8]. However, their routinely use is still compromised by the relatively high cost of the technique.

Circulating cell-free DNA and qPCR measurement

From the blood circulation, it is possible to derive CTC, exosomes or cell-free nucleic acids (Figure 1). Cell-free DNA (cfDNA), consists of DNA fragments released after cell death processes from both tumor and normal cells. The circulating tumor DNA (ctDNA) can be differentiated from the rest of the cfDNA by looking at tumor-specific DNA changes, including mutations, gene amplifications, rearrangements and methylations[9] proving it as a valid non-invasive biomarker to monitor tumor growth, spread, clonal evolution and response to therapies[10]. This can be achieved either by a qualitative way (*i.e.*, type of mutations) or quantitative way (*i.e.*, copy number evaluation of mutated genes). However, the known mutations that can be used in liquid biopsy represent a limited percentage of patients. As an example, the most studied *PI3KCA* mutations all together have been found in about 30%-40% of BC patients[11].

Here, both low-cost and easy-to-be-perform methods that are not bound to one or few specific genetic mutations to predict occurrence and monitor disease progression in BC patients will be described in line of what is currently known in literature.

Briefly, real-time polymerase chain reaction-or quantitative PCR (qPCR) is a powerful advancement of PCR technology that enables the measurement of the starting amount of nucleic acids in the reaction without the need for post-PCR gel analysis. This is achieved by the possibility to detect in a real-time manner the amplification process by fluorescence and to measure the amplification products of samples at exponential phases. Through this technology the expression of a target is measured by fluorescent probes or DNA-labelling dyes. Of note, the qPCR dyes do not discriminate between specific or non- specific amplicon products, thus there is a need for an accurate testing of the annealing conditions and buffer reagents to guarantee specificity of the reaction. The quantification of an unknown sample can be absolute by using an internal amplification standard curve obtained with known DNA quantities or it can be relative by comparison of the difference in cycle threshold values (Ct) of a unknown sample with respect to reference (mainly expressed as $^{\Delta\Delta}$Ct values)[12,13]. Finally, to improve the accuracy of measurements, qPCR offers, together with the basic reagents, a passive fluorescein or ROX dyes to remove well-factors. The fluorescein acts as a passive reference dye, providing sufficient background fluorescence before the amplification reaction occurs, removing in this way the well factors-such as pipetting inaccuracies and fluorescence fluctuations-from the plate with the test samples.

Quantification of total circulating cell-free DNA

Some studies have focused on the quantification of total cfDNA levels using *GAPDH*, *Beta-globin*, *Beta2-Microglobulin*, *hTERT* or *LINE-1* as potential target genes, making the higher levels of cfDNA as a way to distinguish benign from malignant BC[14–18]. Also SYBR Green's fluorescence to measure total serum cfDNA has been investigated[19]. However, in our opinion, it is worth to consider how the total cfDNA levels are susceptible to increase also by the presence of other pathological conditions (*e.g.*, infection, inflammation, *etc.*), thus influencing the results.

Quantification of cell-free DNA integrity

The detection of ctDNA levels using cell-free DNA integrity (cfDI) measurement, as ratio between longer and shorter DNA fragments, is more specific than total serum cfDNA and has been explored in BC by qPCR by many authors using SYBRGreen fluorescent dye (Table 1). In principle, normal cells, undergoing apoptosis, release DNA fragments of about 200 bp as the result of enzymatic cleavage of nucleosome units; whereas, tumor cells undergo many different death processes, including necrosis and autophagy, and they can release DNA fragments of different sizes[20,21]. Umetani *et al*[22], using ALU targets proposed cfDI for the first time as a valuable tool to identify primary BC, showing it could be suitable to define lymph node metastasis in a group of 83 patients compared to 51 healthy controls.

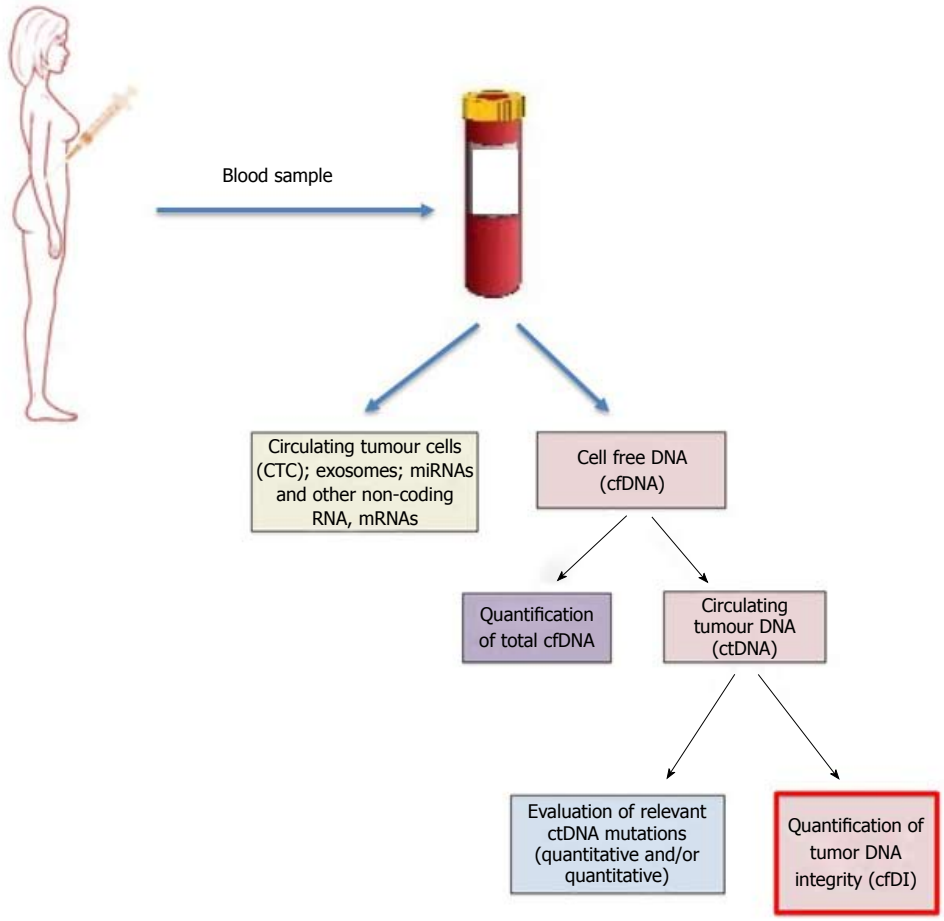

Figure 1 Diagram summarizing the possibility to monitor breast cancer from the blood circulating DNA.

They measured in serum shorter fragments of 115 bp that were considered as derived from apoptotic normal cells and larger ones of 247 bp as ctDNA, derived from necrosis/autophagy of cancer cells. The cfDI value calculated as the ratio quantity of longer over shorter fragments, ALU247/ALU115, was found to be higher in BC patients with high grade cancer compared to healthy controls. Accordingly to Umetani *et al*[22], Agostini *et al*[23] using the same ALU247 bp and ALU115bp targets demonstrated in plasma that cfDI value was twice higher in BC patients (n = 39) *vs* healthy controls (n = 49). Subsequently, Stötzer *et al*[24] proved in plasma that the ratio ALU247/115 were higher in patients with locally confined BC and MBC (n = 47) than benign BC (n = 12) (P < 0.001) but not *vs* healthy controls (n = 28). Moreover, this group evidenced that ALU concentrations alone were very interesting as markers for locally confined BC, while the use of cfDI was limited by the elevated levels found in some healthy controls. However, Iqbal *et al*[25] enrolling a larger number of women (148 patients *vs* 51 healthy controls) confirmed that the cfDI value, represented as ALU247/115 ratio, was significantly higher in serum of patients compared to healthy controls. Moreover, through a multivariate analysis, they showed a correlation between the cfDI value and the tumor size to predict the overall survival (OS) at 5 years and disease-free survival (DFS) at 4 years. Madhavan *et al*[21] also considered cfDI as a useful biomarker for BC in the largest patients' cohort (82 BC and 201 MBC) by using different primer set for ALU sequences and introducing LINE-1 as another DNA repetitive element target. They quantified ALU 260 bp and LINE-1 266 bp amplicons *vs* ALU 111 bp and LINE-197 bp amplicons, respectively. They showed, differently than the other groups, cfDI value was lower in BC patients *vs* healthy control and positively correlated with a decrease in progression-free survival (PFS) (P = 0.0025 for ALU) and OS (P < 0.0001 for both ALU and LINE-1). Similarly, using the same ALU260/111 and LINE-1 266/197 ratios, Cheng *et al*[26] showed that cfDI was significantly lower in recurrent BC (n = 37) *vs* non-recurrent BC (n = 175) (P < 0.001 for both ALU and LINE-1 cfDI values) but they did not provide as an extra measure healthy controls. Interestingly, this latter research group showed that a higher risk of developing recurrence could be predicted by the reduction of cfDI value (P = 0.020 for ALU and P = 0.019 for LINE-1 cfDI values, respectively). Finally, it should be mentioned that Cheng *et al*[27] recently observed that higher cfDI values for both ALU and LINE-1 targets in MBC patients correlated

Table 1 cfDI evaluation for the monitoring of breast cancer

Targets, length of the amplicons and primers' sequences	Patients with primary BC	Results	Ref.
ALU, 115 bp FW: 5′-CCTGAGGTCAGGAGTTCGAG-3′ RV: 5′-CCCGAGTAGCTGGGATTACA-3′ ALU, 247 bp FW: 5′-GTGGCTCACGCCTGTAATC-3′ RV: 5′-CAGGCTGGAGTGCAGTGG-3′	Healthy females (n = 51) and BC patients (n = 83) DNA from serum	The ratio ALU247/115 was higher in 51 patients with stage II (P = 0.005), stage III (P < 0.0001), stage IV (0.002) compared to healthy controls but not in 32 patients with stage 0 or I	Umetani *et al*[22], 2006
ALU, 115 bp FW: 5′-CCTGAGGTCAGGAGTTCGAG-3′ RV: 5′-CCCGAGTAGCTGGGATTACA-3′ ALU, 247 bp FW: 5′-GTGGCTCACGCCTGTAATC-3′ RV: 5′-CAGGCTGGAGTGCAGTGG-3′	Healthy females (n = 49) and BC patients (n = 39) DNA from plasma	In the group of patients the ratio ALU247/115 was twice higher (P < 0.0001) than in the group of healthy controls	Agostini *et al*[23], 2012
ALU, 115 bp FW: 5′-CCTGAGGTCAGGAGTTCGAG-3′ RV: 5′-CCCGAGTAGCTGGGATTACA-3′ ALU, 247 bp FW: 5′-GTGGCTCACGCCTGTAATC-3′ RV: 5′-CAGGCTGGAGTGCAGTGG-3′	Healthy females (n = 28), benign breast disease patients (n = 12), locally confined BC patients (n = 65) and MBC patients (n = 47) DNA from plasma	The ratio ALU247/115 was higher in patients with locally confined BC and metastatic BC than in benign BC (P < 0.001), but not *vs* healthy controls	Stötzer *et al*[24], 2014
ALU, 111 bp FW: 5′-CTGGCCAACATGGTGAAAC-3′ RV: 5′-AGCGATTCTCCTGCCTCAG-3′ ALU, 260 bp FW: 5′-ACGCCTGTAATCCCAGCA-3′ RV: 5′-CGGAGTCTCGCTCTGTCG-3′ LINE-1, 97 bp FW: 5′-TGGCACATATACACCATGGAA-3′ RV: 5′TGAGAATGATGGTTTCCAATTTC-3′ LINE-1, 266 bp FW: 5′-ACTTGGAACCAACCCAAATG-3′ RV: 5′-CACCACAGTCCCCAGAGTG-3′	Healthy females (n = 100), primary BC patients (n = 82) and MBC patients (n = 201) DNA from plasma	Both the ratios ALU 260/111 and LINE-1 266/97 were lower in primary BC patients (ALU: P = 0.046; LINE-1 P = 0.041) In MBC patients the lower values of cfDI were related to both a decrease in PFS (P = 0.0025 for ALU) and OS (P < 0.0001 for both ALU and LINE-1 fragments)	Madhavan *et al*[21], 2014
ALU, 115 bp FW: 5′-CCTGAGGTCAGGAGTTCGAG-3′ RV: 5′-CCCGAGTAGCTGGGATTACA-3′ ALU, 247 bp FW: 5′-GTGGCTCACGCCTGTAATC-3′ RV: 5′-CAGGCTGGAGTGCAGTGG-3′	Healthy females (n = 51) and BC patients (n = 148) DNA from serum	The ratio ALU 247/115 was significantly higher in patients compared to controls (P < 0.001)	Iqbal *et al*[25], 2015
Beta-actin, 100 bp FW: 5′-GCACCACACCTTCTACAATGA-3′ RV: 5′-GTCATCTTCTCGCGGTTGGC-3′ Beta-actin, 400 bp FW: 5-GCACCACACCTTCTACAATGA-3′ (common primer) RV: 5′-TGTCACGCACGATTTCCC-3′	Healthy females (n = 70), benign lesions (n = 95) and BC patients (n = 95) DNA from plasma	cfDI value calculated as difference between 400 bp and 100 bp fragments Higher cfDI values were obtained in BC compared to benign lesions and healthy subjects (P < 0.001)	Kamel *et al*[20], 2016
HER2, 126 bp FW-5-CCAGGGTGTTCCTCAGTTGT-3′ RV-5- -GGAGTTCCTGCAGAGGACAG-3′ HER2, 295 bp FW-5′-CCAGGGTGTTCCTCAGTTGT-3′ RV-5′-TCAGTATGGCCTCACCCTTC-3′ MYC, 128 bp FW-5-GGCATTTAAATTTCGGCTCA-3′ RV-5-AAAAGCCAAATGCCAACTT-3′ MYC, 264 bp FW-5′-TGGAGTAGGGACCGCATATC-3′ RV-5′-ACCCAACACCACGTCCTAAC-3′ BCAS1, 129 bp FW-5-GGGTCAGAGCTTCCTGTGAG-3′ RV-5-TATCATGCCTTGGAGAACCA-3′ BCAS1, 266 bp FW-5′-GGGTCAGAGCTTCCTGTGAG-3′ RV-5′-CGTTGTCCTGAAACAGAGCA-3′ PIK3CA, 129 bp FW-5′CTCCACGACCATCATCATCAGGT-3′ RV-5′-TGGTTATTAATGAGCCTCACGG-3′ PIK3CA, 274 bp FW-5′-CTC CACGAC CAT CATCAGGT-3′ RV-5′-CGAAGGTCACAAAGTCGTCT-3′	Healthy females (n = 10), BC patients (n = 79) DNA from serum	The ratios BCAS1 266/129, MYC 264/128, PIK3CA 274/129 were significantly higher in patients compared to controls (P = 0.002, P = 0.030 and P = 0.004, respectively) No significant values for HER2 targets	Maltoni *et al*[28], 2017

ALU, 111 bp FW: 5′-CTGGCCAACATGGTGAAAC-3′ RV: 5′-AGCGATTCTCCTGCCTCAG-3′ ALU, 260 bp FW: 5′-ACGCCTGTAATCCCAGCA-3′ RV: 5′-CGGAGTCTCGCTCTGTCG-3′ LINE-1, 97 bp FW: 5′-TGGCACATATACACCATGGAA-3′ RV: 5′-TGAGAATGATGGTTTCCAATTTC-3′ LINE-1, 266 bp FW: 5′-ACTTGGAACCAACCCAAATG-3′ RV: 5′-CACCACAGTCCCCAGAGTG-3′	Non-recurrent BC patients (n = 175) *vs* recurrent-BC patients (n = 37) No healthy females reported DNA from plasma	Both the ratios ALU260/111 and LINE1-266/97 were significantly lower during follow-up in recurrent BC *vs* non recurrent BC (P < 0.001 for both ALU and LINE-1 cfDI), Moreover, BC patients with a lower cfDI had higher risk of developing recurrence compared to patients with higher cfDI (P = 0.020 for ALU cfDI and P = 0.019 for LINE-1 cfDI, respectively)	Cheng *et al*[26], 2017
ALU, 111 bp FW: 5′-CTGGCCAACATGGTGAAAC-3′ RV: 5′-AGCGATTCTCCTGCCTCAG-3′ ALU, 260 bp FW: 5′-ACGCCTGTAATCCCAGCA-3′ RV: 5′-CGGAGTCTCGCTCTGTCG-3′ LINE-1, 97 bp FW: 5′-TGGCACATATACACCATGGAA-3′ RV: 5′TGAGAATGATGGTTTCCAATTTC-3′ LINE-1, 266 bp FW: 5′-ACTTGGAACCAACCCAAATG-3′ RV: 5′-CACCACAGTCCCCAGAGTG-3′	MBC patients (total n = 268) No healthy females DNA from plasma	Both the ratios ALU260/111 and LINE1-266/97 significantly increased in 268 MBC patients treated with one cycle of chemotherapy (MBCLB) compared to MBC at baseline (MBC1C) (P = 0.00017 for ALU -0.053 *vs* 0.063- and P = 0.0016 for LINE-1-0.45 *vs* 0.49) Moreover, in both MBCBL and MBC1C patients with a higher cfDI (for both ALU and LINE-1) correlated with a higher PFS and OS *vs* lower cfDI MBC patients	Cheng *et al*[27], 2018

BC: Breast cancer; cfDNA: Cell-free DNA; cfDI: Cell-free DNA integrity; ctDNA: Circulating tumour DNA; DFS: Disease free survival; MBC: Metastatic breast cancer; PFS: Progression-free survival; OS: Overall survival; qPCR: Quantitative real-time PCR; ddPCR: Droplet digital PCR.

with longer PFS and OS. However, Kamel *et al*[20] measuring the 400 bp and 100 bp amplicons of the *Beta-actin* from the DNA derived from plasma of 95 BC and 95 benign lesions *vs* 70 healthy controls estimated a cfDI- as difference between longer and shorter fragments- accordingly to Umetani *et al*[22] and the other authors[23-25], while yet differently from Madhavan *et al*[21]. In fact cfDI was found significantly higher in BC samples compared to those of benign and healthy subjects (P < 0.001). Moreover, they related those higher values to TNM stage, suggesting a cut-off to identify the more aggressive BC[20]. In agreement with Kamel *et al*[20], Maltoni *et al*[28] recently showed that tumour cells released longer DNA fragments than normal cells in the bloodstream. They quantified large fragments of 295 bp, 264 bp, 266 bp, 274 bp and short amplicons of 126, 128, 129, 129 bp from *HER2*, *MYC*, *BCAS1* and *PIK3CA,* respectively, from the serum of healthy females (n = 10), non-recurrent BC (n = 58) and recurrent BC (n = 21). They estimated cfDI as the ratio between longer and shorter amplicons of these genes and demonstrated that *BCAS1*, *MYC* and *PIK3CA* long/short amplicons were significantly higher in patients compared to healthy controls (P = 0.002, P = 0.030 and P = 0.004, respectively). On the other hand, there was no significant difference for long/short amplicons of *HER2*[27].

DISCUSSION

The overall literature on cfDI is intriguing as it has an extraordinary potential for the monitoring of BC, but it remains to be clarified what is the expected value of cfDI: some authors claimed that ctDNA is made of longer amplicons than normal cfDNA, explaining why the cfDI increased in BC[20,22-25,27], whereas other research groups, using different primers, claimed the exact opposite[21,26].

Most of the authors, in their measurement of cfDI through the ALU sequences, decided to use a standard DNA curve, as for Umetani *et al*[22], to derive quantifications of their DNA[21-25,27], and used the fluorescein or ROX passive reference dyes to improve the quality of their results[23,25]. Additionally, the specificities of the amplification reactions for the different couple of primers described in the papers have been controlled by means of denaturation curves or gel electrophoresis. This implies that the different results by qPCR hardly can be attributable to the laboratory's methodology, although we cannot completely exclude some variability in sample collection in the studies here described. Of note, differently than the other groups, Stötzer *et al*[24] have adopted a slightly different protocol for ALU amplifications by introducing UDP-DNA glycosidase.

Higher cfDI values in BC *vs* healthy controls were found in larger patients' cohorts derived from independent clinical settings and by using more different targets compared to studies claiming lower cfDI values in the tumor (Figure 2). Of note, higher cfDI in tumor than healthy controls were found in those studies that have analyzed mainly BCs, which did not reach the metastatic setting[22,23,25], whereas lower cfDI than healthy controls were reported in a study using the largest MBC patients' cohort up-to-date[21]. It is interesting to note that Umetani *et al*[22] proposed an increased cfDI value to predict local micrometastasis and recently Cheng *et al*[28] observed that cfDI value particularly decreased in BC patients with visceral metastasis. Thus we

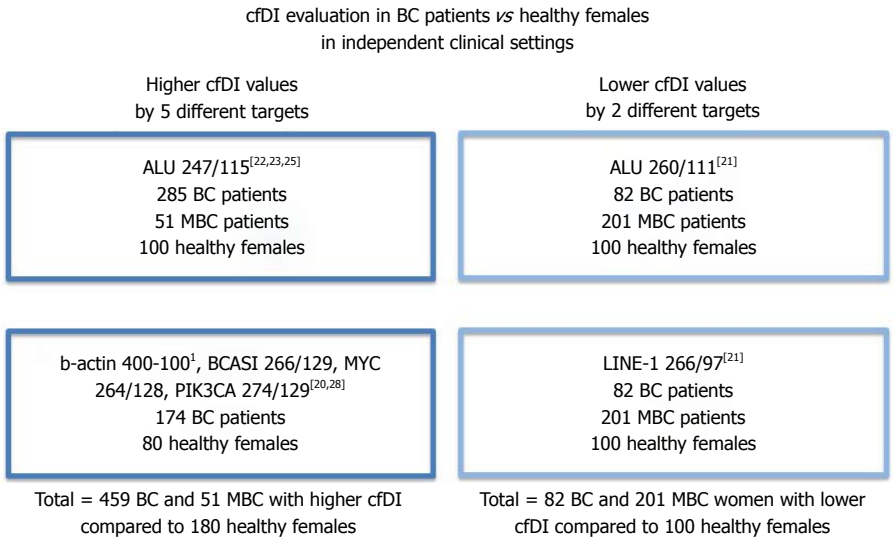

Figure 2 Summary of the literature data on cfDI determination in primary breast cancer *vs* healthy females. [1]Note that cfDI by β-actin was evaluated as difference between large and short amplicons and not as ratio longer to shorter amplicons. BC: Breast cancer; MBC: Metastatic breast cancer.

suggest that cfDI value can increase at initial stages of the BC and decrease in MBC. Surely, the most promising targets for the measurement of cfDI are represented by repetitive elements such as ALU and LINE-1 sequences, accounting for nearly 10% and 17% of the total genome, respectively. It is worth nothing that reproducible results were obtained when independent groups used the same ALU primer pairs, either those demonstrating higher cfDI[22-25] and those demonstrating lower cfDI in BC[21,26]. In our opinion, the methods of DNA extractions merely could have influenced the results. Interestingly, by looking with BLASTN genomic RefSeqGene Human at the target sites of ALU primers' pairs used by the research groups obtaining divergent results, we observed different target sites for ALU247/115 pairs compared to the ALU260/111 ones. We cannot exclude that this could contribute to the opposite cfDI values obtained by the different research groups comparing BC *vs* healthy controls. Moreover, we would like to point out that the qPCR methodology by SYBR Green is not very sensitive in quantifying very small DNA fragments in diluted solutions[29], as it could be in liquid biopsy, and that the variability of amplification efficiency of a sample can be overtaken by many replicates and independent experiments, that are hard to performed with samples derived from liquid biopsy. In this respect, the determination of cfDI in liquid biopsy samples would benefit by more sensitive and accurate technologies such as digital droplet PCR (ddPCR).

In conclusion, monitoring primary and MBC through a non-invasive analysis such as that of circulating DNA remains one of the most interesting goals to achieve. Surely, the mutations in liquid biopsy are of paramount importance for targeted therapies and for monitoring response to treatment. However, the most interesting benefit-to-cost analysis for the follow-up of BC and its recurrence seems to be the evaluation of circulating cfDI. Future investigations for cfDI by ddPCR are warranted for the (1) testing for the choice of best targets; (2) clarification of the clinical significance of larger and shorter DNA fragments origin in serum/plasma; and (3) a better understanding of the potential clinical impact of cfDI in anticipating recurrence and responsiveness to therapies for all patients, independently from the mutational signature of BC.

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ORIGINAL ARTICLE

Observational Study

Clinicopathological predictors of long-term benefit in breast cancer treated with neoadjuvant chemotherapy

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Institutional review board statement: This study was reviewed and approved by the Instituto Nacional de Enfermedades Neoplasicas Institutional Review Board. Personal and filiation data including identity of every patient was protected with an added code in the Excel table. This is a retrospective case series that did not have any activity or contact with the patients.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Abstract

AIM

To investigate the survival impact of clinicopathological factors, including pathological complete response (pCR) and tumor-infiltrating lymphocytes (sTIL) levels according to subtypes, in breast cancer (BC) patients who received neo-adjuvant chemotherapy (NAC).

METHODS

We evaluated 435 BC patients who presented and received NAC at the Instituto Nacional de Enfermedades Neoplasicas from 2003 to 2014. sTIL was analyzed as the proportion of tumor stroma occupied by lymphocytes, and was prospectively evaluated on hematoxylin and eosin-stained sections of the preNAC core biopsy. pCR was considered in the absence of infiltrating cancer cells in primary tumor and axillary lymph nodes. Analysis of statistical association between clinical pathological features, sTIL, pCR and survival were carried out using SPSSvs19.

RESULTS

Median age was 49 years (range 24-84 years) and the most frequent clinical stage was ⅢB (58.3%). Luminal A, Luminal B, HER2-enriched and (triple-negative) TN phenotype was found in 24.6%, 37.9%, 17.7% and 19.8%, respectively. pCR was observed in 11% and median percentage of sTIL was 40% (2%-95%) in the whole population. pCR was associated to Ct1-2 (P = 0.045) and to high sTIL (P = 0.029) in the whole population. There was a slight trend towards significance for sTIL (P = 0.054) in Luminal A. sTIL was associated with grade Ⅲ (P < 0.001), no-Luminal A subtype (P < 0.001), RE-negative (P < 0.001), PgR-negative (P < 0.001), HER2-positive (P = 0.002) and pCR (P = 0.029) in the whole population. Longer disease-free survival was associated with grade Ⅰ-Ⅱ (P = 0.006), cN0 (P < 0.001), clinical stage Ⅱ (P = 0.004), ER-positive (P < 0.001), PgR-positive (P < 0.001), luminal A (P < 0.001) and pCR (P = 0.002). Longer disease-free survival was associated with grade Ⅰ-Ⅱ in Luminal A (P < 0.001), N0-1 in Luminal A (P = 0.045) and TNBC (P = 0.01), clinical stage Ⅱ in Luminal A (P = 0.003) and TNBC (P = 0.038), and pCR in TNBC (P < 0.001). Longer overall survival was associated with grade Ⅰ-Ⅱ (P < 0.001), ER-positive (P < 0.001), PgR-positive (P < 0.001), Luminal A (P < 0.001), cN0 (P = 0.002) and pCR (P = 0.002) in the whole population. Overall survival was associated with clinical stage Ⅱ (P = 0.017) in Luminal A, older age (P = 0.042) in Luminal B, and pCR in TNBC (P = 0.005).

CONCLUSION

Predictive and prognostic values of clinicopathological features, like pCR and sTIL, differ depending on the evaluated molecular subtype.

Key words: Breast cancer; Subtype; Tumor-infiltrating lymphocytes; Neoadjuvant therapy; Pathological complete response; Survival

Core tip: The authors evaluated a series of 435 breast cancer (BC) patients who received neoadjuvant chemotherapy. They evaluated the association between stromal tumor-infiltrating lymphocytes levels and pCR in preneoadjuvant chemotherapy samples according to molecular subtypes. The results confirm differences in the predictive and prognostic role of stromal tumor-infiltrating lymphocytes and pathological complete response depending on the tumor subtype. Additionally, the authors evaluate the value of traditional prognostic features in every BC subset. The results increase the understanding of biomarkers in the heterogeneous scenario of BC.

Galvez M, Castaneda CA, Sanchez J, Castillo M, Rebaza LP, Calderon G, De La Cruz M, Cotrina JM, Abugattas J, Dunstan J, Guerra H, Mejia O, Gomez HL. Clinicopathological predictors of long-term benefit in breast cancer treated with neoadjuvant chemotherapy. *World J Clin Oncol* 2018; 9(2): 33-41 Available from: URL: http://www.wjgnet.com/2218-4333/full/v9/i2/33.htm DOI: http://dx.doi.org/10.5306/wjco.v9.i2.33

INTRODUCTION

Breast cancer (BC) is the second most common cancer in the world and the most frequent cancer among women, with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers), and is the fifth cause of death from cancer overall (522000 deaths)[1]. Neoadjuvant chemotherapy (NAC) is the standard therapy for locally advanced BC and could improve both surgical options and long-term outcome[2]. Response to NAC is considered an *in vivo* test of tumor sensitivity to NAC, and the achievement of a pathological complete response (pCR) is associated with longer disease-free survival (DFS) and greater overall survival (OS)[3-7]. Tumor-infiltrating lymphocytes (TILs) serve to evaluate the host immune system response against a tumor and also constitutes a valuable predictive biomarker of NAC response and survival[8-11].

BC is a heterogeneous disease, and intrinsically different subtypes of BC have been identified in the past years based on gene expression profiles and on the combined immunohistochemical status of hormone and HER2 receptors. Responsiveness to preoperative therapies and outcome after surgery can be predicted by BC subtypes[12-14].

In this study, we investigated the survival impact of different clinicopathological factors, including pCR and TIL levels, according to the subtypes in BC patients who received NAC. The predictive role of different clinicopathological features for having high density TIL and obtaining pCR according to subtypes was also

determined.

MATERIALS AND METHODS

We found 435 patients diagnosed with BC at clinical stage ⅡB to ⅢC at the Medical Department of the Instituto Nacional de Enfermedades Neoplasicas from 2003 to 2014. Eligibility criteria for this retrospective study were a histological diagnosis based on a core needle biopsy, having received NAC regimen and having undergone surgery after NAC. Patient characteristics such as age, clinical stage, histological subtype and grade, presence of estrogen receptors (ERs), progesterone receptors (PgRs) and HER2, and molecular subtype was obtained from the pathology report of preNAC core biopsy. pCR was defined as absence of invasive cancer in the breast and axillary nodes, irrespective of carcinoma *in situ* (ypT0/is ypN0), as previously described[4,15]. Phenotype classification was prospectively concluded through the evaluation of ER, PgR, HER2 and Ki67 as well as histological grade (in cases without Ki67 information): Luminal A (ER ⩾ 10%, PgR ⩾ 20%, HER2-negative and Ki67 < 15% or HG-Ⅰ-Ⅱ), Luminal B (ER ≥ 10% and any PgR < 20%, HER2-positive, Ki67 < 15% or HG-Ⅲ), HER2-enriched (ER < 10%, PgR < 10% and HER2-positive) and triple-negative (TN) (ER < 10%, PgR < 10% and HER2-negative). Stromal (s)TIL was prospectively evaluated in preNAC core biopsy and was defined as percentage of stromal area covered by lymphocytes[16].

Follow-up and recurrence information (date and location) were obtained from patient files. Time-from-last-chemotherapy-to-surgery was considered as the number of months from the date of the last NAC administration to surgery of the primary tumor. OS was calculated from surgery date of the primary breast tumor to death or last follow-up date, and DFS was calculated from surgery date of the primary breast tumor to recurrence or last follow-up date.

Statistical analysis

Categorical comparisons and association analysis between clinical pathological features and pCR were carried out using the chi-square statistic or Fisher's exact test. Survival analysis, regarding OS and DFS, was performed using the Kaplan-Meier method, and differences between curves were estimated by log-rank test. In all cases, the level of alpha was set at 0.05 *a priori*. Statistical analysis was performed using SPSS v19 (IBM Corp., Armonk, NY, United States).

RESULTS

Clinicopathological description

There were 435 patients included in this study, with median age at diagnosis of 49 years (range: 24-84 years), median tumor size of 6.5 cm (range: 1.0-24.0 cm), T3 in 27.8% and T4 in 63.9%. Inflammatory disease was found in 29.2%. The most frequent clinical stages were ⅢB (60.5%) and ⅢA (18.6%). Ductal histology was found in 93.3%, high grade in 52.2%, ER+ status in 62.8%, PgR+ status in 51% and HER2+++ in 32.4%. Luminal A, Luminal B, HER2-enriched and TN phenotype was found in 24.6%, 37.9%, 17.7% and 19.8%, respectively. The most frequent NACs were doxorubicin-cyclophosphamide for 4 cycles followed by 12 weekly paclitaxel (67.18%), doxorubicin-cyclophosphamide for 4 cycles followed by every 3 wk paclitaxel in 4 cycles (18.85%) and doxorubicin-cyclophosphamide for 4 cycles alone (7.32%). The median time from the last chemotherapy to surgery was 63 d (maximum: 982 d). pCR was observed in 48 (11%) patients. Median percentage of sTILs was 40% (2%-95%) in the entire population and 70% (60%-95%) in patients with pCR. Recurrence was found in 35.7%. Median DFS was 7.54 and median OS was 5.16 years (95%CI: 4.16-6.15 years) (Table 1).

Clinicopathological factors associated to pCR according to BC subtypes

Association analysis found that pCR was associated with T1-2 (P = 0.045) and to high sTIL level (P = 0.029) in the entire population (Table 1). Higher sTIL level had a slight trend towards association with pCR (P = 0.054) in Luminal A, and smaller tumor size had a trend towards association with pCR (P = 0.098) in Luminal A. Clinical involvement of axillary lymph nodes was not associated to variation of pCR (Table 2). An additional analysis by level of axillary involvement found that N2-3 had lower rates of pCR than N0-1 only in TNBC (P = 0.018).

Clinicopathological factors associated with sTIL according to BC subtypes

Association analysis found that sTIL level was associated with grade Ⅲ (P < 0.001), no-Luminal A subtype (P < 0.001), ER-negative (P < 0.001), PgR-negative (P < 0.001), HER2-positive (P = 0.002) and pCR (P = 0.029) in the entire population (Table 1). Within each BC subtype, sTIL level remained associated with grade Ⅲ in Luminal B (P = 0.011) and TN (P = 0.006) subtypes, as well as cN+ in Luminal B (P = 0.02) (Table 3).

Prognostic clinicopathological factors according to BC subtypes

Survival analysis found longer DFS was associated with grade Ⅰ-Ⅱ (P = 0.006), cN0 (P < 0.001), clinical stage Ⅱ (P = 0.004), ER-positive (P < 0.001), PgR-positive (P < 0.001), Luminal A (P < 0.001) and pCR (P = 0.002). Longer DFS was associated with grade Ⅰ-Ⅱ in Luminal A (P = 0.033), N0-1 in Luminal A (P = 0.045) and TNBC (P = 0.01), clinical stage Ⅱ in Luminal A (P = 0.003) and TNBC (P = 0.038), and pCR in TNBC (P = 0.001) (Table 1).

Table 1 Clinical-pathological features *n* (%)

	Cases	sTIL ⩾ 50%	*P* value	pCR	*P* value	Overall Survival at 5 yr (OS = 50.1%)	*P* value	Progression free survival at 5 yr (DFS = 57.8%)	*P* value
	435	181		48					
Age (yr), median (range)	49 (24-84)	49 (24-84)	0.923	47 (28-80)	0.472		0.512		0.833
< 50	231 (53.1)	96 (35.2)		28 (12.1)		48.8%		59.7%	
⩾ 50	204 (46.9)	85 (36.7)		20 (9.8)		51.7%		55.9%	
Histological subtypes			0.928		0.234		0.512		0.497
Ductal	406 (93.3)	169 (43.6)		43 (10.6)		49.0%		57.5%	
Lobular	21 (4.8)	7 (36.8)		2 (9.5)		61.0%		55.2%	
Others	8 (1.8)	5 (62.5)		3 (37.5)		-		-	
Histological grade			< 0.001		0.170		0.001		0.006
G1-G2	200 (46.0)	59 (32.6)		17 (8.5)		57.1%		64.6%	
G3	227 (52.2)	119 (65.7)		29 (12.8)		42.8%		52.2%	
NR	8 (1.8)	3 (1.7)		2 (25)		83.3%		45.7%	
ER			< 0.001		0.098		< 0.001		0.000
No	162 (37.2)	89 (57.8)		23 (14.2)		36.1%		47.1%	
Yes	273 (62.8)	92 (35.2)		25 (9.2)		58.2%		64.3%	
PgR			0.003		0.246		< 0.001		0.000
No	213 (49)	104 (51.0)		27 (12.7)		41.0%		50.0%	
Yes	222 (51)	77 (36.5)		21 (9.5)		58.4%		64.8%	
HER2			0.002		0.135		0.334		0.135
No	294 (67.6)	106 (38.3)		28 (9.5)		53.7%		60.4%	
Yes	141 (32.4)	75 (54.3)		20 (14.2)		40.8%		52.3%	
Molecular subtypes			< 0.001		0.233		< 0.001		< 0.001
Luminal A	107 (24.6)	30 (29.7)		13 (12)		72.0%		76.1%	
Luminal B	165 (37.9)	61 (38.4)		12 (7)		50.6%		57.7%	
HER2-enriched	77 (17.7)	50 (66.7)		10 (13)		41.5%		54.9%	
Triple-Negative	86 (19.8)	40 (50.0)		13 (15)		32.5%		40.3%	
Tumor size (cm)			0.183		0.019		0.490		0.250
Median (range)	6.5 (1-24)	6.5 (1-16)		6.0 (2-15)					
cT									
cT1-cT2	36 (8.3)	19 (54.3)		8 (22.2)		55.0%		69.2%	
cT3-cT4	399 (91.7)	162 (42.6)		40 (10)		49.6%		56.8%	
cN			0.084		0.743		0.007		0.001
cN0	83 (19.1)	28 (35.0)		10 (12)		65.8%		77.0%	
cN1-cN2-cN3	352 (80.9)	153 (45.7)		38 (10.8)		47.2%		54.2%	
Clinical stage			0.192		0.088		0.155		0.004
Ⅱ	72 (16.6)	26 (36.6)		12 (16.7)		62.1%		74.3%	
Ⅲ	363 (83.4)	155 (45.1)		36 (9.9)		48.1%		55.4%	
sTIL%					0.002		0.598		0.747
Median (range)	40 (2-95)	70 (60-95)		65 (5-95)					
< 50%	266 (61.1)	0 (0)		20 (7.5)		49.6%		55.7%	
⩾ 50%	149 (34.3)	181 (100)		26 (17.4)		53.9%		63.1%	
Missing data	20 (4.6)	20 (0)		2 (10)		-		-	
TLCS (d)			0.411		0.633		0.317		0.156
Median (range)	63 (5-982)	58 (8-982)		65 (8-281)					
Shorter than median	207 (47.6)	91 (45.5)		22 (10.6)		48.5%		55.0%	
Longer than median	211 (48.5)	82 (41.4)		26 (12.3)		56.7%		61.2%	
Missing data	17 (3.9)	8 (47.1)		0 (0)		17.6%		46.3%	
pCR			0.029				0.002		0.002
No	387 (89)	154 (41.7)		0 (0)		47.4%		55.1%	
Yes	48 (11)	27 (58.7)		48 (100)		85.1%		84.9%	
Relapse			0.895		< 0.001		< 0.001		
No	284 (65.3)	118 (43.4)		42 (14.8)		81.6%		-	
Yes	151 (34.7)	63 (44.1)		6 (4)		8.58%		-	

TIL: Tumor-infiltrating lymphocytes; pCR: Pathological complete response; OS: Overall survival; DFS: Disease free survival; PgR: Progesterone; TLCS: Time-From-Last-Chemotherapy-To-Surgery.

Longer OS was associated with grade Ⅰ-Ⅱ (P < 0.001), ER-positive (P < 0.001), PgR-positive (P < 0.001), Luminal A (P < 0.001), cN0 (P = 0.007) and pCR (P = 0.002) in the entire population. It was also associated with older age in Luminal B (P = 0.042), to clinical stage Ⅱ in Luminal A (P = 0.017), and to cN0 (P = 0.045) and pCR in TNBC (P = 0.005) (Figure 1). Differences in TILs did not affect survival in the entire nor molecular subtype populations (Table 1 and Figure 2).

DISCUSSION

The biological heterogeneity of BC has been extensively described, and differences between intrinsic subtypes have been confirmed in the recent decade. We explored differences in the survival impact

Table 2 Association between response and Clinical-pathological features regarding molecular subtype *n* (%)

	Lum A			Lum B			HER2			TN		
	Total 107	pCR 13	*P* value	Total 165	pCR 12	*P* value	Total 77	pCR 10	*P* value	Total 86	pCR 13	*P* value
Age (yr)			1.000			0.315			0.507			0.157
median (range)	47 (28-75)	46 (28-62)		51 (25-84)	52 (39-69)		51 (28-80)	46 (29-80)		49 (26-73)	45 (28-68)	
< 50	72 (67)	9 (13)		78 (48)	4 (5)		37 (48)	6 (16.2)		44 (48)	9 (20)	
⩾ 50	35 (33)	4 (11)		87 (52)	8 (9)		40 (52)	4 (10)		42 (52)	4 (10)	
Histological subtypes			0.349			1.000			0.434			0.392
Ductal	97 (91)	11 (11)		153 (93)	11 (7)		73 (95)	9 (12.3)		83 (97)	12 (14)	
Lobular and others	10 (9)	2 (20)		12 (7)	1 (8)		4 (5)	1 (25)		3 (3)	1 (33)	
Histological grade			-			0.213			0.266			1.000
G1-G2	103 (97)	12 (12)		61 (39)	2 (3)		23 (30)	1 (4.3)		13 (15)	2 (15)	
G3	-	-		102 (61)	10 (10)		53 (69)	9 (17)		72 (85)	10 (14)	
NR	4 (3)	1 (25)		2 (1)	0 (0)		1 (1)	0 (0)		1 (0)	1 (100)	
Tumor size (cm)			0.102			0.213			0.511			0.620
Median (range)	6 (2-15)	5 (2-9)		7 (2-20)	6 (2-12)		7 (2.5-14)	6 (4-12)		7 (1-24)	8 (3-15)	
cT1-cT2	10 (7)	3 (30)		12 (7)	2 (17)		5 (6)	1 (20)		9 (10)	2 (22)	
cT3-cT4	97 (93)	10 (10)		153 (93)	10 (7)		72 (94)	9 (12.5)		77 (90)	11 (14)	
cN			0.306			0.222			0.270			0.021
cN0	27 (23)	5 (19)		28 (18)	0 (0)		53 (69)	5 (9.4)		14 (14)	4 (29)	
cN1-cN2–cN3	80 (77)	8 (10)		137 (82)	12 (9)		24 (31)	5 (20.8)		72 (86)	9 (13)	
Clinical stage			0.471			0.652			1.000			0.122
EC II	23 (20)	4 (17)		21 (12)	2 (10)		11 (14)	1 (9.1)		17 (16)	5 (29)	
EC III	84 (80)	9 (11)		144 (88)	10 (7)		66 (86)	9 (13.6)		69 (84)	8 (12)	
sTIL%			0.054			0.750			0.150			1.000
Median (range)	30 (2-90)	50 (10-90)		40 (5-90)	30 (8-90)		60 (5-95)	80 (30-95)		45 (2-90)	50 (5-80)	
< 50	71 (69)	6 (8)		98 (60)	6 (6)		25 (32)	1 (4)		40 (47)	6 (15)	
⩾ 50	30 (24)	7 (23)		61 (37)	5 (8)		50 (66)	9 (18)		40 (47)	6 (15)	
Missing data	6 (6)	0 (0)		6 (3)	1 (17)		2 (3)	0 (0)		6 (7)	1 (17)	
TLCS (d)			0.233			0.238			0.744			0.500
Median (range)	67 (14-458)	80 (16-281)		61 (5-412)	54 (8-140)		60 (11-240)	66 (37-106)		64 (8-982)	66 (14-122)	
Shorter than median	49 (48)	4 (8)		77 (45)	8 (10)		41 (53)	5 (12.2)		40 (48)	5 (13)	
Longer than median	57 (51)	9 (16)		76 (47)	4 (5)		33 (43)	5 (15.2)		45 (51)	8 (18)	
Missing data	1 (1)	0 (0)		12 (8)	0 (0)		3 (4)	0 (0)		1 (1)	0 (0)	
Relapse			0.121			0.753			0.300			< 0.001
No	87 (79)	13 (15)		109 (65)	9 (8)		46 (60)	8 (17.4)		42 (41)	12 (29)	
Yes	20 (21)	0 (0)		56 (35)	3 (5)		31 (40)	2 (6.5)		44 (59)	1 (2)	

TIL: Tumor-infiltrating lymphocytes; TLCS: Time-From-Last-Chemotherapy-To-Surgery.

of tumor features, including pCR and TIL levels in each of the four molecular subtypes. Rates of pCR are lower in Luminal-A (9.2%), HER2-enriched (13%) and TNBC (15.3%) subtypes. pCR is also associated with longer survival in the entire population as well as in TNBC (pCR = 92.3% *vs* not pCR = 26.5% 5-year OS, $P = 0.005$; and trend in Luminal A, Luminal B and HER2-enriched phenotypic subsets of our series). It is widely assumed that patients who achieve pCR have significantly better DFS and OS rates in all molecular subtypes[12-14,17-19]. von Minckwitz *et al*[6] found pCR was not associated with prognosis only in Luminal A tumors in a series of 6377 patients with anthracycline-taxane-based NAC from 7 randomized trials; some authors claim it is related to the observed continuous tumor shrinkage occurring in their ER-positive tumor group during extended NAC, different than early and short-period tumor shrinkage observed in the ER-negative group[6,18-24].

pCR was more frequent in small tumors for both the entire population and the Luminal A subtype in our series. This finding is concordant with the previously mentioned idea that the effect of chemotherapy in Luminal A is slower than in other subtypes. Besides, Baron *et al*[18] found a similar lower rate of pCR in tumor size larger than 5 cm ($P = 0.022$) in their entire series ($n = 608$), but no association in the Luminal setting ($P = 0.411$). Higher grade of axillary involvement (cN2-3) was associated with lower rates of pCR only in the TNBC subset of our series. This lower response in bulky metastases could explain the previously described TNBC paradox phenomena of higher pCR rates but also higher distant relapse[21].

pCR was associated with higher percentage of

Table 3 Association between percentage of tumor-infiltrating lymphocytes and clinical-pathological features regarding molecular subtype *n* (%)

	Lum A			Lum B			HER2			TN		
	< 50%	⩾ 50%	*P* value	< 50%	⩾ 50%	*P* value	< 50%	⩾ 50%	*P* value	< 50%	⩾ 50%	*P* value
	71	30		98	61		25	50		40	40	
Age (yr)			0.181			0.783			0.624			0.074
Median (range)	47 (28-75)	47 (36-74)		52 (28-73)	50 (25-84)		52 (28-66)	49 (29-80)		51 (26-73)	45 (27-73)	
< 50	50 (70)	17 (57)		46 (47)	30 (49)		11 (44)	25 (50)		16 (40)	24 (60)	
⩾ 50	21 (30)	13 (43)		52 (53)	31 (51)		14 (56)	25 (50)		24 (60)	16 (40)	
Histological subtypes			0.445			1.000			0.597			1.000
Ductal	66 (93)	26 (87)		91 (93)	57 (93)		23 (92)	48 (96)		39 (98)	38 (95)	
Lobular and others	5 (7)	4 (13)		7 (7)	4 (7)		2 (8)	2 (4)		1 (3)	2 (5)	
Histological grade			-			0.011			0.514			0.006
G1-G2	69 (97)	28 (93)		43 (44)	15 (25)		9 (36)	14 (28)		11 (28)	2 (5)	
G3	0 (0)	0 (0)		53 (54)	46 (75)		16 (64)	35 (71)		29 (73)	38 (95)	
NR	2 (3)	2 (7)		2 (2)	0 (0)		0 (0)	1 (2)		0 (0)	0 (0)	
Tumor size (cm)												
Median (range)	6 (3-13)	6 (2-15)		6 (3-20)	7 (2-15)		7 (3-14)	7 (3-14)		7 (4-24)	7 (1-16)	
cT			1.000			0.538			0.659			0.263
cT1-cT2	7 (10)	3 (10)		6 (6)	6 (10)		1 (4)	4 (8)		2 (5)	6 (15)	
cT3-cT4	64 (90)	27 (90)		92 (94)	55 (90)		24 (96)	46 (92)		38 (95)	34 (85)	
cN			0.890			0.020			0.631			0.762
cN0	18 (25)	8 (27)		22 (22)	5 (8)		6 (24)	8 (16)		6 (15)	7 (18)	
cN1-cN2-cN3	53 (75)	22 (73)		76 (78)	56 (92)		11 (44)	27 (54)		34 (85)	33 (83)	
Clinical Stage			0.666			0.141			0.742			0.576
EC II	17 (24)	6 (20)		16 (16)	5 (8)		3 (12)	8 (16)		9 (23)	7 (18)	
EC III	54 (76)	24 (80)		82 (84)	56 (92)		22 (88)	42 (84)		31 (78)	33 (83)	
TLCS (d)			0.631			0.882			0.502			0.141
Median (range)	64 (14-449)	70 (19-458)		61 (5-412)	58 (8-285)		68 (16-234)	56 (11-240)		74 (24-230)	51 (14-982)	
Shorter than median	34 (48)	13 (43)		48 (49)	28 (46)		12 (48)	28 (56)		15 (38)	22 (55)	
Longer than median	36 (51)	17 (57)		44 (45)	27 (44)		12 (48)	20 (40)		24 (60)	18 (45)	
Missing data	1 (1)	0 (0)		6 (6)	6 (10)		1 (4)	2 (4)		1 (3)	0 (0)	
pCR			0.054			0.750			0.150			1.000
No	65 (92)	23 (77)		92 (94)	56 (92)		24 (96)	41 (82)		34 (85)	34 (85)	
Yes	6 (8)	7 (23)		6 (6)	5 (8)		1 (4)	9 (18)		6 (15)	6 (15)	
Relapse			0.450			0.201			0.737			0.502
No	59 (83)	23 (77)		61 (62)	44 (72)		16 (64)	30 (60)		18 (45)	21 (53)	
Yes	12 (17)	7 (23)		37 (38)	17 (28)		9 (36)	20 (40)		22 (55)	19 (48)	

%sTIL was performed over 415 cases. There 20 missed values. TIL: Tumor-infiltrating lymphocytes; TLCS: Time-From-Last-Chemotherapy-To-Surgery.

sTILs in the entire population and also within the HER2-enriched subtype (P = 0.02). A trend towards association was found in Luminal A, Luminal B and TNBC. Different studies have found that high TIL levels in preNAC samples are associated to higher pCR rates in the entire BC population[25-27]. Wang *et al*[28] performed a meta-analysis with 23 studies including 13100 BC patients, and similarly found that high TIL level was associated with improved pCR rate in the entire population, and in HER2 and TNBC. A high TIL level significantly predicted longer OS in the entire population (P < 0.001) and in patients with HER2-positive (P = 0.005) BC and in TNBC patients (P < 0.001).

TIL showed association with grade Ⅲ tumors in the entire population and in Luminal B and TNBC subsets in our series. Similarly, Pruneri *et al*[29] describes that higher TIL levels have a trend towards association with HG3 (P = 0.052) and was associated to Ki67 ⩾ 50% (P < 0.0001) in a series of 897 TNBC cases, and could reflect the appearance of a larger amount of neoantigens that elicit an immunomediated response. Involvement of axillary lymph nodes was associated to higher TIL levels only in the Luminal B subset. High density of TILs has previously been described as associated to absence of lymph node involvement in the entire population of BC, and our results indicate that this association could differ by some subtypes[30]. Higher level of sTILs was not associated to longer survival in the entire population nor in any subtype in our series. This finding could be explained by the small size of our series and because the highest impact of TILs is over pCR instead of survival.

Our study has some limitations. First, because of the retrospective design of the study, different chemotherapy schemas were used depending on the oncologist decision and surgical election depending

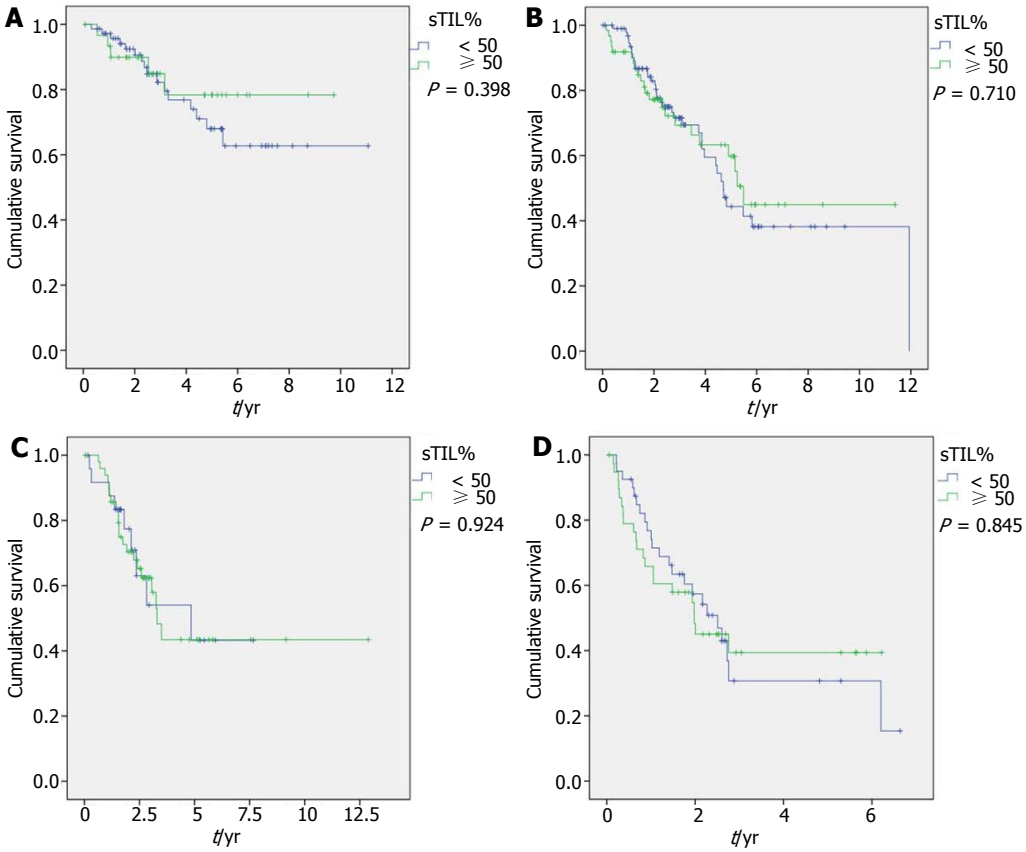

Figure 1 Overall survival regarding tumor-infiltrating lymphocytes (cut-off: 50%) for Luminal A (A), Luminal B (B), HER2-enriched (C) and Triple Negative group (D).

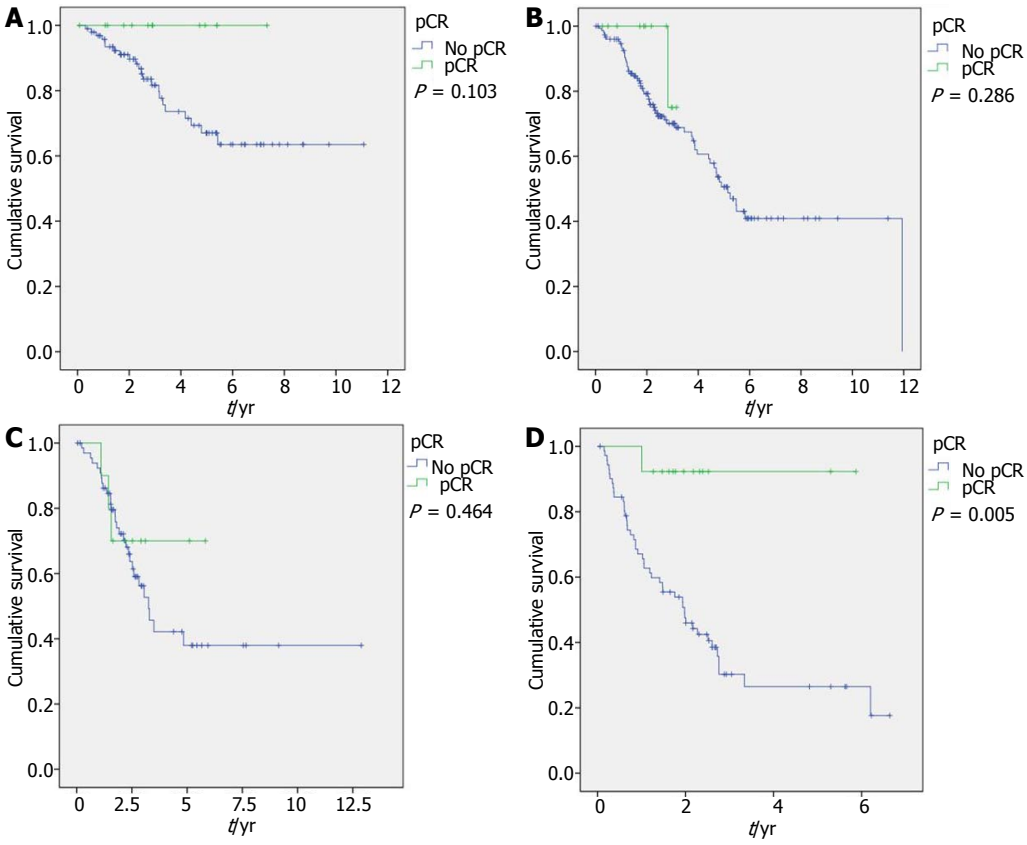

Figure 2 Overall survival regarding pathological complete response for Luminal A (A), Luminal B (B), HER2-enriched (C) and triple negative group (D).

on surgeon. Second, the sample sizes of each BC subgroup are rather small, so the prognostic impact of every clinicopathological feature in each BC subtype should be investigated in a larger population in subsequent studies. Despite these limitations, this is the first comprehensive report of the NAC effect over breast molecular subtypes in a Latin-American population.

ARTICLE HIGHLIGHTS

Research background

Breast cancer can be classified into Luminal A, Luminal B, HER2-enriched and triple-negative. Clinicopathological features can identify breast cancer prognosis and include pathological complete response (tumor sensibility to chemotherapy) and tumor-infiltrating lymphocytes (TILs; host activity against the tumor).

Research motivation

Discussion and new information about molecular breast cancer subtypes have been included in the most relevant cancer-related meeting, and more than 30,000 articles have been published in the last 2 years. Two biomarkers, pathological complete response (pCR) and TILs, have been re-defined and gained pathologist acceptance in the last 3 years.

Research objectives

The main objective is to evaluate the survival impact of different clinicopathological factors, including pCR and TIL levels, according to the subtypes in breast cancer patients who received neoadjuvant chemotherapy.

Research methods

Evaluation of TIL levels was prospectively performed following international guidelines. Breast cancer cases were classified according to 2017 St Gallen Breast Cancer Meeting guidelines.

Research results

pCR was associated with cT1-2 (P = 0.045) and high stromal (s)TILs (P = 0.029) in the entire population. However, this relationship was not found for every molecular subtype, probably because of the small sample size. pCR was associated with longer disease-free survival in the entire population (P = 0.002) and in TNBC (P < 0.001), as well as to longer overall survival in the entire population (P = 0.002) and in TNBC (P = 0.005).

Research conclusions

Predictive and prognostic value of clinicopathological features like pCR and sTIL level differ depending on the molecular subtype being evaluated. Identification of pCR and TIL roles in every molecular subtype will allow for identification of those patients who need more intense chemotherapy and those who will benefit from an immune-modulator treatment.

Research perspectives

No information about the relevance of pCR and TILs in South-American women with breast cancer have been published in. An increase in the knowledge about prognosis impact of pCR and TIL in every molecular breast cancer subtype will allow for obtaining more effective personalized therapies. Furthermore, similar analysis needs to be done with more precise methods to evaluate response to chemotherapy and host immune activity, such as tumor residual burden and CD3/CD8 ratio, respectively.

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