# Analysis of the cytogenetic response in peripheral blood lymphocytes from breast cancer patients following chemotherapy

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**Summary**

The presence of chromosomal aberrations induced in circulating lymphocytes from breast cancer patients during chemotherapy was analyzed. Ten breast cancer patients undergoing neoadjuvant chemotherapy and ten healthy women (controls) were evaluated. Metaphases were obtained from cultures of peripheral lymphocytes stimulated with phytohemaglutinin and metaphase blockage was achieved with colchicine. One hundred metaphases were analyzed for chromosomal aberrations and 1,000 cells for the mitotic index. No significant differences were observed regarding the frequency of chromosomal aberrations, number of cells with chromosomal aberrations and mitotic index between the controls and patients before chemotherapy. However, after the first chemotherapy cycle, the numbers of chromosomal aberrations and cells with them was greater. After the third cycle, the mitotic index was lower, but the fifth cycle produced an increase in relation to the third and fourth cycles. The results suggest that chemotherapy raises the number of chromosomal aberrations and favors persistence of stable chromosomal abnormalities.

*Key words:* Breast cancer; Chemotherapy; Chromosomal changes; Mitotic index.

## Introduction

Breast cancer is a complex disease that results from the interaction of multiple environmental, hormonal and lifestyle risk factors associated with the individual genome [1]. Its heterogenous clinical course results from different risk factors such as ethnicity, diet, age, environmental factors and cumulative exposure to estrogen. These factors are believed to be responsible for differences in tumor grade, degree of invasion, potential for metastasis and other complex signs of cell growth and survival [2].

Combined treatment started to be provided in 1974 consisting of primary (neoadjuvant) chemotherapy followed by surgery and/or radiotherapy and it has become commonly administered to patients with locally advanced breast cancer or those presenting inoperable margins [3]. This type of treatment may increase survival through eradicating distant micrometastases and diminishing the size of the tumor, thereby enabling surgery that is more conservative [4, 5]. Another important advantage of neoadjuvant chemotherapy is that it makes it possible to observe the response of the primary tumor to treatment [6].

Chemotherapy drugs act on cells to interfere with the growth and division process, mostly in a nonspecific manner. Thus, they are usually toxic to rapidly proliferating tissues with high mitotic activity and short cell cycles [6, 7]. Despite the benefits observed following chemotherapy, there is an increased risk of leukemia among breast cancer patients who undergo this type of treatment [8].

Evaluation of chromosomal aberrations is useful for studying radiosensitivity and risk factors. The micronucleus test on breast cancer patients has demonstrated that patients present greater numbers of micronuclei than controls do [9]. In a study conducted by our group, increased numbers of micronuclei were also demonstrated in patients with risk factors for cancer of the uterine cervix [10]. Several studies have evaluated the radiosensitivity of peripheral lymphocytes by means of culturing [11, 12], through new methodologies with cancer risk factor scores [9, 13, 14] and as treatment assessments [15]. Certain chromosomal abnormalities may characterize cancer with a poor prognosis [16].

Therefore, studying genetic aberrations may be used to analyze genetic damage following cancer treatment, and to determine risk factors. Few studies have analyzed the influence of neoadjuvant chemotherapy on treatments for breast cancer and genetic abnormalities. The aim of the present study was to analyze the presence of chromosomal aberrations induced in peripheral blood lymphocytes, in breast cancer patients. For this, patients were compared with a control group before any treatment and were also evaluated after each sequential chemotherapy cycle.

## Patients and Methods

### *Patients and controls*

Ten women with a diagnosis of breast cancer who underwent neoadjuvant chemotherapy without any type of previous antineoplastic treatment or use of immunosuppressor drugs were selected randomly and evaluated prospectively. All of these patients were attended at the Mastology Outpatient Clinic of the Oncological Research Institute (IPON)/Discipline of Gynecol-

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ogy and Obstetrics of the Teaching Hospital of the Federal University of the Triângulo Mineiro (UFTM). The samples were collected between 2004 and 2006. The diagnosis was made by means of clinical and mammographic examinations and confirmed by means of puncture for fine-needle aspiration biopsy and/or core biopsy. The anatomopathological staging followed the recommendations of the American Joint Committee on Cancer (AJCC), together with the Committee of the International Union against Cancer (UICC). This staging reflected the extent of the tumor expressed through the TNM system – tumor size (T), presence of axillary node (N) and/or metastasis (M) – and made it possible to then give priority to the most appropriate treatment [17]. Data such as age, ethnicity, side of the breast affected, drugs used in the chemotherapy and type and stage of the tumor were gathered from the patients' medical files.

The controls were healthy female volunteers from the community, i.e., they did not have any diagnosed disease and were not using immunosuppressor drugs. They were approached and invited to participate at the time when blood samples were being collected from the patients. The control and patient groups were paired with regard to age and presence of smoking habit.

The project was approved by the Research Ethics Committee of UFTM and all the patients who agreed to participate signed a free and informed consent statement.

*Chemotherapy*

The treatment was carried out over six or eight cycles, with 21-day intervals between the cycles and when the total leukocyte count was greater than or equal to 2000/mm$^3$. The latter was evaluated by means of a leukogram, produced on average two to three days before starting each cycle. The chemotherapy regimen consisted of one of the following combinations: (a) AC: adriamycin (50 mg/m$^2$) and cyclophosphamide (500 mg/m$^2$); (b) EC: cyclophosphamide (500 mg/m$^2$) and epirubicin (50 mg/m$^2$); or c) CMF: cyclophosphamide (500 mg/m$^2$), methotrexate (50 mg/m$^2$) and 5-fluorouracil (600 mg/m$^2$). Since the treatment was individualized, its maintenance for periods shorter or longer than what was initially prescribed was dependent on the tumor response.

*Blood collection*

Samples of peripheral venous blood were collected from the patients using disposable sterilized material and following all the principles of asepsis. This was done on two different occasions: (1) before the first chemotherapy cycle; and (2) around 21 days after finishing each cycle, immediately before starting the next cycle. The latter was the amount of time needed for recovery of the medullary aplasia induced by chemotherapy. On each occasion, one sample of 5 ml of blood was collected in a tube containing anticoagulant (heparin, 100 UI/ml), which was used for lymphocyte culturing. After collection, the samples were conserved at 4ºC for a few hours until the cultures were performed.

The same procedure was followed for collecting blood from the healthy volunteers on a single occasion.

*Lymphocyte cultures*

The presence of chromosomal aberrations was analyzed by means of metaphases obtained from lymphocytes [18]. The blood was collected in RPMI 1640 medium (Gibco®) and/or Dulbecco's medium (Gibco®) and was centrifuged (10 min; 1200 rpm) to separate the leukocytes. The lymphocytes were added to cultures containing 70% RPMI 1640 medium (Gibco®) and/or Dulbecco's medium (Gibco), 30% fetal bovine serum (Gibco®), 0.3% phytohemaglutinin (Sigma®) and 0.1% of glutamine. The cultures were incubated for 72 hours at 37°C. The metaphases were blocked by adding to each culture 25 µl of colchicine (0.16%), 60 min before cell collection. Two cultures were made from each sample.

After 72 h of incubation, cells were collected and were subjected to hypotonic treatment with 0.075M KCl, for 25 min. Next, they were fixed using a solution of methanol and acetic acid (3:1), three times. After fixing, the cells on their slides were stained using Giemsa solution and Sorënsen buffer for 5 min.

*Chromosome analysis*

The metaphases were analyzed in a blind test using an optical microscope with an immersion objective lens (magnification of 1000x). To quantify chromosomal aberrations, 100 metaphases were analyzed per individual. Only metaphases with 46 ± 1 chromosomes that were well spread out without overlapping of the chromosomes were used. Structural abnormalities such as gaps, breaks, acentric fragments, rings, dicentric chromosomes, triradial chromosomes, telomeric associations and exchanges were investigated, following the terminology that has been proposed [19, 20]. The mitotic index was determined as the ratio of the number of metaphases per 1,000 cells and was expressed as a percentage.

*Statistical analysis*

The results were evaluated by means of the Sigmastat 3.1 and Statistica 6.0 software. The Kolmogorov-Smirnov test was used to investigate whether the data presented normal distribution and the Levene test was used to investigate the homogeneity of the variance. Since the distribution was normal, the results were presented as means and standard deviations. ANOVA-F analysis was performed, followed by the unpaired Student's t-test, for comparisons between the controls and patients before chemotherapy, and the paired test between the groups before and after treatment. The significance level was 5%.

## Results

*Study population*

Ten patients with breast cancer and ten healthy female volunteers (forming the control group) were evaluated. The patients' mean age (± SD) was 54.10 ± 17.10 years (range 24-86 years) and the mean for the control group was 51.9 ± 17.93 years (range 21-85 years). Nine of the patients (90%) were white and one (10%) was black, while all the women in the control group were white. In six patients (60%), the tumor was in the left breast; three (30%) presented a tumor in the right breast and there was one case of bilateral cancer. In this last patient, 51.7% of the chromosomal aberrations in the metaphases were located in the group E chromosome. None of the members of this patient's family had breast cancer. The histological type most frequently found was ductal carcinoma, in eight cases (80%), while lobular carcinoma was diagnosed in two patients (20%). The chemotherapy regimens used were EC in five cases, AC in four and CMF in one.

*Chromosomal aberrations*

Chromosomal aberrations were analyzed in the metaphases of lymphocytes from the controls and patients. Out of the total of 4,700 metaphases from the patients analyzed, 213 cells with aberrations were found, with a total of 244 aberrations, thus indicating that some cells had more than one aberration. In the control group, 1,000 metaphases were analyzed and 12 cells with chromosomal aberrations were found. The principal aberrations encountered were simple abnormalities such as chromatid breaks, gaps, chromosomal breaks and fragments. Complex abnormalities such as rings and dicentric, triradial and quadriradial chromosomes were only found in the patients, but with lower frequency (Table 1). Before the chemotherapy, the patients presented a higher frequency of cells with chromosomal aberrations and greater number of cells with chromosomal aberrations than observed in the controls, although without reaching statistical significance (Table 2).

Table 1. — *Description of the types of chromosomal aberrations found in metaphases from breast cancer patients and controls.*

| Type of chromosomal aberration | No. of chromosomal aberrations % Controls (%) | Patients (%) |
|---|---|---|
| Chromatid gap | 3 (25.0) | 32 (13.11) |
| Chromosome gap | 2 (16.66) | 39 (15.98) |
| Chromatid break | 1 (8.33) | 38 (15.57) |
| Chromosome break | 1 (8.33) | 67 (27.46) |
| Ring | 0 (0) | 3 (1.23) |
| Dicentric chromosome | 0 (0.0) | 6 (2.46) |
| Fragment | 5 (41.66) | 57 (23.36) |
| Triradial chromosome | 0 (0) | 1 (0.41) |
| Quadriradial chromosome | 0 (0) | 1 (0.41) |
| Total number of chromosomal aberrations | 12 (12.0) | 244 (244.0) |
| Number of cells with chromosomal aberrations | 12 (12.0) | 213 (213.0) |
| Number of metaphases analyzed | 1,000 | 4,700 |

Table 2. — *Number of chromosomal aberrations (NCA), number of cells with chromosomal aberrations (NCCA) and mitotic index (MI) in the controls and in breast cancer patients who underwent chemotherapy. Values are expressed as means and standard deviations, with minimum and maximum values in between brackets.*

| Groups (n) | NCA | NCCA | MI |
|---|---|---|---|
| Controls | 1.2 ± 1.2 | 1.2 ± 1.2 | 1.9 ± 0.9 |
| (10) | (0-3) | (0-3) | (0.7-4.3) |
| Before chemotherapy | 3.5 ± 3.6* | 3.5 ± 3.6* | 2 ± 0.8*** |
| (10) | (0-12) | (0-12) | (1-3) |
| 1st cycle | 5.2 ± 4.6 | 4.8 ± 3.8 | 1.5 ± 0.6*** |
| (10) | (0-16) | (0-13) | (0.2-2.6) |
| 2nd cycle | 7.4 ± 5.0** | 5.1 ± 3.2 | 2 ± 2.1 |
| (8) | (0.17) | (1-9) | (0.3-7.1) |
| 3rd cycle | 5.5 ± 4.2 | 4.9 ± 3.0 | 1.1 ± 0.4# |
| (8) | (1-14) | (1-9) | (0.7-2) |
| 4th cycle | 7.0 ± 4.9 | 6.2 ± 4.2 | 1.5 ± 0.6# |
| (5) | (3-15) | (3-13) | (1.1-2.6) |
| 5th cycle | 3.2 ± 1.5 | 3.2 ± 1.2 | 2.1 ± 1 |
| (6) | (2-5) | (2-5) | (1-3.5) |

n = number of individuals; mean ± standard deviation; $V_{MIN}$ and $V_{MAX}$ = minimum and maximum values, respectively.
ANOVA-F for repeated measurements (F = 3.171; $p < 0.05$); paired t test, * $p < 0.01$ in comparison with the fourth cycle; ** $p < 0.05$ in comparison with the third cycle; *** $p < 0.01$ in comparison with the third cycle; # $p < 0.05$ in comparison with the fifth cycle.

Increases in the total number of chromosomal aberrations and the number of cells with chromosomal aberrations were also observed between the first and fourth treatment cycles, in comparison with the findings before chemotherapy. Statistical significance was reached after the fourth cycle. After the fifth treatment cycle, there was a return to close to baseline values (Table 2). There was no statistically significant difference in mitotic index between the control and patient groups before the chemotherapy, but there was a significant decrease in mitotic index after the third treatment cycle, in relation to before the chemotherapy and after the first cycle, and there was an increase after the fifth cycle (Table 2).

## Discussion

The purpose of this study was to analyze the effect of chemotherapy on the frequency of chromosomal aberrations in lymphocytes from women with breast cancer. Cytogenetic studies are a classical means of evaluating mutagenicity and clastogenicity because of their sensitivity of response to agents that induce DNA damage. The frequency of chromosomal aberrations in lymphocytes from human peripheral blood has been used as a marker for the initial effects induced by occupational exposure or chemotherapy in specific types of tissue. Assuming that the mechanisms for chromosomal damage are similar in different types of tissue, the level of damage to lymphocytes may reflect the damage induced in other types of tissue [21]. An accumulation of chromosomal abnormalities may affect critical genes involved in cell proliferation, differentiation and survival and thus direct the processes of the multiple stages in the development and progression of cancer [22].

Among the patients evaluated in this study, 70% were aged between 40 and 69 years, which was in accordance with data showing that the greatest incidence of breast cancer affects women within this age group. The left breast was the one most affected and, since this was more voluminous than the right breast, increased volume of breast tissue might be associated with a greater likelihood of mutations [6]. Only one of the patients was black, and this is in line with other studies that have demonstrated greater incidence among white women [6, 23]. Cytogenetic studies have demonstrated that white women with breast cancer present greater numbers of chromosome abnormalities than women of other ethnicity do [24]. Ductal carcinoma was more frequent than lobular carcinoma, and this was concordant with other studies [25] and with data obtained at our clinic, at which 90.6% of the cases diagnosed were ductal carcinoma [26].

Blood was not collected from all of the patients in relation to all cycles for a variety of reasons, such as cases that received blood transfusions because of leukopenia, difficulty in performing venous puncture and patient debilitation. Despite the small number of patients, the study was shown to be representative, with characteristics similar to those of studies with greater study populations.

The abnormalities in group E chromosomes that were

seen in one patient may have related to inactivation of tumor suppressor genes or activation of proto-oncogenes such as *p53, BRCA, E-cadherin and HER-2,* which are found in the chromosomes of this group [27-30].

To evaluate the residual chromosomal aberrations induced by chemotherapy and obtain the greatest number of metaphases, the blood collection was performed immediately before each cycle. In this way, it was expected to find stable chromosomal aberrations that had not been eliminated by the cells during the repair. The chromosomal aberrations observed most frequently, both in the control group and in the breast cancer patients, were simple abnormalities such as gaps, chromatid breaks, chromosome breaks and fragments. Studies have correlated the presence of chromatid breaks as a response to the action of chemotherapy agents [21]. In tests on G2 radiosensitivity to chemotherapy, greater frequency of breaks and gaps was also observed [31].

The frequency of chromosomal aberrations found in the control group was within the baseline frequency range for healthy individuals, i.e., 1-2% [32]. Both greater frequency of cells with aberrations and greater numbers of aberrations were observed in the patients before the chemotherapy, in relation to the controls, although without any statistically significant difference. This was concordant with other studies [33] and suggests that the cells of cancer patients present a higher frequency of abnormalities. Patients with breast cancer present more DNA damage than is seen in control groups, according to the comet test [34]. They also present greater lymphocyte sensitivity to induction of chromosome damage by means of radiation, as shown by the micronucleus test [35, 36].

Following chemotherapy there was an increased frequency of chromosomal aberrations, reaching significance after the fourth cycle, compared with before the treatment. However, after the fifth cycle, there was a reduction in the frequency of aberrations, such that values close to baseline were reached, thus demonstrating that the cell damage was probably undergoing repair. Among groups of patients with lung cancer and ovarian cancer, increased frequency of micronuclei during the first half of the therapy have been reported, with a peak in the second or third cycle and subsequent decline with continuing treatment, thereby reaching values lower than found before the treatment. Two possible mechanisms may be involved in these results: 1) repopulation with leukocytes may have occurred faster than the formation of cytogenetic damage; or 2) the lymphocytes became resistant to chemotherapy drugs [37]. Other studies have detected accumulations of chromosomal aberrations over the last two cycles of chemotherapy, thus demonstrating the difficulty of recovering the damage induced by chemotherapy [6].

Analysis of cell proliferation and progression in tissues exposed to clastogenic agents may be used to observe these agents' influence on the cell cycle. Any disturbance to the events controlling the progression of cell division may stop the cells from following their normal course. They might remain halted in one phase, or apoptosis might be induced [38]. No statistically significant difference in mitotic index was found between the controls and breast cancer patients, perhaps because an increase in the number of chromosomal aberrations occurred between these groups. There was a significant reduction in the mitotic index after the third cycle, in relation to before the chemotherapy and after the first cycle, along with an increase in the number of chromosomal aberrations, thus demonstrating that the cells were not undergoing repair yet. However, an increase in the mitotic index was also found in the fifth cycle, compared with the third and fourth cycles, thereby showing that the cells had started to undergo repair of the damage caused by the chemotherapy and taking the number of chromosomal aberrations towards pretreatment values.

The variation between individuals may have been due to differences in how the chemotherapy drugs were metabolized [39] and differences in the degree of reduction of leukocyte numbers [37]. The persistence of high frequencies of cells with rearrangements that seem to be stable for many years after finishing the cyclophosphamide, methotrexate or 5-fluoracil therapy suggests that although these cells present severe aberrations, they become viable progenitors in that they enable survival and cell proliferation. Within this context, there may be increased incidence of hematological diseases secondary [40] or immunological changes [41, 42] after chemotherapy administration for cancer treatment.

In conclusion, the results suggest that evaluation of the cytogenetic damage to lymphocytes from breast cancer patients may be able to estimate the sensitivity to chemotherapy, considering that persistence of stable chromosomal aberrations may lead to increased risk of secondary neoplasia.

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