D-dimer as a possible prognostic marker of operable hormone receptor-negative breast cancer

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Background: Breast cancer is the most common cause of death in women by neoplasia. The mechanisms related to recurrence are unclear, specially the hemostatic alterations that occur during the development of the disease. Plasma D-dimer is a hypercoagulability and fibrinolytic system marker and is increased in patients with various solid tumors. The purpose of this study was to evaluate the hemostatic status assessed by plasma D-dimer in operable breast cancer patients and to investigate its value as a prognostic marker.

Materials and methods: The study comprised 32 patients with operable hormone receptor-negative breast cancer and a control group with 43 healthy women. Variables included presence and absence of breast cancer, clinical and histopathology findings, and overall survival.

Results: Plasma D-dimer level was normal in the control group and significantly higher in breast cancer patients ($P = 0.001$), as well as in nonsurvivors compared with survivors ($P = 0.025$). The results showed that plasma D-dimer levels were not correlated with clinical and histopathology findings ($P > 0.213$).

Conclusions: The results taken together indicate the presence of a hypercoagulability state in women with operable hormone receptor-negative breast cancer given the increased levels of D-dimer in this group. Therefore, considering higher levels of D-dimer in patients with a poor outcome, its evaluation may be a promising tool for prognosis in women with operable hormone receptor-negative breast cancer.

Key words: D-dimer, hormone receptor negative, hypercoagulability, operable breast cancer, prognostic

introduction

Breast cancer, the most common female malignancy, represents a heterogeneous group of tumors, which present both varied behaviors and response to therapy. The development of distant metastases is the primary cause of death in breast cancer patients. The involvement of axillary lymph nodes, larger size of tumor, hormone receptor negative, presence of the mutation of protein 53 (p53), and vascular invasion determine poor prognosis and treatment options upon initial diagnosis [1, 2]. Upon diagnosis, ~50% of the patients present no involvement of the axillary lymph nodes and can therefore be considered cured after primary locoregional treatment. However, ~20%–30% will experience distant relapse within 5–10 years, indicating an outgrowth of disseminated tumor cells present upon diagnosis yet undetectable by the current diagnostic tools [3].

The three strongest prognostic determinants in operable breast cancer used in routine clinical practice today are lymph node status, primary tumor size, and tumor grade. Previous studies have shown independent prognostic significance for histological grades in breast cancer, providing strong evidence for the importance of routine assessment of histological grades in invasive ductal carcinoma (IDC) [2, 4–6].

Overexpression of HER2, a transmembrane protein with tyrosine kinase activity and a member of the human epidermal growth factor receptor gene family is associated with a poorer clinical outcome [7, 8]. Twenty-five to thirty percent of human breast malignancies with a more aggressive phenotype showed HER2 overexpression [9].

More recently, studies have reported the association between HER2 status and the hormone receptor negative. Patients with the hormone receptor-negative and HER2-negative status tend to present a less favorable prognosis than those with HER2-positive status. In fact, HER2-positive patients have been benefited by target therapy. As a consequence, patients with a breast cancer estrogen receptor (ER) negative, progesterone receptor (PgR) negative, and HER2 negative (triple-negative breast cancer) are considered a subset of poorer prognosis [10].
Many studies have already analyzed HER2 and p53 protein expression in ductal carcinoma in situ and IDC [11, 12]. Both the functional loss of p53 and the overexpression of HER2 are important for breast cancer progression in postmenopausal women [13]. The p53 mutation is more frequent in ER-negative tumors with a worse prognosis when compared with ER-positive tumors [14]. Overexpression of nuclear proteins, such as the Ki-67 antigen, may also indicate a poorer prognosis, especially when considering its association with a cell’s capacity to proliferate [15].

Epidemiologic studies in postmenopausal women have consistently showed that reproductive steroid hormones contribute to the primary risk of breast cancer. However, breast cancer is more aggressive in premenopausal than in postmenopausal women, mainly due to poor prognoses for young women [2].

The association of breast cancer and hemostasis activation has been previously reported and runs in-line with disease progression. Fibrin in the tumor may increase endothelial motility, worsen angiogenesis, and contribute to an increased risk of thrombosis in patients with breast cancer [16, 17].

Currently, studies concerning the blood factors involved in angiogenesis and coagulation are scarce [18–20]. It has been indicated that an interaction between angiogenesis and hemostasis may facilitate metastasis in breast cancer and that plasma D-dimer levels are a measure of matrix remodeling in the tumor [21]. Elevated levels of circulating D-dimer have been correlated with an enhanced progression of the disease and a reduced overall survival in metastatic breast cancer [17]. D-dimer assessment constitutes a first attempt to consider a product of fibrin degradation as a specific marker related to the extent of disease in human breast cancer. Several studies have confirmed this finding, correlating regulated fibrinolytic activity and increased D-dimer levels in the metastatic disease [12, 21–23].

Although hormone receptor-negative patients present a worse prognosis than do those with hormone receptor-positive tumors, few studies have attempted to establish possible differences and new perspectives for the treatment of these patients. The purpose of this study was to investigate the significance of the D-dimer as a prognostic marker in a group of Brazilian women with hormone receptor-negative breast cancer and its relationship with other variables, such as histological classification, tumor size, menopausal status, postoperative survival, lymph node status, and the immunohistochemistry receptors p53, HER2, and Ki-67.

There are few studies focusing on hemostatic changes in patients with hormone receptor-negative breast cancer and no study has been conducted in Brazil to date. This study may determine the relevant characteristics for the target population, thus bringing insights into the better management of treatment of these patients.

materials and methods

subjects and analysis

In our study, 32 patients with hormone receptor-negative and operable breast cancer (average age of 48.5, ranging from 29 to 71 years of age) were enrolled. As a control group, 43 healthy patients (average age of 45.4, ranging from 31 to 67 years of age) with no previous family history of thrombosis or cancer were studied. Only nonusers of hormone therapy or contraceptives were included in both the groups. The participants were recruited from an oncology unit (Centro de Pesquisas Oncológicas), in Itajai, Santa Catarina, Brazil. This study was approved by the Universidade Vale do Itajaí and Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais Ethical Research Committees, and written informed consent was obtained from all subjects.

blood collection

Blood samples from women with operable breast cancer were obtained at least 2 weeks after surgery. The blood samples from the control group were collected at the beginning of the study. Five milliliters of peripheral blood were collected with citrate (trisodium citrate 0.129 mol/l, whole blood cell ratio 1 : 9) and centrifuged at 2000 g for 20 min. Next, all plasma samples were divided into aliquots, frozen, and preserved at −70°C until assays were carried out.

quantitative plasma D-dimer level determination

Quantitative D-dimer levels were measured using the Immunoclonel® D-Dimer ELISA (American Diagnostic Inc., CT) and enzyme-linked immunosorbent assay plate reader (SpectraMax-340), according to the manufacturer’s instructions. The results were obtained by a standard curve prepared according to the manufacturer’s instructions. The D-dimer in normal human plasma is usually <400 ng/ml.

histological and immunohistochemical analysis

For histological evaluation, tissue sections (3 mm) were deparaffinized with xylene and stained using hematoxylin and eosin. Tumors were graded histologically according to the modified Bloom–Richardson grading system [6]. Immunohistochemical detection of ER, PgR, p53, Ki-67, and HER2 was assessed in the sample sections (5-μm slices), using monoclonal mouse anti-ER (1 : 100) (DAKO Carpinteria, CA), monoclonal mouse anti-PgR (1 : 200) (DAKO), monoclonal mouse anti-p53 (1 : 500) (DAKO), monoclonal mouse anti-Ki-67 (1 : 300) (DAKO), and polyclonal mouse anti-HER2 (1 : 1500) (Novoceastra Laboratories Ltd, Newcastle upon Tyne, UK; 1 : 200), respectively. High-temperature antigen retrieval was carried out by immersing the slides in a water bath at 95–98°C in 10 mM trisodium citrate buffer (pH 6.0) for 45 min. The nonspecific binding was blocked by incubating sections for 1 h with goat normal serum diluted in phosphate-buffered saline (PBS). After overnight incubation at 4°C with primary antibodies, the slides were washed with PBS and incubated with the secondary antibody LSAB plus (DAKO), following the manufacturer’s instructions. The sections were washed in PBS, and the visualization was completed by the use of 3,3’-diaminobenzidine (DAKO) in a chromogen solution and counterstained lightly with Harris’s hematoxylin solution. The immunohistochemical staining was evaluated upon visual inspection using an optical microscope (Nikon Eclipse 50i®, Nikon Instruments Inc., NY).

survival

The number of survivors was evaluated 8 months after the diagnosis and after patients had finished chemotherapy.

statistical analysis

Statistical analysis was carried out using SigmaStat (version 3.5; Systat Software, Inc., Washington, DC) and GraphPad Prism (version 4.0; Graphpad Software, Inc., San Diego, CA). The D-dimer results did not follow a normal distribution and were expressed as medians and interquartile ranges (IQRs). Differences between groups were tested using the Mann–Whitney U test and Kruskal–Wallis test for comparison between two and three groups, respectively; P <0.05 was considered significant.
results

The most widely used grading system of breast cancer is the Nottingham combined histological grade (Elston–Ellis modification of the Scarff–Bloom–Richardson grading system). The sum of these scores stratifies breast tumors into three grades (grade 1, well differentiated; grade 2, moderately differentiated; and grade 3, poorly differentiated). According to the histological grade tumor differentiation, 9.4% of the patients were GI, 25% were GII, and 65.6% were GIII. 46.9% of the patients were at a postmenopausal status (Table 1).

The tumor’s histologic characteristics identified 81.2% as IDC. Seventeen patients (53.1%) presented lymph node negative, whereas 15 (46.9%) patients presented lymph node positive. The immunohistochemical characteristics showed receptor HER2 positive in 8 patients (25%), while 7 patients were inconclusive (21.9%) and 17 patients (53.1%) presented receptor HER2 negative. Mutation p53 was observed in 10 patients (31.3%), while in 23 (68.7%) this mutation was not observed. According to the cell’s capacity to proliferate, 15 patients presented Ki-67 <30% (45.9%), while 17 presented Ki-67 >30% (53.1%).

Plasma D-dimer levels in women with breast cancer were analyzed according to several clinical variables. Upon comparing women with breast cancer to the control group, a statistically significant difference between the groups (P < 0.001) was observed. Median plasma D-dimer levels in patients with breast cancer and the control group were 502.8 pg/ml (IQR 264.4–730.9) and 285.7 pg/ml (IQR 202.0–367.8), respectively (Figure 1A).

Regarding survival, D-dimer results were significantly lower in survivors (470.7 pg/ml; IQR 244.1–603.3) when compared with nonsurvivors (1042.4 pg/ml; IQR 590.8–1916.1) (P = 0.025; Figure 1B). The nonsurvivors (six patients) died within a period of 8 months after diagnosis.

The D-dimer median for women with negative and positive axillary lymph nodes, whose values were 446 pg/ml (IQR 238.6–612.8) and 585.0 pg/ml (IQR 339.0–893.3), respectively (Figure 2A), presented no significant difference (P = 0.260). In addition, no difference between the triple-negative (18 patients) and non-triple-negative groups (14 patients) could be observed (P = 0.970). The median for the triple-negative group was 502 pg/ml (IQR 261–754), while for the non-triple-negative group it was 610 pg/ml (IQR 273–1231) (Figure 2B).

The D-dimer median for women with receptor HER2 negative was 503.4 pg/ml (IQR 269.9–701.6), while for those with inconclusive HER2 it was 368.1 pg/ml (IQR 260.3–581.0). In contrast, HER2-positive women presented a D-dimer median of 759.4 pg/ml (IQR 289.1–1249.2). No statistically significant difference among groups could be observed (P = 0.823) (Figure 3A). Regarding the p53 variable, the D-dimer’s results showed a median of 571.6 pg/ml (IQR 203.7–1203.0) for mutation p53 versus a median of 524.5 pg/ml (IQR 290.7–672.3) when the mutation was absent, with no statistically significant difference (P = 0.984) (Figure 3B).

Considering the proliferate marker Ki-67, patients were classified into two groups: Ki-67 <30% and Ki-67 >30%. No statistically significant difference between them was found since the Ki-67 <30% group presented a D-dimer median 384.5 pg/ml (IQR 257.3–600.2), while the Ki-67 >30% group was 759.4 pg/ml (IQR 289.1–1249.2). No statistically significant difference among groups could be observed (P = 0.823) (Figure 3A).

Table 1. Clinicopathological characteristics of 32 women with hormone receptor-negative breast cancer

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
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<th>%</th>
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<tbody>
<tr>
<td>Survivors</td>
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</tr>
<tr>
<td>Nonsurvivors</td>
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<tr>
<td>Menopausal status</td>
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<tr>
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<tr>
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<tr>
<td>GII</td>
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<td>25.0</td>
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<tr>
<td>GIII</td>
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<td>65.6</td>
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<tr>
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<tr>
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<tr>
<td>Positive</td>
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<td>25</td>
</tr>
<tr>
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<tr>
<td>Ki-67 &gt; 30%</td>
<td>17</td>
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IDC, invasive ductal carcinoma.

Figure 1. Plasma D-dimer levels, expressed as medians and interquartile ranges, in patients with breast cancer according to different variables and control group. (A) Breast cancer versus control group (P < 0.001); (B) breast cancer survivors versus nonsurvivors (P = 0.025).
presented a D-dimer median of 576.4 pg/ml (IQR 308.0–1342.2) ($P = 0.213$) (Figure 4A). A total of 12 patients presented metastasis. The median plasma D-dimer level for this group was 583.6 pg/ml (IQR 267.4–1042.4), but no difference was found when compared with the group with no metastasis (median = 474.1 pg/ml; IQR 250.2–927.9) ($P = 0.580$; Figure 4B).

**discussion**

The literature describes a strong correlation between cancer, angiogenesis, and hemostasis activation [24]. The association between markers of fibrin degradation in patients with progressive breast cancer indicates that the D-dimer level is a clinically important marker for progression and points toward a relation between hemostasis and tumor progression [17, 24]. This study is in partial agreement with this relation since plasma D-dimer levels were higher in patients with operable hormone receptor-negative breast cancer as compared with those without breast cancer (Figure 1A).

Elevated levels of plasma D-dimer have been correlated with enhanced progression kinetics and have commonly reduced the overall survival rates of patients with metastatic breast cancer. These results strongly indicate interactions between angiogenesis and hemostasis to facilitate metastasis in breast cancer [21]. Preoperative D-dimer levels have provided support to the role of increased plasma D-dimer levels in predicting progression.

![Figure 2](image1.png)

**Figure 2.** Plasma D-dimer levels, expressed as medians and interquartile ranges. (A) Patients with negative and positive axillary lymph nodes ($P = 0.260$); (B) tumor characteristic triple negative versus non-triple negative ($P = 0.970$).

![Figure 3](image2.png)

**Figure 3.** Plasma D-dimer levels in patients with breast cancer according to different variables expressed as medians and interquartile ranges. (A) D-dimer in human epidermal growth factor receptor 2 (HER2)-negative, HER2-inconclusive, and HER2-positive patients ($P = 0.823$); (B) D-dimer in p53-negative and -positive patients ($P = 0.984$).

![Figure 4](image3.png)

**Figure 4.** Plasma D-dimer levels in patients with breast cancer according to different variables expressed as medians and interquartile ranges. (A) D-dimer in Ki-67 <30% versus Ki-67 >30% patients ($P = 0.213$); (B) metastatic versus nonmetastatic breast cancer ($P = 0.580$).
vascular invasion, advanced tumor stage, and poor postoperative survival in cancer patients [19]. In agreement, in the present study, significant higher levels of D-dimer were observed among patients who died during the first year after surgery, when compared with those who survived \( (P = 0.025) \). This finding indicates that D-dimer may be an important prognostic marker (Figure 1B) since it is understood that their levels increase as the disease worsens. Moreover, it has been indicated that detectable fibrin degradation, as measured by the plasma D-dimer, is a clinically important marker for lymphovascular invasion and early tumor metastasis in operable breast cancer [25].

Clinical and biologically relevant factors in breast cancer have confirmed increased D-dimer levels as unfavorable predictive factors, but these should not be considered alone [25]. Some factors, such as an age <45 years, lymph nodes positive, tumor location, ER-negative status, histological grade, menopausal status, and a positive family history of breast cancer, have been reported as independent factors associated with locoregional recurrence risk [26]. Plasma D-dimer levels in our study showed no significant difference when clinical factors, including development of regional recurrences, menopausal status, age, family history, and histopathological findings were compared (Figure 2; \( P > 0.213) \). Therefore, no inference can be drawn regarding the correlation between D-dimer levels and clinical and histopathological findings. Although the literature describes the lymphonodal status as an important prognostic marker [27], the lymphonodal status did not significantly affect D-dimer levels (Figure 2A), indicating that this result may not be analyzed solely.

More recently, studies have reported that patients with ER negative, PgR negative, and HER2 negative (triple-negative breast cancer) showed a significantly shorter survival following the first metastatic event [10, 28]. According to previous studies [12, 21, 23], elevated plasma levels of D-dimer are associated with breast cancer progression and, consequently, a poorer outcome. Our results are in agreement with this finding, i.e. nonsurvivors presented significantly higher plasma D-dimer levels when compared with that from survivors. However, the present study showed no difference in D-dimer levels when comparing triple-negative and non-triple-negative patients (Figure 2B). This is at least a curious issue if we take into account that triple-negative patients may present a poorer prognosis. To our knowledge, this is the first study in which a clot activation marker has been used to compare hypercoagulability in triple- and non-triple-negative breast cancer patients. Nevertheless, this preliminary result calls for evidence from more vigorous studies involving a greater number of triple- and non-triple-negative patients.

In the present study, the immunohistochemical markers of p53, HER2, and Ki-67 were not correlated with plasma D-dimer levels (Figures 3 and 4). However, other authors have described the relation between poor prognosis and axillary node-negative breast cancer patients and ER negativity, grade III, and p53 positivity [29, 30]. Although not statistically significant, increased D-dimer levels were present in HER2-positive patients (Figure 3A). This finding is not surprising since the HER2-amplified tumors had higher angiogenesis [25]. In addition, D-dimer appears to be associated with other markers of tumor neovascularization [31] and with an up-regulation of urokinase plasminogen activator and in turn with an increase in fibrin degradation [32].

In conclusion, in this study, a correlation between breast cancer and hypercoagulability was observed as well as a relationship between the increase of plasma D-dimer levels and overall survival in operable hormone receptor-negative breast cancer patients, although plasma D-dimer levels were correlated neither with clinical and histopathological findings nor with the presence of metastases. To our knowledge, this is the first study of its kind in which D-dimer levels are assessed as a possible prognostic marker in hormone receptor-negative breast cancer patients according to different clinical and histopathological characteristics.

Altogether, these data indicate that the D-dimer is a promising prognostic marker related to breast cancer survival. This proposal may be suitable for laboratorial practice since D-dimer tests are not time consuming, require only a small plasma aliquot, and are low in cost. The main limitation of this study is the small number of women assayed. Therefore, additional prospective studies are required and may identify more effective uses of D-dimer as a prognostic marker for breast cancer, especially in women with hormone receptor-negative breast cancer.

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**disclosure**

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**references**