Methylation profiling in promoter sequences of \( \text{ATM} \) and \( \text{CDKN2A} \) (\( p14^{\text{ARF}}/p16^{\text{NK4a}} \)) genes in blood and cfDNA from women with impalpable breast lesions

LUCAS DELMONICO\(^1\), MAURICIO AUGUSTO SILVA MAGALHÃES COSTA\(^2\), ROMARIO JOSÉ GOMES\(^3\), PÂMELLA DE OLIVEIRA VIEIRA\(^3\), ANA BEATRIZ PASSOS DA SILVA\(^3\), MARCIA V. FOURNIER\(^4\), LUCIANO RIOS SCHERRER\(^5\), CAROLINA MARIA DE AZEVEDO\(^6\), MARIA HELENA FARIA ORNELLAS\(^3,7\) and GILDA ALVES\(^3,7\)

\(^1\)LaRBio-Radiation Laboratory in Biology, Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro 21941-902; \(^2\)Americas Medical City, Barra da Tijuca, Rio de Janeiro 22775-001; \(^3\)Circulating Biomarkers Laboratory, Department of General Pathology, Faculty of Medical Sciences, Rio de Janeiro State University, Rio de Janeiro 20550-170, Brazil; \(^4\)Dimensions Sciences, Washington, D.C. 20003, USA; \(^5\)Kennedy College, Belo Horizonte, Minas Gerais 31535-040; \(^6\)Department of Radiology, Gaffrée-Guinle University Hospital, Rio de Janeiro 22270-003; \(^7\)Postgraduate Program in Medical Sciences, Rio de Janeiro State University, Rio de Janeiro 20550-170, Brazil

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Abstract. The objective of the present study was to evaluate the epigenetic changes occurring in early stages of breast cancer. The present study investigated the methylation profile of the \( \text{ATM}, p14^{\text{ARF}} \) and \( p16^{\text{NK4a}} \) promoters in total blood and plasma cell-free DNA (cfDNA) from women with impalpable breast lesions compared with in total blood of a control cohort of women without breast lesions. The samples were evaluated using the methylation-specific PCR method. The Fisher’s exact test was used to evaluate statistical significance between the methylation and clinical variables. A total of 111 women were evaluated, including 56 women with impalpable breast cancer (39/56 also had paired plasma cfDNA) and 55 women in the control cohort (55 blood DNA). For blood DNA from women with malignant impalpable breast lesions, \( p16^{\text{NK4a}} \) exhibited the greatest percentage of methylation (48%), followed by \( \text{ATM} \) (37.5%) and \( p14^{\text{ARF}} \) (27%) promoters, regardless of age variation. For plasma cfDNA, the methylation rates for \( \text{ATM}, p14^{\text{ARF}} \) and \( p16^{\text{NK4a}} \) were 26, 26 and 10%, respectively. The methylation rates for the blood DNA of controls were the lowest for \( \text{ATM} \) (9%), \( p14^{\text{ARF}} \) (7%) and \( p16^{\text{NK4a}} \) (7%). The women with impalpable breast lesions (benign and malignant lesions) exhibited the highest methylation rate, regardless of age, compared with the paired plasma cfDNA and controls. This epigenetic change was statistically significant for the promoters of \( \text{ATM} \) (P=0.009) and \( p16^{\text{NK4a}} \) (P=0.001) (impalpable breast lesions vs. control). The present study demonstrated that epigenetic changes occurring in the \( \text{ATM} \) and \( \text{CDKN2A} \) genes detectable in liquid biopsy were associated with the development of impalpable breast lesions.

Introduction

In recent decades, the detection of impalpable breast lesions has increased due to the dissemination of mammographic screening programs and the improved resolution and accuracy of imaging tests (1,2). In general, impalpable breast lesions are small (<2 cm) with an initial histopathologic phenotype, for example, lesions in situ associated with infiltrative lesions, and positive estrogen and/or progesterone receptors (luminal) (3,4). Although the pathological scenario is favorable, surgical management has become a challenge. With conservative surgery, the residual tumor cells became a risk, increasing the possibility of relapses over the years. There is no estimation of the impalpable breast lesion relapse rate (4-7), however, for initial breast lesions, the risk of relapse for treated and not treated radiotherapy cases may reach 7 to 26%, respectively. Also, mortality over 15 years may reach 36% (8) which is higher than expected given the favorable prognosis.
Shedding of tumor cells in the blood circulation can occur simultaneously with primary tumor. This process which is part of metastasis may take weeks, or even decades, to develop and varies depending on the tumor type (9). During this period, tumor cells initiate the process of cellular plasticity and motility promoting their detachment from the primary site. On the other hand, an immune response is initiated to eliminate not only the tumor cells but possible circulating tumor cells (CTCs). In this battle between immune system and tumor, many apoptotic and necrotic tumor cells are phagocytized, increasing the concentration of cell-free DNA (cfDNA) in the blood which can be used as liquid biopsy. In some cases, this process starts very early, even in the absence of the primary site formed (10-13). In this context, in the course of carcinogenesis and invasion of tumor cells in the bloodstream, white blood cells (WBC) are constantly undergoing molecular alterations.

Hypomethylation and silencing of tumor suppressor gene expression by hypermethylation have been recognized as important markers for different cancers (14). Especially hypermethylation has been studied in DNA from WBC and revealed potential signatures to detect and predict breast cancer evolution (15,16). In this way, the p14[ARF]/p16[INK4a] and ATM genes are potential targets for epigenetic study, as they are described as hypermethylated in breast carcinogenesis (17-23). ATM plays a critical role in DNA double-break repair, involved in DNA damage recognition, recruitment of repair proteins, cell signaling for checkpoints, transcriptional regulation, and apoptosis activation (24). Hypermethylation in the ATM promoter has been reported in different types of cancers, including breast (20,24-26), glioma (27), gastric lymphoma (28), and colorectal neoplasia (29). The p14[ARF] and p16[INK4a] tumor suppressor genes (TSGs) are encoded within the CDKN2A locus on chromosome 9q21 (29). The encoded proteins are kinase-dependent inhibitors, and regulate the cell cycle under interference with the actions of p53 and Rb (29). Genetic and epigenetic alterations have been described in these genes in some cancers, including breast cancer (30), cervical intraepithelial neoplasia (31), follicular lymphoma (32), non-small cell lung cancer (33), and others (34,35).

To contribute to the understanding of the epigenetic changes detected by liquid biopsies of women with impalpable breast lesions, we analyzed the methylation pattern of ATM and CDKN2A (p14[ARF]/p16[INK4a]) promoter genes in total blood DNA and plasma cfDNA from women with impalpable breast lesions, and compared this with the blood DNA from a control cohort of women without breast lesion.

**Materials and methods**

**Study population.** The women with impalpable breast lesions were recruited in 2015-2016 at Americas Barra Medical City, in the city of Rio de Janeiro, Brazil. The control cohort of women with nipple aspirate fluid (NAF) without breast lesions were recruited in 2008-2012 from the Radiology Service at Hospital Universitário Gafré-Guinle (HUGG). The subjects enrolled in this study signed an informed consent and protocols were approved by ethics committee approval, Rio de Janeiro State University Hospital, no. CAAE:43560115.5.0000.5259 and HUGG-07/2007-80/2012. The control cohort was part of a previous study from our group (36) and were followed up to the year 2015, and none of them had developed benign or malignant breast lesions. All participants were subject to clinical evaluation, mammography, and/or breast ultrasonography. The patients with impalpable breast lesions were classified as Breast Imaging Reporting and Data System (BI-RADS)3 or 4. The NAF control subjects were classified by their macroscopic characteristics, including whether they were watery, citrine, serous, bloody, or mixed (seropurulent). Subjects were excluded from the study if they showed immunodeficiency or genetic syndromes or were previously diagnosed as cancer patients and in treatment. The patients’ clinical data were obtained from hospital/clinic records. The lesion histological classification was graded according to current (2012) World Health Organization (WHO) criteria (37), and nuclear grade was defined as grades I-III according to Elston and Ellis (38). For more details, the social demographic profile and clinical data of the cases are shown in Table I.

**DNA preparation.** Blood-4 ml of blood from controls and impalpable breast lesion patients was collected in EDTA and transferred to a 15 ml tube and then centrifuged at room temperature for 10 min at 2,000 x g. The plasma was discarded and 10 ml of erythrocyte lysing solution (4°C) was added to the cells (10 mM Tris-HCl, 5 mM MgCl2, 10 mM NaCl). The DNA extraction was performed by the Phenol-Chloroform method. The DNA samples were stored at -20°C until further analysis. For cfDNA extraction, 10 ml of blood from impalpable breast lesion patients were collected in EDTA before surgery, and centrifuged at room temperature for 10 min at 2,000 g. Supernatants were centrifuged at 16,000 x g for 10 min at 20°C to remove debris. Plasma was harvested and stored at -80°C. When DNA was to be analyzed, 2 ml was used to obtain cfDNA using the QIAamp® Circulating Nucleic Acid Kit (Qiagen), according to the manufacturer’s protocol. The blood DNA and cfDNA samples were quantified with the Qubit dsDNA HS Assay Kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol.

**Evaluation of ATM and CDKN2A promoter methylation.** For DNA modification reaction, the Epitect Bissulfite (Qiagen) and EZ DNA Methylation (Zymo Research) kits were used according to the manufacturer’s protocol. PCR amplification was performed in a reaction mixture containing 50 ng of modified genomic DNA, STR 1X buffer, 200 mM dNTPs; 3 mM of MgCl2, primers for each promoter (10 pmol/µl each), and 0.2 units Platinum® Taq DNA Polymerase (all from Invitrogen; Thermo Fisher Scientific, Inc.) in a final volume of 25 µl. The primers used for methylation specific polymerase chain reaction (MSP-PCR) have been previously described (39-41). Universal Methylated DNA Standard (Zymo Research) and DLD-1 cell line were used as positive controls for ATM and CDKN2A genes, respectively. PCR assays were performed in the Veriti™ DX Thermal Cycler (Thermo Fisher Scientific, Inc.). The PCR program consisted of a pre-denaturation at 94°C for the first 10 min, followed by 35 cycles at 94°C for 45 sec, 60°C (methylated and non-methylated primers) for 45 sec, and 72 for 1 min. The final extension was performed at 72°C for 7 min. MSP products were resolved in 10% polyacrylamide gel.
Table I. Social demographic and clinical data of the cases available.

| Characteristic                                      | Patients, n (%) (n=111) |
|-----------------------------------------------------|-------------------------|
| Age, years                                          |                         |
| Benign cases                                        |                         |
| Mean                                                | 45.5                    |
| SD                                                  | 7.12                    |
| Malignant cases                                     |                         |
| Mean                                                | 61                      |
| SD                                                  | 11.6                    |
| NAF cases                                           |                         |
| Mean                                                | 50                      |
| SD                                                  | 11.3                    |
| NAF classification                                  | N=55                    |
| Watery                                              | 4 (7)                   |
| Bloody                                              | 4 (7)                   |
| Mixed (seropurulent)                                | 4 (7)                   |
| Serous                                              | 18 (33)                 |
| Citrine                                             | 18 (33)                 |
| NI                                                  | 7 (13)                  |
| Malignant lesions                                   | N=48                    |
| IDC                                                 | 19 (40)                 |
| DCIS                                                | 5 (10)                  |
| IDC-DCIS                                            | 17 (35)                 |
| LCIS                                                | 1 (2)                   |
| ILC-LCIS                                            | 4 (8)                   |
| Micropapillary carcinoma                            | 2 (5)                   |
| Benign lesions                                      | N=8                     |
| Fibroadenoma                                        | 1 (12)                  |
| Ductal ectasia/apocrine metaplasia                   | 4 (50)                  |
| Hyperplasia of columnar cells with and without atypia| 3 (8)                   |
| Nuclear grade                                       | N=48*                   |
| I                                                    | 11 (23)                 |
| II                                                  | 26 (54)                 |
| III                                                 | 10 (21)                 |
| Unknown                                             | 1 (2)                   |
| TNM/Stage                                           | N=48*                   |
| T1N0M0 (stage I)                                    | 35 (73)                 |
| T1N1M0 (stage Ia)                                   | 6 (13)                  |
| T2N1M0 (stage Ia)                                   | 1 (2)                   |
| TisN0M0 (stage Ia)                                  | 6 (12)                  |
| ER status                                           | N=48*                   |
| Positive                                            | 40 (83)                 |
| Negative                                            | 2 (4)                   |
| Unknown                                             | 6 (13)                  |
| PR status                                           | N=48*                   |
| Positive                                            | 33 (69)                 |
| Negative                                            | 9 (19)                  |
| Unknown                                             | 6 (12)                  |

Table I. Continued.

| Characteristic                                      | Patients, n (%) (n=111) |
|-----------------------------------------------------|-------------------------|
| HER2 status                                         | N=48*                   |
| Positive                                            | 7 (14)                  |
| Negative                                            | 33 (69)                 |
| Unknown                                             | 8 (17)                  |
| Ki 67                                               | N=48*                   |
| Low (<20%)                                          | 21 (44)                 |
| Intermediate/High (≥20%)                            | 10 (21)                 |
| Unknown                                             | 17 (35)                 |
| BC subtype                                          | N=48*                   |
| Luminal A                                           | 33 (69)                 |
| Luminal B                                           | 7 (15)                  |
| Triple negative                                     | 2 (4)                   |
| Unknown                                             | 6 (12)                  |

*Malignant lesions. NAF, nipple aspirate fluid; NI, not informative; IDC, infiltrative ductal carcinoma; DCIS, ductal carcinoma in situ; ILC, infiltrative lobular carcinoma; LCIS, lobular carcinoma in situ; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal receptor 2; BC, breast cancer.

Statistical analysis. Contingency tables were used to associate the hypermethylation of each promoter ($ATM, p14^{ARF}, p16^{INK4a}$) with the specimens evaluated herein. For the purpose of statistical analysis and evaluation of the correlation between age and methylation, women were divided into age groups of ≤50 and >50 years old. The Fisher's exact test was adopted to test the statistical significance. The receiver operating characteristic (ROC) curve was used to evaluate the correlation between positive methylation and age for each gene analyzed. The survey data were processed in Predictive Analytics Software (PASW), version 20. In all statistical tests, a 5% significance level was considered. Thus, statistically significant associations were considered as those whose P-value was ≤0.05.

Results

Clinical data. One hundred and eleven women were included in this study, being 55 and 56 (plus 39 cfDNA) women with NAF and impalpable breast lesions, respectively. The women with benign breast lesions ranged in age from 27 to 49 years ($M=45.5$ years, $SD=7.12$), while the patients with malignant breast lesions ranged in age from 33 to 90 ($M=61$ years, $SD=11.62$). The NAF patients ranged in age from 30 to 82 years ($M=50$ years, $SD=11.32$). Following surgery, the histopathological diagnosis revealed 8/56 (14%) benign lesions and 48/56 (86%) malignant lesions. Malignant lesions were 40% infiltrative ductal carcinomas (IDC) and 35% mixed lesions (IDC with ductal carcinoma in situ). Immunohistochemistry revealed 83% ER-positive, 69% PR-positive, and 69% HER2-negative tumors. Thus, 69% of the malignant tumors were Luminal A gels at 160 V in 1 X TBE buffer (Tris-Borate-EDTA), stained by silver.
In relation to the staging, 83% of lesions were initials (T1N0M0) (Table I).

**Table II. DNA methylation pattern in promoters of ATM, p14^{ARF} and p16^{INK4a} genes.**

| DNA type                  | Gene     | Benign lesions, n (N=8) (%) | Malignant lesions, n (N=48) (%) |
|---------------------------|----------|-----------------------------|---------------------------------|
| Blood DNA from women with impalpable breast lesions | ATM      | 0                           | 18/48 (37.5)                   |
|                          | p14^{ARF}| 4/8 (50)                    | 13/48 (27)                      |
|                          | p16^{INK4a} | 3/8 (37.5)               | 23/48 (48)                      |

| DNA type                  | Gene     | Benign lesions, n (N=6) (%) | Malignant lesions, n (N=39) (%) |
|---------------------------|----------|-----------------------------|---------------------------------|
| cfDNA from women with impalpable breast lesions | ATM      | 2/6 (33.3)                  | 10/39 (26)                      |
|                          | p14^{ARF}| 0                           | 10/39 (26)                      |
|                          | p16^{INK4a} | 0                          | 4/39 (10)                       |

| DNA type                  | Gene     | Cases, n (N=55) (%) |
|---------------------------|----------|---------------------|
| Blood DNA from women with NAF | ATM      | 5/55 (9)            |
|                          | p14^{ARF}| 4/55 (7)            |
|                          | p16^{INK4a} | 4/55 (7)            |

**Table III. Distribution of DNA methylation pattern in promoters of ATM, p14^{ARF} and p16^{INK4a}, according to the two different age groups (≤50 and >50 years old).**

| DNA type                  | Gene     | ≤50 years Benign (N=8) (%) | ≤50 years Malignant (N=9) (%) | >50 years Malignant (N=39) (%) |
|---------------------------|----------|----------------------------|------------------------------|--------------------------------|
| Blood DNA from women with impalpable breast lesions | ATM      | 0                          | 4/9 (44)                     | 14/39 (36)                      |
|                          | p14^{ARF} | 4/8 (50)                  | 3/9 (33.3)                  | 10/39 (26)                      |
|                          | p16^{INK4a} | 3/8 (37.5)               | 5/9 (55.5)                  | 18/39 (46)                      |

| DNA type                  | Gene     | ≤50 years Benign (N=6) (%) | ≤50 years Malignant (N=6) (%) | >50 years Malignant (N=27) (%) |
|---------------------------|----------|----------------------------|------------------------------|--------------------------------|
| cfDNA from women with impalpable breast lesions | ATM      | 2/6 (33.3)                 | 1/6 (16.7)                  | 9/27 (33)                      |
|                          | p14^{ARF} | 0                          | 1/6 (16.7)                  | 9/27 (33)                      |
|                          | p16^{INK4a} | 0                          | 1/6 (16.7)                  | 3/27 (11)                      |

| DNA type                  | Gene     | ≤50 years (N=28) (%) | >50 years (N=27) (%) |
|---------------------------|----------|---------------------|---------------------|
| Blood DNA from women with NAF | ATM      | 3/28 (11)            | 2/27 (7.4)          |
|                          | p14^{ARF} | 2/28 (7.1)           | 2/27 (7.4)          |
|                          | p16^{INK4a} | 2/28 (7.1)          | 2/27 (7.4)          |

NAF, nipple aspirate fluid.

and 15% Luminal B. In relation to the staging, 83% of lesions were initials (T1N0M0) (Table I).

**Methylation analysis.** The DNA methylation pattern was assessed in the promoters of ATM, p14^{ARF}, and p16^{INK4a} in
56 blood DNA samples and 39 paired plasma cfDNA samples from the women with impalpable lesion and 55 blood DNA samples from women without breast lesion were. Further, in relation to blood DNA from women with malignant impalpable breast lesions, among the 3 promoter genes assayed, p16\textsuperscript{INK4a} showed the greatest percentage of methylation, regardless of age variation, followed by ATM and p14\textsuperscript{ARF} promoters. However, the p14\textsuperscript{ARF} gene had the highest rate of methylation for benign cases (Table II). This same result can be observed in the distributions by age (Table III). For the cfDNA samples, the methylation rates showed a pattern of positivity for all age groups and genes analyzed here (Table II). However, there was a slight increase in methylation rates for the promoters of ATM and p14\textsuperscript{ARF} in cases older than 50 years old (Table III).

Table IV. Association of the variable methylation of the ATM, p14\textsuperscript{ARF} and p16\textsuperscript{INK4a} genes among the groups of women with impalpable breast lesions and nipple aspirate fluid.

| Genes/Methylation | Groups (>50 years old) | NAF, n (%) | P-value |
|-------------------|------------------------|------------|---------|
| ATM               |                        |            |         |
| No                | 25 (64.1)              | 25 (92.6)  | 0.009   |
| Yes               | 14 (35.9)              | 2 (7.4)    |         |
| p14\textsuperscript{ARF} |                      |            |         |
| No                | 29 (74.3)              | 25 (92.6)  | 0.102   |
| Yes               | 10 (25.7)              | 2 (7.4)    |         |
| p16\textsuperscript{INK4a} |                    |            |         |
| No                | 21 (53.8)              | 25 (92.6)  | 0.001   |
| Yes               | 18 (46.2)              | 2 (7.4)    |         |

NAF, nipple aspirate fluid.

Table V. Association of the variable methylation of the ATM, p14\textsuperscript{ARF} and p16\textsuperscript{INK4a} genes among the groups of women with IBLs (blood and cfDNA).

| Genes/Methylation | Group (>50 years old) | IBL, n (N=39) | cfDNA, n (N=27) | P-value |
|-------------------|-----------------------|---------------|-----------------|---------|
| ATM               |                        |               |                 |         |
| No                | 25 (64.1)              | 18 (66.7)     | >0.999          |
| Yes               | 14 (35.9)              | 9 (33.3)      |                 |         |
| p14\textsuperscript{ARF} |                      |               |                 |         |
| No                | 29 (74.3)              | 18 (66.7)     | 0.584           |
| Yes               | 10 (25.7)              | 9 (33.3)      |                 |         |
| p16\textsuperscript{INK4a} |                    |               |                 |         |
| No                | 21 (53.8)              | 24 (88.9)     | 0.003           |
| Yes               | 18 (46.2)              | 3 (11.1)      |                 |         |

IBL, impalpable breast lesions; cfDNA, circulating free DNA.

Regarding the control subjects, there was no difference in the methylation positivity for p14\textsuperscript{ARF} and p16\textsuperscript{INK4a} (Table II). Two cases were methylated, for each age group or gene analyzed (Table III). For the ATM gene three cases showed methylation positivity for the group with ≤50 years, and two for >50 years old (Table III). For details of the histological types of hypermethylated cases shown in Tables II and III and SI.

The hypermethylation present in the group of malignant cases (blood DNA from cases with impalpable breast lesions) was higher than that found in the group of women without lesions (Tables II and III). From this association between groups, the promoters of ATM and p16\textsuperscript{INK4a} presented significant P-values of 0.001 and 0.009, respectively (Table IV). When comparing the same group of malignant cases with their respective cfDNA, only hypermethylation of the p16\textsuperscript{INK4a} promoter showed a significant P-value of 0.003 (Table V).

Nine cases had a methylation correlation between blood DNA and the respective cfDNA: Among these cases, 7/9 (77%), 4/9 (44%), 2/9 (22%), and 1/9 (11%) for ATM, p14\textsuperscript{ARF}, p14\textsuperscript{ARF}/ATM, and p16\textsuperscript{INK4a}, respectively. For clinical and histopathological details (Table SII).

The individual correlations (benign, malignant lesions, cfDNA of benign cases, cfDNA of malignant cases, and control subjects) between the methylation of each promoter (ATM, p14\textsuperscript{ARF}, and p16\textsuperscript{INK4a}) and age (ROC curve) did not reveal statistically significant values. For all analyses, P-value was >0.05 (data not shown).

**Discussion**

In the present study, we describe epigenetic changes occurring in liquid biopsies from women with impalpable breast lesions, compared to a control cohort of women without lesions. The women with impalpable breast lesions (benign and malignant lesions) had the highest methylation rate, regardless of age, compared to the cfDNA and control groups (Tables II and III). This change was statistically significant for the promoters of ATM (P=0.009) and p16\textsuperscript{INK4a}}
In our previous study (3), the methylation rates in 39 blood DNA samples from women with impalpable breast lesions were similar to those found here: Frequencies for the ATM, p14ARF and p16INK4a genes in the previous study were 41, 26, and 41%, respectively, whereas in the current study frequencies were 37.5, 27 and 48% (Table I), respectively. Both studies show the ATM and p16INK4a genes with high hypermethylation rate in malignant cases, suggesting the silencing in the repair pathways, senescence, and cell cycle control in the impalpable breast lesions establishment.

The ATM gene involvement in mammary carcinogenesis has been described by several studies, but presented with controversies (3,19,20,25,26). In the study by Cao et al (19) the authors evaluated more than 30 CpG islands of ATM gene (using MassARRAY Epithelial Assay and Infinium HumanMethylation450 BeadChip array) in peripheral blood from women with breast lesions, similar to those analyzed here, and reported 62% of mammary tumor stage I/II (N=229) (78% IDC, 69% ER positive, 63% PR positive, and 72% HER negative); interestingly, the authors did not find any significant difference in the ATM methylation levels between the breast cancer patients and the healthy controls. Brennan et al (20), evaluating ATM intragenic regions (ATMmpv2a and ATMmpv2b) in sporadic breast cancer cases (N=501), familial breast cancer cases (N=166), and controls (N=769), found a strong association of ATM methylation levels in the family group (P=4.87x10⁻⁶), and also in cases up to 59 years old (P=0.01). In relation to the young and familiar cases, it is not possible to compare with the data described here, because we analyzed sporadic breast cancer and the number of women under 50 years old is small, making statistical analysis unfeasible.

Regarding ATM hypermethylation in breast tissue, although not evaluated in this study, methylation rates have been described ranging from 36 to 78% (3,25,39). In the study by Begam et al (26) the frequency of methylation in sporadic mammary tumors was 59%, while in adjacent non-tumor tissue 4%. Further, the authors found association between promoter hypermethylation and lower ATM mRNA expression (P=0.035). For malignant and benign impalpable lesions, we found proximal frequencies of 63.4 and 33.3%, respectively, in our previous study (3).

In the study by Askari et al (22) p14ARF and p16INK4a hypermethylation in blood from women with breast cancer was 11 and 22%, respectively. Further, the authors found a significant association between hypermethylation for p14ARF (P=0.004) and p16INK4a (P=0.000) in women over 50 years old. The hypermethylation found in our study was superior, with 27 and 48% of methylated cases for p14ARF and p16INK4a, respectively. In addition, the significant association between hypermethylation of p16INK4a and ATM was revealed in women over 50 years old (Tables IV and V). These data show that hypermethylation of p14ARF and p16INK4a promoters significant association with breast cancer, hence indicating involvement in the breast tumor pathogenesis.

The methylation findings found in cfDNA in our study should be interpreted with caution. Despite the presence of circulating tumor DNA (ctDNA) in the bloodstream and metastases in the bone marrow in early cases (11,12), the concentration of cfDNA in these cases is lower than in metastatic cases (42). Hypothetically, in malignant cases, methylation in blood DNA may be a result of CTCs. In this study we found nine cases with cfDNA that coincided with the methylation pattern of the WBCs. Of these cases, seven were from women with malignant lesions, and only one with lymph node infiltration (Table SII). In this context, it is not possible to affirm that the hypermethylation found in these cases originated from the CTCs.

The p16INK4a gene has been reported methylated in the cfDNA of mammary tumors at a rate of 22% (23). In our study, this rate was 10% lower. In the study by Shan et al (23) the p16INK4a methylation in cfDNA associated with five other genes (SFN, hMLH1, HOXD13, PCDHGB7 and RASSF1a) reached sensitivity (79.6-72.4%) and specificity (82.4-78.1%) for the distinction of initial malignant lesions (N=268) of controls (N=245) and benign lesions (N=236), respectively. However, the authors emphasized the importance of study expansion, since the methylation found in the genes was associated with cases with a family history (P=0.0249), low proliferation index (Ki67) (P=0.0356), and luminal tumors (P=0.0314). These data corroborate with those presented here, since both studies used similar populations (Table I) except for cases with a family history.

Further, the evaluation of methylation through high technology platforms (MassARRAY Epityper assay, Illumina Infinium array, Infinium HumanMethylation450 BeadChip array, and Pyrosequencing) has shown higher sensitivity values (20,23,25,31,32,35), when compared to other conventional methods, as performed here. Thus, larger multicenter prospective study cohorts are needed to validate the findings here.

To our knowledge this is the first study that evaluates the methylation positivity in the promoters of the ATM and CDKN2A genes in liquid biopsies from women with impalpable breast lesions compared to women without lesions. This study is in progress and further analyses should be performed for molecular description of the factors involved with the development of impalpable lesions. Although methylation levels may be associated with environmental (43) and social (44,45) factors, the percentages disclosed here and by other studies (17,18,20,22,23,25) show that methylation levels of WBC are high in the presence of breast lesions.

In conclusion, we found high rates of methylation in blood from women with benign and malignant breast lesions. Regardless of nature, the breast lesions presence is capable of promoting epigenetic responses in liquid biopsies. The alterations detected here represent the systematic heterogeneity of every woman in front of the impalpable breast lesion installation. We believe that epigenetic changes in liquid biopsy may reveal potential biological biomarkers capable of complement biopsy results and predict the risk of lesion invasion or tumor response to treatment.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
LD, MHFO and GA conceived and designed the study. LD, RJG, PODOV and ABFPS conducted the experiments. LD, MASM, MVF, CMDA and GA recruited the cases, acquired pathological and radiological reports, and analyzed and interpreted the data. LD, LRS, MVF and GA obtained statistical data, and analyzed and interpreted data. LD, MVF, MHFO and GA were involved in drafting the manuscript or revising it critically for important intellectual content. All authors read and approved the final version.

Ethics approval and consent to participate
The present study was approved by the Institutional Ethics Committee of the Hospital Universitário Gafuré-Guinle (HUGG) and Rio de Janeiro State University Hospital (approval nos. H UGG-07/2007-80/2012 and CAAE: 43560115.5.0000.5259). The subjects enrolled in the present study signed the informed consent.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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