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Lucas Delmonico Rodrigues da Silva

Biomarcadores circulantes em mulheres com lesões impalpáveis da mama

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Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-Graduação em Ciências Médicas, da Universidade do Estado do Rio de Janeiro.

Orientadora: Prof.ª Dra. Gilda Alves Brown

Coorientadora: Prof.ª Dra. Maria Helena Faria Ornellas de Souza

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DEDICATÓRIA

À todas as mulheres portadoras de câncer de mama, que cada uma em sua individualidade, história e personalidade, buscam diariamente força, perserverança, e esperança para sobreviver. Eu desejo à todas: amor, alegria, paciência, resiliência, e o mais importante: a cura.

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O período de maior ganho em conhecimento e
experiência é o período mais difícil da vida de alguém.
Dalai Lama

RESUMO

SILVA, Lucas Delmonico Rodrigues da. **Biomarcadores circulantes em mulheres com lesões impalpáveis da mama**. 2019. 178 f. Tese (Doutorado em Ciências Médicas) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2019.

O câncer de mama representa a primeira causa de morte por câncer no mundo para mulheres. Neste contexto, a detecção precoce do câncer de mama aumenta consideravelmente as chances de cura. Entretanto a caracterização das lesões iniciais e impalpáveis tem se tornado um desafio. Histologicamente estas lesões mesclam células agrupadas *in situ* e infiltrativas, sendo difícil a predição do seu potencial de evolução. A busca por marcadores tumorais circulantes vem sendo amplamente explorada na tentativa de minimizar procedimentos invasivos, repetitivos e tratamentos agressivos nestes casos. O objetivo do trabalho foi detectar candidatos a marcadores circulantes envolvidos no desenvolvimento de lesões impalpáveis mamárias BIRADS-3 e 4. Para este fim, foi realizado o perfil de metilação dos promotores dos genes *CDKN2A (p14^{ARF}* e *p16^{INK4A}*) e *ATM*; a detecção de mutações somáticas nos genes *TP53* (éxons 4 ao 9), *CDKN2A* (éxons 1 ao 3), *PIK3CA* (éxons 9 e 20) no DNA de tecido tumoral e circulante e ainda, a identificação de proteínas diferencialmente expressas na saliva e no plasma das pacientes com lesões impalpáveis da mama. Em conjunto, estes objetivos geraram cinco publicações. Resumidamente duas coortes de mulheres (N=62 e N=56) foram avaliadas para a detecção de metilação nos promotores de *CDKN2A* (*p14^{ARF}* e *p16^{INK4A}*) e *ATM*. Para as duas coortes, as frequências de metilação no DNA do sangue foram de 41%-37,5%, 26%-27% e 41%-48% para os genes *ATM*, *p14^{ARF}* e *p16^{INK4a}*, respectivamente. A frequência de metilação encontrada nos tumores (N=62) foram de 1/62 (1,6%), 3/62 (4,8%) e 33/62 (53,2%) para os genes *p14^{ARF}* e *p16^{INK4a}* e *ATM*, respectivamente. Em relação a avaliação mutacional, os genes *PIK3CA*, *TP53* e *CDKN2A* foram sequenciados (PCR-Sanger) em 58 mulheres com lesões impalpáveis (49 malignas e 9 benignas) com o respectivo DNA circulante livre. Um total de 37 mutações foram encontradas, sendo 8/58 (14%), 18/58 (31%) e 11/58 (19%) para os genes *PIK3CA*, *TP53* e *CDKN2A*, respectivamente. Em relação aos dados proteômicos as principais proteínas diferencialmente expressas na saliva dos pacientes em comparação com os controles foram: α2-macroglobulina, ceruloplasmina, *leukocyte elastase inhibitor*, α-enolase, e *deleted in malignant brain tumors 1*. Em relação ao plasma, a alfa-2-macroglobulina e a ceruplasmina estão em super expressão, enquanto outras proteínas, como haptoglobina, hemopexina e proteína de ligação à vitamina D estavam em baixa expressão comparadas com o controle. Baseados nestes resultados, a vitamina D foi dosada no plasma de 65 pacientes com lesões mamárias impalpáveis e em 20 controles. A prevalência de deficiência e/ou insuficiência de vitamina D em mulheres com lesões malignas e nos controles foram de 84% e 60%, respectivamente. Neste estudo os resultados são inéditos, entretanto os candidatos a biomarcadores descritos aqui carecem de maiores investigações para descrição e validação do seu envolvimento no desenvolvimento das lesões mamárias impalpáveis.

Palavras-chave: Lesões mamárias impalpáveis. Detecção precoce. TP53. CDKN2A. PIK3CA. ATM.

ABSTRACT

SILVA, Lucas Delmonico Rodrigues da. **Circulating biomarkers in women with impalpable breast lesions.** 2019. 178 f. Tese (Doutorado em Ciências Médicas) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2019.

Breast cancer is the leading cancer death cause in the world for women. In this context, the breast early detection increases the cure chances greatly, however the initial characterization of the impalpable lesions has become a challenge. Histologically, these lesions contain *in situ* and infiltrative cells, which makes it difficult to predict their potential for evolution. The search for circulating tumor biomarkers has been extensively explored in an attempt to minimize invasive, repetitive procedures and aggressive treatments in these cases. The aim of this study was to detect circulating biomarkers involved in the development of impalpable breast lesion with classification BIRADS-3 and 4. For this purpose: the methylation profile of *CDKN2A* (*p14ARF* and *p16INK4A*) and *ATM* promoters; the detection of somatic mutations in *TP53* (exon 4 to 9), *CDKN2A* (exons 1 to 3), *PIK3CA* (exons 9 and 20) genes in tumor and circulating free DNA; and the identification of proteins differentially expressed in the saliva and plasma from patients with impalpable breast lesions were performed. Together, these results generated five publications. Briefly, two cohorts (N=62 and N=56) were evaluated for the methylation detection in the promoters of *CDKN2A* (*p14ARF* and *p16INK4A*), and *ATM*. For the two cohorts, methylation frequencies in blood DNA were 41%-37.5%, 26%-27%, and 41%-48% for the *ATM*, *p14ARF* and *p16INK4a* genes, respectively. The methylation frequency found in tumors (N=62) was 1/62 (1.6%), 3/62 (4.8%) and 33/62 (53.2%) for *p14ARF* and *p16INK4a* and *ATM*, respectively. Regarding the mutational evaluation, the *PIK3CA*, *TP53* and *CDKN2A* genes were sequenced (PCR-Sanger) in 58 women with impalpable lesions (49 malignant and 9 benign) with their respective free circulating DNA. A total of 37 mutations were found, being 8/58 (14%), 18/58 (31%) and 11/58 (19%) for the *PIK3CA*, *TP5*3 and *CDKN2A* genes, respectively. Regarding the proteomic data the main proteins differentially expressed in the saliva from the patients compared to the controls were: α2-macroglobulin, ceruloplasmin, leukocyte elastase inhibitor, α-enolase, and deleted in malignant brain tumors 1. In relation to plasma, alpha -2-macroglobulin and ceruplasmin are in high expression, whereas other proteins such as haptoglobin, hemopexin and vitamin D binding protein were in low expression compared to the control. Based on these results, we measured plasma vitamin D in 65 patients with impalpable breast lesions and in 20 controls. The prevalence of vitamin D deficiency and/or insufficiency in women with malignant lesions and in the controls were 84% and 60%, respectively. In this study the results are unpublished, however the biomarkers candidates described here need further investigation to describe and validate their involvement in the development of impalpable breast lesions.

Keywords: Impalpable breast lesions. Early detection. TP53. CDKN2A. PIK3CA. ATM.

LISTA DE QUADROS

LISTA DE ABREVIATURAS E SIGLAS

ACR	*American College of Radiology*
ACS	Sociedade Americana de Câncer
ASCO	Sociedade Americana de Oncologia Clínica
AT	Ataxia Telangiectasia
BIRADS	*Breast Imaging Reporting and Data System*
CCR	Câncer colorretal
CDIS	Carcinoma Ductal *In Situ*
CDI	Carcinoma Ductal Infiltrante
CEA	Antígeno Carcinoembrionário
cfDNA	DNA circulante livre
CI	Carcinoma Intraductal
CLI	Carcinoma Lobular Infiltrante
CLIS	Carcinoma Lobular *In Situ*
CMI	Carcinoma Micropapilar Invasivo
DNA	Ácido Desoxiribonucléico
DNMTs	DNA Metiltranferases
CTCs	Células Tumorais Circulantes
ctDNA	DNA tumoral circulante
EDTA	Etilenodiamino Tetra-Acético
FDA	*Food and Drug Administration*
HA	Hiperplasia Atípica
HBOC	Hereditariedade para câncer de mama e ovário
HE	Hematoxilina Eosina
HER-2	*Human Epidermal growth factor receptor- 2*
HR	Risco Relativo
HUGG	Hospital Universitário Gaffrée e Guinle
HUPE	Hospital Universitário Pedro Ernesto
IARC	*Internacional Agency for Research on Cancer*
INCA	Instituto Nacional de Câncer José Alencar Gomes da Silva
M100	Marcador de 100 pares de base

MA	Mamografia
$MgCl_2$	Cloreto de Magnésio
mRNA	*Messenger Ribonucleic Acid*
MS	Ministério da Saúde
MSP	*Methylation Specific PCR*
NaCl	Cloreto de Sódio
NCBI	*National Center Biotechnology Information*
NCCC	*National Comprehensive Cancer Network*
NGS	*Next Generation Sequencing*
OMIM	*Online Mendelian Inheritance in Man*
OMS	Organização Mundial da Saúde
OR	*Odds Ratio*
pb	pares de base
PCR	Reação em Cadeia pela Polimerase
RE	Receptor de Estrogênio
RH	Receptor Hormonal
RP	Receptor de Progesterona
SDS	Dodecil Sulfato de Sódio
TAE	Tampão Tris-Amino-EDTA
TCLE	Termo de Consentimento Livre e Esclarecido
UICC	*Union for International Cancer Control*
WBC	*White blood cells*
WHO	*World Health Organization*

LISTA DE SÍMBOLOS

G	Unidade de rotação gravitacional
L	Litro
M	Concentração Molar
mM	Concentração em mili molar
ml	Mililitro
rpm	Rotações por minuto
UI	Unidade internacional
V	Volts
µl	Microlitro
α	Alpha
β	Beta

SUMÁRIO

INTRODUÇÃO

O câncer de mama representa a primeira causa de morte por câncer no mundo para mulheres. No total, um quarto de toda a população feminina (13 em 100 mil mulheres) diagnosticada com câncer de mama terá seu desfecho na estatística da mortalidade. Mundialmente, a incidência do câncer de mama é estimada em 46 mil casos para mulheres (1). Já no Brasil esta incidência é superior, sendo 60 mil novos casos esperados para o ano de 2019 (2).

As lesões mamárias são histologicamente diferentes entre si, com perfis moleculares e evoluções diferentes. Consequentemente, o tratamento unificado das mulheres com câncer deveria ser personalizado. Neste contexto, com o rastreamento e as melhorias no diagnóstico por imagem, as lesões infraclínicas, ou denominadas impalpáveis, passaram a ser achados comuns, por outro lado, tornaram-se um dilema oncológico. Estas lesões intercalam entre o estadio inicial e evolução do tumor, podendo apresentar microscopicamente lesões mistas que entremeiam com focos de tecidos tumorais dispostos *in situ* e/ou infiltrativos (3,4). Por serem lesões pequenas, a biópsia não é capaz de representar histologicamente a lesão como um todo, além dos marcadores de imunohistoquímica não serem suficientes para elucidação molecular e clínica destas lesões (3).

Diante da complexidade e evolução, estas lesões são retiradas cirurgicamente; porém tardiamente estes casos podem recidivar, ou surgir um tumor ou *denovo*. A cinética metástatica para cada tipo de tumor e diferente e, em geral, variam com o fenótipo das células tumorais (5). A metástase a longa distância pode ocorrer simultaneamente com o tumor primário e levar semanas, a décadas para o seu desenvolvimento. Este período de intervalo entre o diagnóstico e o desenvolvimento da metástase e denominada de latência (5). Durante este período, o câncer de mama que é dependente, em maioria, dos receptores hormonais (RH), inicia o processo de plasticidade e motilidade celular promovendo o desprendendimento das células tumorais. Em alguns casos, este processo se inicia precocemente, ainda na ausência do sítio primário formado, ou com lesões *in situ*, mas com sitios metástaticos na medula óssea (7-10).

Os mecanismos celulares, bioquímicos e moleculares por trás da sobrevivênvia das células tumorais ainda são parte de um quebra cabeça. Entretanto, durante a latência e a migração das células tumorais circulantes (CTCs), estas secretam componentes capazes de promover sinalizações sistêmicas, como reações imunes e modificações ao nível molecular.

Neste cenário, e formado um microambiente rico capaz de revelar inúmeros biomarcadores (11-13).

A utilização de marcadores moleculares pode ser empregada para a detecção precoce, promoção da vigilância e resistência imunológica do paciente. A investigação de alterações genéticas e epigenéticas nas lesões impalpáveis poderão constituir uma ferramenta útil, a ponto de se entender os mecanismos envolvidos no processo inicial da carcinogênese, auxiliar no diagnóstico e procedimentos terapêuticos.

A glândula mamária

A glândula mamária é constituída de um conjunto de unidades acinares, células epiteliais, entre luminais, mioepiteliais e progenitoras, que se conformam em arquiteturas denominadas de lóbulos. Dos lóbulos formam se ramificações ductais que se finalizam no mamilo. No arcabouço mamário estas unidades acinares são envoltas por tecido adiposo e fibroso (14,15) (Figura 1).

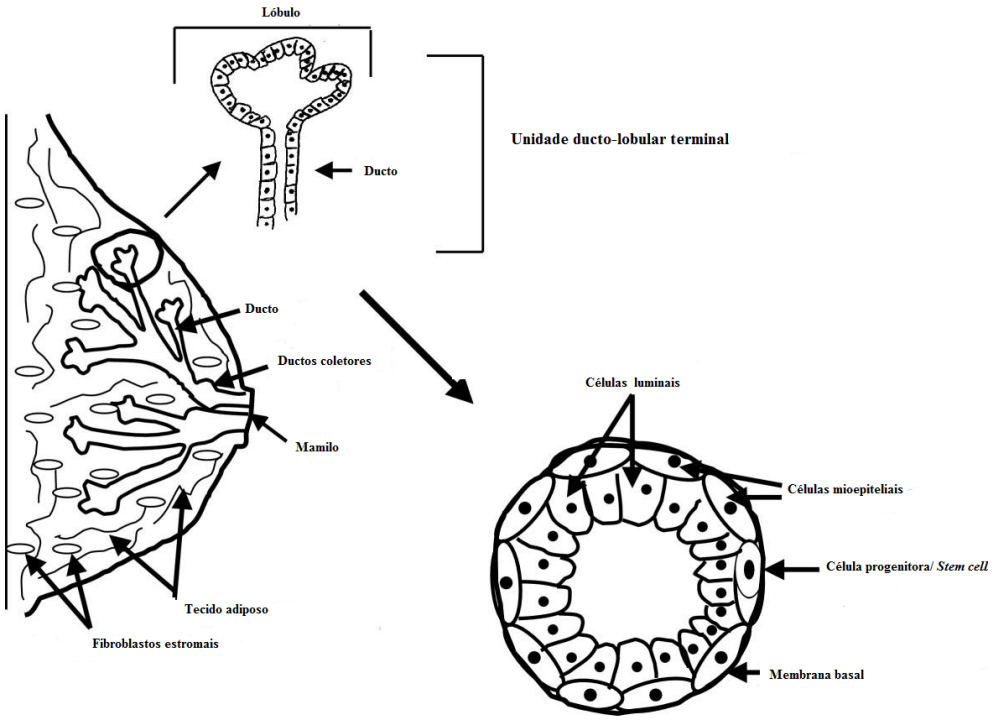

Figura 1: Estrutura mamária e divisões funcionais. Modificado de Dimri *et al.*, 2005 (15).

Além das estruturas acinares, uma rede linfática funcional age como dreno de todo o complexo mamário. Entremeado ao tecido mamário é possível encontrar plexos linfáticos subepiteliais e papilares que se confluem com os linfonodos subepiteliais. Cerca de 97% do fluxo linfático da mama tem destino a rede linfática das axilas, fazendo desta via, a principal rota das células tumorais (16).

Lesões benignas da mama

As lesões benignas podem ser do tipo não-neoplásicas e neoplásicas, e algumas destas associadas ao maior risco para o desenvolvimento de lesões malignas. Segundo a Sociedade Americana do Câncer (ACS), as lesões benignas podem ser resumidas em doze grupos, que se ramificam, dependendo da sua histopatologia (Quadro 1) (17).

Quadro 1 – Grupo de lesões benignas segundo a ACS

• Fibrose e cistos simples	• Fibroadenomas	• Necrose adiposa e cistos de óleo
• Hiperplasia ductal e lobular	• Tumores filoides *	• Mastites
• Carcinoma lobular in Situ (CLIS)*	• Papilomas intraductais	• Ectasia ductal
• Adenose	• Tumores de células granulares	• Outras condições não cancerosas da mama (cicatrizes, lipoma, hamartoma, hemangioma, hematoma, adenomioepitelioma, neurofibroma)

Fonte: Adaptada de ACS (17)

*grupos denominados benignos, porém com porcentagens que podem ser diagnosticadas como malignas, dependendo da histopatologia

Entre as lesões benignas com maior fator de risco para o desenvolvimento de câncer de mama são as hiperplasias atípicas e o carcinoma lobular in *situ* (CLIS) (17).

As hiperplasias são conhecidas por um crescimento excessivo das células que revestem os ductos, ou os lóbulos. Podem ser divididas em três grupos (17):

- Hiperplasia leve do tipo usual: não aumenta o risco de câncer de mama;

- Hiperplasia moderada ou florida do tipo usual (sem atipia), também conhecida como hiperplasia habitual: o risco de câncer de mama é elevado a 1,5 a 2 vezes;

- Hiperplasia atípica (hiperplasia ductal atípica ou hiperplasia lobular atípica): o risco de câncer de mama é aumentado em cerca de 4 a 5 vezes.

Já o CLIS também chamado de neoplasia lobular faz margem à classificação benigna e maligna, dependente do seu estadiamento e avaliação histopatológica. Restrige-se aos lóbulos, e pode adquirir o potencial de invasão, caso não tratado. Dentre as patologias benignas é a lesão com maior risco de evolução para câncer, além de aumentar o risco relativo para o desenvolvimento de câncer de mama em 7 a 12 vezes. O acompanhamento e o rastreamento nestes casos deve ser regular (17).

Por outro lado, as lesões benignas mais comuns da mama são os fibroadenomas. Caracterizados pela composição de tecido glandular e tecido estromal (conectivo), os fibroadenomas são mais comuns em mulheres na faixa dos 20 e 30 anos, variáveis de tamanho, podendo ir de massas pequenas e indetectadas à palpação até a massas tumorais de centímetros de diâmetro. Os fibroadenomas tendem a ser redondos e possuem bordas bem definidas, firmes a palpação e com aspecto emborrachado, mas não macios (17).

Mulheres com fibroadenomas simples têm um risco ligeiramente aumentado de 1 ½ vezes para o desenvolvimento de câncer de mama (17).

Lesões malignas

As lesões malignas da mama têm em sua maioria, origem nas células epiteliais que revestem os lóbulos e ductos mamários. Por esta razão, os tumores mais comuns são os ductais *in situ*, os carcinomas ductais e os lobulares infiltrativos. Há aqueles menos comuns, tais como os sarcomas, tumores filoides, lesões decorrentes da doença de Paget e angiossarcomas que se iniciam nas células do músculo, tecido adiposo ou tecido conjuntivo (18,19).

Carcinomas *in situ*

Os tumores *in situ* da mama são determinados por grupamentos celulares bem diferenciados, com localização restrita que confirmam a ausência de invasão das células tumorais no estroma mamário (18-23). São divididos em dois grupos:

Carcinoma lobular *in situ* (CLIS) - Representam cerca de 20-30% de todos os carcinomas *in situ*. Atualmente intercalam entre o grupo das lesões benignas e malignas, devido a sua característica de risco e evolução. A conformação celular é multicêntrica, com baixo grau nuclear e citoplasma bem delimitado, com aspectos em sinete. As células tumorais se distendem entre os lóbulos e são bastante pequenas, redondas e poucos coesas, devido a perda de expressão gradativa da proteína E-caderina (18-22).

Carcinoma ductal *in situ* (CDIS) – Estima-se que um em cada cinco novos cânceres de mama diagnosticados será carcinoma ductal ou carcinoma ductal *in situ*. As células que revestem os ductos iniciam o processo da carcinogênese, porém a massa tumoral desenvolvida não possue a capacidade de se infiltrar no tecido mamário adjacente (23).

Carcinomas infiltrativos

Os cânceres de mama que se espalham são conhecidos como infiltrativos, sendo os mais comuns os lobulares e os ductais.

Carcinoma lobular infiltrativo (CLI) - O carcinoma lobular invasivo (CLI) inicia-se nos lóbulos mamários, espalhando-se para o tecido adjacente. Estima-se que a cada dez tumores diagnosticados, um será CLI. O CLI é de difícil detecção no exame físico, assim como na mamografia. Em comparação com outros carcinomas invasivos, há uma tendência a bilateralidade, com estimativa de uma a cada cinco mulheres com CLI ter câncer em ambas as mamas (23).

Carcinoma ductal infiltrativo (CDI) - Este é o tipo mais comum de câncer de mama. Cerca de 8 dos 10 cânceres de mama invasivos são CDI. O CDI inicia-se nas células que revestem o ducto e se infiltra no tecido mamário próximo.

Os outros tipos especiais de câncer de mama infiltrativo correspondem a menos de 5% de todos os cânceres de mama, e são variáveis segundo a sua conformação histológica, assim como a origem do tumor (23). Eles podem ser divididos em dois grupos, considerando o prognóstico em relação ao CLI e CDI:

- Bom prognóstico: carcinoma adenoide cístico, carcinoma metaplásico de baixo grau, carcinoma medular, carcinoma mucinoso, carcinoma papilar e carcinoma tubular;
- Mau prognóstico: carcinoma metaplásico de alto grau e variantes (exceto baixo grau), carcinoma micropapilar e carcinoma misto.

Lesões impalpáveis da mama

Nas últimas décadas, a frequência das lesões mamárias impalpáveis tem aumentado devido à disseminação de programas de rastreamento mamográfico e melhora na resolução e precisão dos exames de imagem (24,25).

Atualmente há um aumento no número de casos diagnosticados com lesões pequenas, e ainda, redução daqueles com invasão de linfonodo (26). Por outro lado, o manejo cirúrgico nestes casos, tornou-se um dilema. A multifocalidade destas lesões, pode promover o risco de permanência de células tumorais no tecido mamário, e levar a recidivas ao longo dos anos. Não há dados estimados para recidivas das lesões impalpáveis (4,26,27,28), entretanto, para lesões mamárias iniciais, o risco de recidiva para casos tratados e não tratados radioterapicamente, pode atingir de 7 a 26%, respectivamente (29). Estendendo a análise, o risco de mortalidade para quinze anos pode atingir 36% (29).

Em geral, lesões impalpáveis são pequenas (<2cm) com fenótipo histopatológico inicial ou seja, podendo ser lesões *in situ* associadas com início de lesões infiltrativas e com receptores de estrógeno e/ou progesterona positivos (luminais) (4,30). Poucos são os casos com receptores hormonais negativos, e nestes cenários deve-se analisar as variáveis individuais, incluindo histórico familiar e idade (4,30).

Independente de serem lesões iniciais, podem apresentar invasão de linfonodo axilar (4,31), entretanto, metástases a longa distância são raras, variando de 0.3% a 1% (32).

Câncer de mama e fatores de risco

O câncer de mama e causado por uma combinação de fatores: hormonais (fisiológicos e terapêuticos), genéticos, fisiológicos não-hormonais (idade), de meio ambiente e de estilo de vida (Quadro 2). Segundo o relatório da Organização Mundial da Saúde (OMS), que reúne os fatores de riscos para câncer de mama, estima-se que 50% dos casos novos são resultados de fatores hormonais, e somente 5 a 10% de todos os cânceres de mama sejam consequência da herança genética (*BRCA 1/2*, *ATM*, *BARD1*, *BRIP1*, *CDH1*, *CHEK2*, *PALB2*, *PMS2*, *PTEN*, *RAD50*, *RAD51C*, *TP53*, entre outros (33-36).

Quadro 2 – Fatores de risco para câncer de mama

Fatores reprodutivos	Fatores hormonais	Nutricional/Estilo de vida	Outros fatores
Idade precoce para menarca (+) Idade da primeira gestação (>35 *versus* <20) ++ Ausência de filhos (0 *versus* 1 crianca) + Amamentação (> 1 ano *versus* nenhum) -	Uso de contraceptivo oral (corrente *versus* nenhum) + Reposição hormonal (+ de 10 anos *versus* nenhum) + Reposição hormonal, estrógeno + progesterona (> 5 anos *versus* nenhum) ++ Alta concentração de estrógenos ou andrógenos (pós-menopausa) +++ Alta concentração de prolactina no sangue ++	Obesidade (>30 índice de massa corporal *versus* <25) Pré-menopausa – Pós-menopausa + Ganho de peso adulto/sobrepeso (pós-menopausa) ++ Álcool (1 ou mais dose/dia *versus* nenhuma) + Altura (>170 cm) + Atividade física (> 3 horas/semana) – Gordura monosaturada *versus* gordura saturada) – Baixa ingestão de frutas e vegetais (especialmente para câncer de mama receptor de estrógeno negativo) +	Histórico familiar (mãe e irmã)[a] +++ Histórico familiar[b] (primeiro grau) ++ Herança judia (sim *versus* não) + Radiação ionizante (sim *versus* não) + Doença benigna da mama[c] (Diagnosticada e retirada) ++ Densidade mamográfica (mais alta densidade *versus* baixa densidade) +++

Fonte: Adaptada de WHO (33). Nota: +, risco relativo (RR) = 1.1-1.4; ++, RR = 1.5-2.9; +++, RR = 3.0-6.9; -, RR = 0.7-0.8.

[a] Histórico familiar de primeiro grau com dois casos diretos de câncer de mama antes dos 65 anos de idade *versus* sem histórico familiar

[b] Histórico familiar de primeiro grau com um caso direto de câncer de mama antes dos 65 anos de idade *versus* sem histórico familiar

[c] Clínicamente diagnosticado como doenca cística crônica, fibrocística ou outra doença benigna da mama *versus* nenhuma

Diagnóstico do câncer de mama por imagem

Os exames de imagem exercem uma grande função no rastreamento e na detecção das patologias mamárias. Os exames de imagem mais usados e comuns para essa finalidade são: mamografia, ultrassonografia e ressonância magnéctica (Quadro 3).

Quadro 3 - Examens de imagem usados para detecção de patologias mamárias

Mamografia	Ultrassonografia	Ressonância Magnética
Mecanismo: o exame mamográfico é realizado a partir de aparelhos de baixa intensidade de emissão de raios-x. A mama é comprimida sob um receptor de imagem (podendo ser de filme ou digital), e uma base transparente de compressão da mama. Os raios-x não absorvidos marcam o filme, ou são revelados pelo detector digital, produzindo uma fotografia similar a um negativo (37).	Mecanismo: a técnica baseia-se nos transdutores de ondas com frequência média que varia de 5 para 15 MHz. A resolução da técnica permite sua visualização em profundidade, garantindo sua aplicação em mamas densas e em estágio de lactação (36, 38).	Mecanismo: a técnica consiste na secção do tecido analisado em quadros de imagens, com a recriação do movimento dos prótons, de acordo com o relaxamento do tecido (36).
Recomendação: rastreamento de patologias mamárias. O Ministério da Saúde (MS) recomenda um exame mamográfico, pelo menos a cada dois anos, para mulheres de 50 a 69 anos (37)	Recomendação: técnica complementar a mamografia, em especial, para casos de mamas densas, e lesões de difícil visualização, ou não palpáveis e ocultas clinicamente.	Recomendação: é usado principalmente para a) detecção multifocal, multicêntrica e de patologia contralateral, ou quando a detecção por mamografia e ultrassonografia não é suficiente para reconhecer a anormalidade mamária; b) detectar a possibilidade de invasão tumoral em CDIS; c) avaliar a resposta à quimioterapia neoadjuvante; d) detectar massas tumorais ocultas na mama, principalmente quando presentes inicialmente nos linfonodos axiliares; e e) detecção de lesões impalpáveis em mamas densas (39)

Sensibilidade e especifificadade 40-98% (37). Estes valores são dependentes da qualidade do aparelho, e se a mamografia é convencional, ou digital (37).	Sensibilidade e especificidade: variando entre 90-96% (39).	Possui sensibilidade superior, ou mesmo equiparada a US e especificidade inferior aos demais exames com 88% (39).

Classificação *BI-RADS*

Para uniformização dos achados nos exames de mamografia, o *American College of Radiology* (ACR) criou em 1993 (40) um sistema de seis categorias para classificação das lesões mamárias, o *Breast Imaging-Reporting and Data System* (BI-RADS) (Quadro 4).

Ao longo dos anos, com o avanço das tecnologias e relatórios médicos (41), a classifição BI-RADS se estendeu aos demais exames de imagem, incluindo a ultrassonografia e a ressonância magnética. Atualmente na sua quinta versão (publicada no ano de 2013), a classificação trouxe uma unificação das categorias para os exames (42).

Quadro 4 - Classificação BI-RADS

Avaliação	Resultado
Categoria 0:	Mamografia incompleta – avaliação adicional e/ou comparação com as mamografias feitas anteriormente; Ultrassonografia e ressonância magnética incompleta – necessária a avaliação adicional
Categoria 1	Negativo
Categoria 2	Benigno
Categoria 3	Provavelmente benigna
Categoria 4	Mamografia e ultrassonografia (não se aplica a ressonância magnética): Categoria 4A: Baixa suspeita para malignidade Categoria 4B: Suspeita moderada para malignidade Categoria 4C: Alta suspeita para malignidade
Categoria 5	Altamente sugestivo de malignidade
Categoria 6	Malignidade – deve ser realizada biópsia

Fonte: Quadro resumido e adaptado de Sickles, D'Orsi e Basset, 2013 (43).

Marcadores de imunohistoquímica e bioquímicos para câncer de mama

Estrógeno e progesterona

Os principais estrogênios circulantes no corpo humano são: estradiol, estrona, e o 16-hidroxiestradiol (estriol) (44). São produzidos, em maioria, nos ovários e na placenta, porém são necessários para o crescimento normal e desenvolvimento do tecido mamário (45).

Os dois receptores de estrogênio (receptor-α (RE-α) e receptor-β (RE-β)) compartilham de estruturas funcionais, contudo, RE-β possui poucos dados que demonstrem algum papel clínico, sendo o ER-α utilizado normalmente para avaliação (44,45).

Cerca de 75% dos casos de câncer de mama são ERα positivos (45). Dessa forma, a avaliação da positividade do receptor é fundamental, pois é um parâmetro utilizado como fator preditivo e de prognóstico. Os fármacos moduladores da via do ER podem atuar no próprio receptor, agindo como moduladores seletivos (como é o caso do tamoxifeno); como antagonistas puros que causam a degradação do receptor (no caso do fulvestranto); e também como inibidores da produção de estrogênios (como o letrozol) (45,46).

A progesterona é um hormônio esteróide ovariano chave na fase lútea e diferenciação glandular do endométrio (47). Assim como o estrogênio, a progesterona tem seu ciclo de exposição hormonal, que se inicia na menarca e termina na menopausa. Durante este período, regula o crescimento e a diferenciação de tecidos especializados dentro do trato reprodutivo e dos tecidos mamários (48). Na gravidez este ciclo é interrompido e se elevam os níveis de progesterona, que são necessários para o desenvolvimento fetal, desenvolvimento da mama para a lactação, manutenção da integridade uterina/placentária e quiescência miometrial (47,48). Os receptores de progesterona se assemelham bastante com os de estrogênio, possuindo duas isoformas, o receptor de progesterona-α (RP-α) e o receptor de progesterona-β (RP-β). Ambos, possuem a região de extremidade C-terminal e o sítio de ligação ao DNA. Em resposta à ligação do hormônio, o RP regula a expressão de genes atuantes no controle do desenvolvimento, na diferenciação e na proliferação de tecidos-alvo (47-49).

Apesar da progesterona ser um hormônio proliferativo e um fator de risco para câncer de mama, a presença de PR em câncer de mama primário é um marcador de prognóstico favorável, e está associado a um fenótipo mais diferenciado e menos invasivo do que os tumores negativos. Os tumores ER+/PR+ respondem a terapias endócrinas melhor do que os

tumores ER+/PR- tanto em mulheres pré e pós-menopáusicas, consequentemente a positividade de RP é preditiva de uma melhor sobrevida global (50).

Fator de crescimento epidérmico humano tipo-2 (HER2)

O receptor do fator de crescimento epidérmico humano 2 (HER2) é um dos quatro receptores tirosina quinases (PTKs) de membrana (*HER1* - EGFR, *HER3* - erbB3 e *HER4* - erbB4 (51). O oncogene *HER2* localizado no cromossomo 17q12 (52) codifica uma proteína de 185 kDa de localização transmembrana responsável pela emissão da sinalização de crescimento celular, diferenciação e migração das células epiteliais (53).

Estima-se que cerca de 15-20% dos tumores mamários sejam HER2+, e em maioria, respondem ao Trastuzumabe® como primeira linha de tratamento (53). O anticorpo humanizado, age diretamente sob a porção extracelular do receptor, inibindo a progressão do tumor (53). Os tumores que respondem ao Trastuzumabe® possuem bom prognóstico e melhores taxas de sobrevida global. Por outro lado, aqueles tumores HER2 resistentes são agressivos, com rápido crescimento e evolução para metástases a longa distância (54).

Classificação molecular do câncer de mama

No ano de 2000, o câncer de mama foi classificado em cinco grupos moleculares, baseados na diferença de expressão gênica: luminal A, luminal B, basal-*like*, superexpressão de HER2 e normal *breast-like* (55). Um ano mais tarde, os detalhes de prognóstico para cada grupo foi publicado (56).

O grupo luminal (A e B) é marcado pela alta expressão gênica das células luminais do epitelio mamário, incluindo expressão das citoqueratinas tipo 8 e 18, genes relacionados ao ER, além de apresentarem bom prognóstico. Os tumores luminais tipo B apresentam altas taxas de proliferação, tipo histológico agressivo com prognóstico pior que os luminais A (55-57).

O subtipo superexpressão do HER2, cujo fenótipo é RH+ ou RH- e HER2+, apresentam uma amplificação do oncogene *HER2* e, concomitantemente, a superexpressão de

sua proteína (55,56). O valor do HER2 como fator prognóstico é controverso, com definição de bom valor preditivo para casos sensíveis ao Trastuzumabe®, e pior valor preditivo para aqueles resistentes (54).

O subtipo *normal breast-like* foi caracterizado por meio do aumento da expressão de genes do tecido adiposo e por outros tipos de células epiteliais (55). Este subtipo está pobremente caracterizado e a sua significância clínica é limitada (55).

O subtipo basal-*like* podem apresentar positividade, mas em maioria são negativos para os RHs e HER2. Possuem poucas semelhanças com os demais grupos, consequentemente apresentando a maior diversidade intrínseca entre tumores (55,57). Pacientes com esse subtipo de tumor possuem pior prognóstico, e cerca de 40% dos casos apresentam recaída/recidiva dentre 5 anos após o diagnóstico (58). No ano de 2011, Lehmann e colaboradores (59), analisando o padrão de expressão gênica de 587 tumores *basal-like* descreveram mais quatro subgrupos: 2 tipos basais (basal *like*-1 e basal-*like* 2), um mesenquimal e um subtipo de receptor de andrógeno luminal.

Outras mais duas classificações moleculares foram descritas nos anos de 2005 e 2007 (60-62), apócrina e *claudin-low*, respectivamente. Os tumores apócrinos são RE- com expressão androgênica aumentada e compartilham características com o subtipo HER2 e basal (60,61). O subtipo *claudin-low* apresenta baixa expressão gênica das proteínas de junção, em destaque para as claudinas 3, 4 e 7 e E-caderina (62).

Alterações genéticas e epigenéticas envolvidas na carcinogênese mamária

Aproximadamente 5% a 10% dos cânceres de mama são relacionados à mutações hereditárias nos genes *BRCA1* ou *BRCA2*, e os demais por mutações de média e intermediária penetrância nos genes: *ATM*, *BARD1*, *BRIP1*, *CDH1*, *CHEK2*, *PALB2*, *PMS2*, *PTEN*, *RAD50*, *RAD51C*, *TP53*, entre outros (63,64).

Mutações em *BRCA1* e *BRCA2* são encontradas aproximadamente em 1 de 300 indivíduos na população geral, e 1 em 40 indivíduos descendentes de judeus Ashkenazi (65). Essas mutações estão associadas ao risco elevado de câncer de mama (risco de vida de aproximadamente 70% aos 80 anos) e câncer de ovário (45% para portadores de mutação *BRCA1* e 20% para *BRCA2*) (65). Aumento para o risco de câncer pancreático e próstata de alto grau também é visto, particularmente em portadores de mutação em *BRCA2*.

As recomendações do *National Comprehensive Cancer Network* (NCCC) (66) para rastreamento mutacional nos genes *BRCA1* e *BRCA2* continuam a se expandir e incluem (mas não estão limitadas): a) todas as mulheres com câncer de ovário; câncer de mama diagnosticado com menos de 45 anos; b) mulheres com câncer de mama triplo negativo com menos de 60 anos, ou com ascendência judaica Ashkenazi; c) todos os indivíduos com câncer pancreático; e d) todos os homens com câncer de mama ou câncer de próstata metastático. Com o aumento das opções de testes genéticos e o custo mais acessivel, é provável que mais mulheres sejam identificadas como portadoras de mutações *BRCA1* e *BRCA2* e enfrentem decisões em relação à mastectomia redutora de risco (67).

Por outro lado, o outro grupo de casos de câncer de mama, denominados esporádicos, (cerca de 90%) são resultado de mutações genéticas somáticas (64). As principais anormalidades genéticas observadas nestes casos incluem a expressão aumentada de proto-oncogenes, inativação de genes supressores de tumor, instabilidades cromossômicas, alterações nos genes de reparo do DNA, reativação da telomerase e alterações epigenéticas (68). Ao longo dos anos, muitos genes têm sido pontuados com elevada frequência de alteração na carcinogênese mamária. Entre estas alterações podem ser citadas mutações pontuais, como inserções, deleções, substituições; e alterações epigenéticas, como metilações nas regiões promotoras, responsáveis por controlar a expressão gênica (69).

TP53 (OMIM–191170)

TP53 é um gene supressor de tumor localizado na região cromosssômica 17p13, e codifica uma fosfoproteína de 53-kd, a p53. A proteína p53 foi descoberta em 1979, ligada à proteína transformante do vírus SV40, T-Ag (70). Passado quase quatro décadas da sua descoberta, o gene *TP53* vem sendo descrito com múltiplas funções, e as descobertas do seu envolvimento no processo tumoral ainda não se limitaram (71).

O gene atua na regulação e proliferação celular em respostas aos danos ocorridos no DNA, através de duas maneiras: causando a parada do ciclo celular nos pontos de checagem e dando chances a célula de consertar a lesão no DNA, ou induzindo a célula a entrar em apoptose (71,72). Ao longo dos anos, o papel do *TP53* em outros processos celulares, tais como o metabolismo, a angiogênese, as respostas imunes, a manutenção de células-tronco e a comunicação entre tumor e o estroma tem sido desvendado (73).

No câncer de mama as modificações genéticas em *TP53* são um evento molecular comum. Alterações em *TP53* têm sido demonstradas em menos de 10% nas hiperplasias ductais atípicas, mas são muito mais frequentes nos carcinomas *in situ* e cânceres invasivos, variando de 20-85% dependendo do subtipo molecular. Tumores luminais tendem a apresentar menores frequências de mutação, em comparação aos tumores basais e HER2 (74). As mutacões pontuais são as mais comuns no gene, seguidas por pequenas inserções e deleções e os rearranjos gênicos mais complexos são menos frequentes. O espectro de mutação de *TP53*, em todos os cânceres, incluindo mama e ovário, é dominado por mutações de perda de sentido, ou "*missense*", predominantemente localizadas nos exons 5–8, nos chamados *"hotspots"* mutacionais em determinados códons (por exemplo, 175, 245, 273, 285, 220, 306). Mutações de mudança de leitura, ou *"frameshift"*, estão espalhadas sob a região de codificação e mostram uma distribuição uniforme no gene (72).

Ao examinar as associações entre mutação e prognóstico, a mutação em *TP53* parece estar fortemente correlacionada à pior sobrevida global em pacientes ER positivos, mas não em pacientes ER negativos. De fato, a presença de uma mutação parece eliminar o efeito benéfico de prognóstico da positividade do ER (72). Pacientes com *TP53* mutante podem, portanto, não responder tão bem à terapia endócrina quanto pacientes com *TP53* sem mutações (72). Em um estudo recente, o acúmulo da proteína p53 foi identificado como um forte preditor de recorrência em pacientes ER positivos tratados com inibidor de aromatase (75).

CDKN2A (*p14^{ARF}*/*p16^{INK4a}*) (OMIM–600160)

O gene *CDKN2A* está localizado na região cromossômica 9p21.3. Consiste em três éxons codificantes, sendo o primeiro éxon (E1), contendo 125 pb, E2, 307 pb, e E3, com apenas 12 pb (76). O gene codifica duas proteínas através de *splicing* alternativo no éxon 1, que são responsáveis pelo controle do ciclo celular): a p16 (*INK4*) que inibe as ciclinas dependente de quinase CDK4/CDK6; e a p14 (*ARF*), que liga à proteína p53, induzindo efeito apoptótico (77).

Vários estudos sugerem que os baixos níveis de expressão de *p14^{ARF}* e *p16^{INK4a}* desencadeados pela hipermetilação do promotor sejam associados ao desenvolvimento do câncer de mama e sua progressão (78,79). Por outro lado, a superexpressão de *p16^{INK4a}*

também pode influenciar no aumento progressivo do tumor, naqueles cuja função do gene *RB* foi perdida. Embora haja essa positividade de expressão e relação de progressão, nestes casos não houve relação com o pior prognóstico (80-82). Meseure *et al*. (81) encontrou uma diferença significativa na sobrevida livre de metástases entre os pacientes com superexpressão da proteína p16 (P = 0,025). Os pacientes p16 positivos tiveram uma melhor resposta à quimioterapia neoadjuvante em comparação aquelas p16 negativo. Esses achados suportam a possibilidade de que o P16 possa ser usado como um fator prognóstico para o desfecho e resposta ao tratamento dos pacientes com câncer de mama.

Em outro contexto, quando há sincronia elevada de expressão das duas proteínas, este efeito promove um pior cenário clínico. Um estudo envolvendo 1.080 pacientes com CDI invasivo, os autores detectaram que o nível de expressão de p16 e p14 foi maior no tecido maligno comparado ao tecido normal, e quando combinada essa expressão, há um aumento de risco para recorrência do tumor (83).

ATM (OMIM–607585)

O gene *ATM* possui 66 éxons e está localizado na posição cromossômica 11q22.3 (84,85). O seu transcrito possui cerca de 13 Kb com uma sequência de codificação de 9.168 pb. Dentre a sua extensão existem transcritos alternativos e sua proteína codificada possui 350 kDa (84,85).

O gene *ATM* é um dos iniciadores do mecanismo de reparo de quebra dupla do DNA. Neste mecanismo de sinalização há uma série de fosforilações que tornam o complexo formado ativo. Em resultado, mecanismos do ciclo celular são recrutados para indução da apoptose e⁄ou para o reparo celular (86).

Mutações hereditárias no gene *ATM* são a causa da instabilidade rara do genoma que é denominada síndrome da Ataxia Telangiectasia (AT). Casos de AT possuem taxas de até 40% de risco para o desenvolvimento de leucemias e linfomas primários, justamente pela deficiência em reparo genético no sistema imunológico (87). Embora, não existam muitos estudos que comprovem a influência da heterozigosidade no risco para desenvolvimento de câncer de mama, Swift *et al.* (88) demonstraram que carregar o alelo recessivo pode aumentar em cerca de 5 vezes o risco para o seu desenvolvimento.

Stredrick *et al.* (89) avaliando a presença da mutação Ser49Cyc em 2.800 casos, incluindo casos com câncer de mama e controles, encontraram a sua presença em 2% dos indivíduos, sendo mais comum no câncer de mama, com *odds ratio* (OR) de 1,69 (*P*=0,004 (89). Entretanto, as mutações em *ATM* não são específicas para risco do desenvolvimento de câncer de mama, pois Dombernbowsky *et al.* (90) avaliando o genótipo S49C e a heterozigosidade Ser707Pro em mais de 10.000 indivíduos controles, revelaram que aproximadamente 20% dos quais possuiam estes genótipos desenvolveram câncer durante a observação prospectiva. O genótipo S49C foi associado com um aumento da incidência de melanoma (risco relativo (HR) de 4,8), câncer de próstata (HR 2,3) e câncer de orofaringe (HR 3,4). O genótipo S707P foi associado a um risco aumentado de câncer de tireoide ou endócrino (HR 10) (90). No entanto, essas mutações de perda de sentido não foram associadas com um aumento do risco de câncer de mama em comparação com a população geral (HR 0,8 e 0,6 para S49C e S707P, respectivamente) (90).

Fletcher *et al.* (90) expandiram a análise das variantes de *ATM* para mais de 26.000 casos e controles de câncer de mama (91). Nesta população a heterozigosidade para uma das 5 variantes de *ATM* avaliadas (S49C e S707P como acima, e também F858L, P1054R e L1420F) foi associada a um pequeno aumento do risco de câncer de mama (OR 1,05). O risco foi maior para homozigotos raros (OR 1,51). Coletivamente, esses estudos apoiam um papel de mutações pontuais em *ATM* na predisposição ao câncer de mama, embora esse efeito possa estar limitado a um pequeno subconjunto de casos, como pacientes jovens com doença familiar ou bilateral (91).

Vo *et al.* (92) demostraram a redução de expressão da proteína ATM em resultado da metilação da região promotora em cânceres de mama esporádicos. Outros estudos também relatam a relação da redução de expressão do gene *ATM* em casos de câncer de mama com uma menor sobrevida livre da doença, estadio mais agressivo, necrose, invasão vascular e perineural (93,94).

PIK3CA **(OMIM – 171834)**

O gene *PIK3CA* foi mapeado em 1994 na posição cromossômica 3q26.3 (95). É classificado como um oncogene e atua como mediador em funções biológicas importantes, como sobrevivência, diferenciação e proliferação celular (96). Esse gene é responsável pela

codificação de duas subunidades, a p100-alfa e p85-alfa, porém que podem se dividir em diferentes isoformas. As subunidades p110α, p110β e p110δ são resultados da transcrição dos genes *PIK3CA, PIK3CB* e *PIK3CD* (97).

Amplificações, deleções e mutações somáticas no gene *PIK3CA* foram relatadas em diferentes tipos de câncer, incluindo câncer de cólon, mama, cérebro, fígado, estômago e pulmão (96). Essas mutações somáticas resultam no aumento da atividade quinase de *PIK3CA*, contribuindo para a transformação celular (96).

Ao longo dos anos, a frequência de mutações em *PIK3CA* para câncer de mama foram equiparadas aquelas encontradas em *TP53*, com frequência de até 45% nos tumores luminais A (97). A maioria são resultado de mutações de perda de sentido nos domínios helicoidais (E524K e E545K) e catalítico (H1047R) (98). Recentemente, a avaliação do benefício do uso da droga everolimus nos pacientes com câncer de mama (receptor hormonal positivo e HER negativo – estudo BOLERO-2) *versus* controle (99) revelou que casos que carreavam mutações em *PIK3CA* no cfDNA e fizeram uso da everolimus tiveram sua mediana de progressão livre da doença prolongada, tanto em pacientes com mutação H1047R (HR 0,37; IC95%, 0,24–0,56) quanto aqueles com E545K/E542K (HR 0,30; IC95%, 0,18–0,51) (99). Neste mesmo contexto, Kalinsky *et al.* (100) descreveram que aqueles casos de câncer de mama com mutações em *PIK3CA* tiveram seus desfechos clinicopatológicos melhores, incluindo benefícios de sobrevida.

Por outro lado, o significado prognóstico das mutações em *PIK3CA* ainda não é bem elucidado. De acordo com o *Cancer Genome Atlas Network* (97), embora nos tumores triplo negativos as mutações em *PIK3CA* sejam observadas em baixa frequência – a atividade de expressão da via PI3K é, na verdade, a mais alta nestes tumores. Por exemplo, Sakr *et al.* (101) descrevem que as alterações moleculares que afetam a via PI3K podem desempenhar um papel na progressão de CDIS de alto grau para CDI em um subconjunto de casos específicos (por exemplo, lesões RE positivas e HER2 negativas).

Mais além, tem sido descrito que as mutações em *PIK3CA* levam à resistência em tumores HER2 positivos, ou RH positivos. Neste contexto, Berns *et al.* (102) avaliando 55 pacientes com câncer de mama, descreveram que os casos resistentes ao Herceptin estão associados a mutações em *PIK3CA*, ou a baixa expressão de *PTEN* (102). Da mesma forma, em modelos de cultura celular que continham as mutações E545K e H1047R e sensíveis a GDC-0941 (inibidor pan-PI3K), as mutações em *PIK3CA* definiram resistência ao Herceptin (103,104).

Metilação no DNA e o câncer de mama

Pelo menos três sistemas constituem o mecanismo epigenético: a metilação do DNA, a modificação de histonas e cromatina e o silenciamento gênico associado ao RNA não codificador (ncRNA) (104). Os mecanismos epigenéticos são essenciais para o desenvolvimento normal do organismo como um todo, controlando e regulando o padrão de expressão gênica. Qualquer modificação, ou alteração neste mecanismo, pode desencadear transformações celulares que levam a malignidade (105).

O mecanismo epigenético mais estudado no processo da carcinogênese, é a metilação no DNA. As reações de metilação são modificações químicas, classificadas em hipometilações e hipermetilações, sendo estas comuns aos dinucleotídeos CpG (106). Um dinucleotídeo CpG consiste em uma citosina que precede a uma guanina, e ilhas CpG são sucessões de 500 para para 2.000 pares de bases em comprimento de dinucleotídeos CpG principalmente localizadas nas regiões promotoras de regulação dos genes (107). A metilação do DNA acontece pela ação das enzimas dimetiltransferases (DNMTs), que acrescentam o grupo metil ao quinto carbono do anel pirimidínico. Cerca de 75% destas regiões são metiladas em algum momento do genoma dos mamíferos, e se localizam preferencialmente em regiões promotoras da regulação gênica (108).

A metilação do DNA no tecido mamário está relacionada, em parte aos fatores de risco à carcinogênese. Entre os principais, podem ser citadas: a idade avançada, que permite o acúmulo de mutações somáticas, acarretando na disfunção da telomerase permitindo o silenciamento gênico de várias regiões promotoras, e ainda, as exposições a níveis aumentados de estrogênio que favorecem o crescimento celular do epitélio mamário, levando consequentemente a modificações epigenéticas no DNA (109).

Dentre os genes que sofrem hipermetilações no tecido mamário incluem: os de crescimento, envolvidos na invasão e apoptose (*DAPK*, *TWIST1*, *HOXA5*, $RAR\beta_2$), regulação no ciclo celular (*CDKN2A* (*$p14^{ARF}$*/*$p16^{INK4a}$*), *CCND2*, *RASSF1A*), invasão celular e metástase (*CDH1* e *APC*), reparo do DNA (*BRCA1* e *GSTP1*) e sinalização celular (*ER* e *RARb2*), entre outros (109-112).

Tanto a hipometilação global, quanto a hipermetilação do promotor podem acompanhar o desenvolvimento do tumor, e ambos têm sido reconhecidos como eventos comuns em muitos cânceres (106). Já nos primeiros estudos de avaliação do perfil de metilação de tumores mamários, Yan *et al.* (113) avaliando cerca de 1104 ilhas CpGs em 28

tumores mamários pareados com tecido normal, revelou que tumores pobremente diferenciados apresentaram ser mais hipermetilados do que aqueles moderados, ou bem diferenciados (P = 0,041). Em outro estudo, van Hoesel *et al.* (114) avaliando a expressão dos genes *RARβ2*, *RASSF1A*, *MINT17* e *MINT13* em epitélio normal da mama, hiperplasia ductal, CDIS, e CDI estágio 1, os autores detectaram que os níveis de metilação aumentaram significativamente durante os estágios progressivos do desenvolvimento do câncer de mama. Os valores de P foram 0,0012, 0,0003, 0,012, <0,0001 e <0,0001 para *MINT17*, *MINT31*, *RARβ2*, *RASSF1A*, respectivamente (114).

Mais além, a avaliação de 57 locus de promotores gênicos em 20 carcinomas ductais invasivos (IDC) pareados aos tecidos mamários normais, revelou a hipermetilação de 15 genes específicos (*DLEC1*, *GRIN2B*, *HOXA1*, *MT1G*, *SFRP4*, *TMEFF2*, *APC*, *GSTP1*, *HOXA10*, *IGF2*, *RARβ*, *RASSF1A*, *RUNX3*, *HIN-1* e *SFRP1*) (115). Expandindo essa avaliação de metilação para quatro diferentes espécimes: atipia epitelial plana (AEP), hiperplasia ductal atípica, (HDA), DCIS e CDI, o número de genes metilados aumentou progressivamente da mama normal para AEP/HDA e CDIS, enquanto o IDC não diferiu do DCIS. Os níveis de metilação e as freqüências das ilhas CpG dos promotores de *APC*, *DLEC1*, *HOXA1* e *RASSF1A* foram significativamente maiores no AEP/HDA do que no tecido mamário normal. Os autores concluíram que o processo de metilação dos promotores mudaram significativamente nas lesões pré-invasivas, e foi semelhante em CDI e CDIS, sugerindo que a metilação das ilhas CpG de genes relacionados ao tumor é um evento precoce, e está envolvido na progressão do câncer de mama.

De fato, a metilação aberrante encontrada nos promotores dos genes é uma característica da carcinogênese. Dessa forma, essas alterações epigenéticas podem ser encontradas no DNA do sangue, o que sugere a possibilidade de biomarcadores de metilação no DNA circulante livre (cfDNA) (116,117). Até o momento, um número considerável de estudos com foco na metilação do DNA derivado do câncer, em comparação com o DNA obtido do sangue (sangue total ou glóbulos brancos), ou cfDNA isolado do soro ou plasma, tem revelado diferenças entre o casos com câncer de mama e controles (118-125). Por outro lado, estes estudos têm revelado resultados controversos, e ainda faltam evidências claras de que as alterações de metilação no DNA podem servir como biomarcadores para o diagnóstico de câncer de mama, estratificação de estágios tumorais, predição do prognóstico, ou na recorrência do câncer.

Biópsia líquida e câncer de mama

O termo biópsia líquida vem da análise, ou busca por biomarcadores a partir de espécimes que não o tecido biológico (tumor), ou seja, fluidos, em especial, o sangue. Ao contrário das biópsias tradicionais, é um procedimento sem risco, minimamente invasivo, que não requer cirurgias, e pode reduzir custo e tempo no diagnóstico (126).

Muitos tumores são de difícil acesso, e mesmo quando possível, a heterogeneidade temporal e espacial do tumor não são explícitas em uma biópsia. Em demais exemplos, na necessidade de monitoriamento de evolução, ou recidiva, os casos são expostos constantemente a biópsias e a radiação, ou outros exames de imagem (MRI), que serão inconclusivos, ou insuficientes (126-128). Diante do respectivo cenário, a biópsia líquida emergiu como uma esperança na oncologia. Entretanto, por conta da limitação e conclusão dos estudos ja realizados, poucos são os resultados que de fato foram traduzidos para a clínica médica (126).

Atualmente a biópsia líquida é focada na análise das CTCs, DNA circulante tumoral (ctDNA), cfDNA, e vesículas extracelulares (exossomos); materiais estes derivados do tumor primário, ou sítio metástatico, que podem ser encontrados na corrente sanguínea, saliva, urina, líquido cefalorraquidiano, entre outros fluidos periféricos (126,129).

Células tumorais circulantes (CTCs)

CTCs são células tumorais liberadas do tumor primário, ou do sítio metástatico (130). O pesquisador australiano Thomas Ashworth relatou-as pela primeira vez em 1869 no sangue de um caso com câncer metastático de abdômen (131). Inicialmente as CTCs foram descritas como achados acidentais, e que ultrapassavam a barreira dos vasos sanguíneos a partir de forças homeostáticas, decorrentes do crescimento natural do tumor e estresses mecânicos da própria cirurgia (132). Ao longo dos anos, foi revelado que as CTCs, na realidade, não são exclusivas de tumores avançados. As CTCs com potencial de formação metastática podem ser liberadas ainda na fase inicial do tumor, e acompanham o seu crescimento. Além disso, a sua liberação é variável, e em maioria, são dependentes de fenótipo imune e genético do indivíduo (126,133,134).

O isolamento das CTCs ainda é um desafio, pois a sua detecção é mascarada por milhões de células imunes e bilhões de células vermelhas da corrente sanguínea (135). Os métodos de isolamento são baseados na biologia da célula tumoral, incluindo: captura por marcação de anticorpo específico (136-139), filtros por seleção de tamanho (140), depleção das células vermelhas e brancas da corrente sanguínea (141), dielotroforese (142), entre outras (143,144). Entretanto, o sistema *CellSearch system* (Menarini Silicon Biosystems) é o único sistema aprovado pelo FDA (*Food and Drug Administration*) para isolamento e detecção de CTCs de pacientes com tumor de mama, próstata e colorretal (136-139). O sistema consiste no enriquecimento das células epiteliais que expressam moléculas de adesão, do inglês *epithelial cell* (EpCAMs), e eliminam aquelas que expressam CD45 (antígeno comum aos leucócitos). Os primeiros estudos de validação clínica utilizando do *CellSearch* revelaram que a presença de ≥ 5 CTCs em 7,5 mL de sangue é um forte preditor para reduzir o tempo de sobrevida livre da doença (PFS), e < 5 CTCs em 7,5 mL de sangue é um indicador de melhora na sobrevida global para os cânceres de mama (145), pulmão (146) e colorretal (147). Já para tumores de colorretal metástaticos, a presença de ≥ 3 CTCs em 7,5 mL sugerem uma progressão livre da doença menor, enquanto < 3 CTCs em 7.5 mL de sangue sugere uma melhor sobrevida global (148).

DNA circulante livre (cfDNA) e DNA circulante tumoral (ctDNA)

O cfDNA é definido como todo o conjunto e qualquer DNA livre das estruturas subcelulares ou moleculares (148). Já o ctDNA faz parte de uma fração mínima do cfDNA total encontrado em indivíduos com câncer. Em condições fisiológicas normais, os detritos celulares necróticos e apoptóticos são removidos do tecido por fagócitos infiltrados; entretanto, este processo biológico não é eficaz no microambiente tumoral, levando a um aumento exponencial de cfDNA na corrente sanguínea nos casos com câncer (126). Atualmente o ctDNA tem sido o alvo da oncologia personalizada, pois pode trazer as mesmas alterações genéticas e mutações existentes no tumor primário (149), consequentemente oferecendo uma possibilidade de avaliação não invasiva e prognóstica do câncer.

Uma vantagem particular do ctDNA como biomarcador em comparação as CTCs é que ele pode ser informativo sobre o crescimento da massa tumoral, pois pode refletir a extensão da doença ou, predizer a uma resposta à uma determinada terapia (150). Uma

relação linear entre o volume do tumor e a frequência do alelo variante presente no ctDNA isolado do plasma foi encontrada em casos com câncer de pulmão de células não pequenas e em casos com câncer de ovário seroso de alto grau (151-153). Além disso, a alta concentração do ctDNA na corrente sanguinea após cirurgia curativa e/ou quimioterapia em casos com câncer de ovário (153), cólon (154), e pulmão (155), demonstrou ter forte valor indicativo de recorrência da doença. Intessantemente, o cfDNA tem mostrado importância no diagnóstico precoce, já que mutações em *KRAS* e *TP53* foram detectadas em controles saudáveis em até 14 e 20 meses, respectivamente, antes do diagnóstico de câncer. Dos 1648 cfDNAs de controles analisados, 33 apresentaram mutações nos genes *KRAS2* e/ou *TP53*. Entre os 137 indivíduos que desenvolveram câncer de bexiga, 5 tinham mutações em *KRAS2* [*odds ratio* (OR), 4,25; IC 95%, 1,27-14,15) e 7 tinham mutações em *TP53* (OR, 1,81; CI 95%, 0,66-4,97) (156).

As limitações do uso do ctDNA na clínica oncológica ainda está diretamente relacionada a três razões: 1) fácil degradação (dependente do rápido processamento após coleta), b) baixa concentração que limitam o seu uso e 3) custos altos para seu estudo que envolvem tecnologias de ponta, como exemplo, uso de sequencimento de nova geração ou PCR digital para detecção de mutações em baixa frequência. Dessa forma, os estudos em sua maioria avaliam os casos com tumores metastáticos (149). Atualmente existem dois testes no mercado aprovados pelo FDA: o *Cobas EGFR Mutation Test v2* (*Roche Molecular Diagnostics*), que detecta mutações de *EGFR* (157) em cfDNA de pacientes com câncer de pulmão (158). O teste também pode ser usado para detectar a mutação T790M de *EGFR* em pacientes cujo câncer progrediu após o tratamento com inibores de tirosina quinases com alvo à *EGFR.* Essa mutação leva à resistência à terapia e a sua detecção indica que o tratamento pode ser mudado do erlotinibe para o medicamento de terceira geração osimertinibe. O segundo teste, o Epi proColon® (*Epigenomics* AG) detecta o status de metilação do promotor *SEPT9* no cfDNA de pacientes submetidos a triagem para câncer colorretal (CCR) (159). As validações do teste revelaram que a hipermetilação de *SEPT9* é um forte preditor de ocorrência de CCR, porém de baixa sensibilidade para detecção de casos ainda em estágio inicial (160,161).

Recentemente o FDA recebeu dois pedidos de aprovação para painéis de rastreamento de mutações em mais de 70 genes, um da empresa *Foundation Medicine*, e outro da *Guardant Health*, utilizando do cfDNA como material de pesquisa. Dessa forma, ambos os testes podem ser os primeiros painéis de biópsia líquida a obter possíveis aprovações de regulamentação, e direcionadas a determinar o tratamento personalizado e alvo específico (162,163).

Outros materiais genéticos circulantes

Entre os outros materiais genéticos circulantes devem ser citados: os RNA tumorais circulantes (ctRNAs), que incluem os RNAs longos não codificantes (lncRNAs), com mais de 200 pares de bases (pb), e os microRNAs (miRNAS) menores que 200 pb (148,164).

Os lncRNA estão presentes no núcleo e podem interagir com o DNA e proteínas do complexo nuclear, consequentemente agindo sob a expressão gênica (165). Os lncRNAs também estão envolvidos no splicing regulatório de proteínas no citoplasma, e mantêm a estabilidade dos mRNAs na dependência de fatores de tradução (166). Ainda há pouca informação sobre os lncRNAs, e somente uma fração deles tem sido analisada experimentalmente. Os lncRNAs derivados de tumores têm sido apontados como candidatos à biomarcadores, com a possibilidade de oferecer diferentes abordagens terapêuticas (167).

Os microRNAs (miRNA) são pequenos RNAs endógenos de fita simples (18 a 25 nucleotídeos) (168). São responsáveis pela modulação de expressão de quase 30% dos genes codificadores de proteínas em humanos. Caso haja, alterações na estrutura deste material, eles podem provocar modificações de expressão ou disfunções, desencadeando processos biológicos que podem levar a tumorigênese e/ou afetar a progressão da doença (169).

Os miRNAs podem ser disseminados pelas células para a corrente sanguínea em uma forma estável e a análise de alguns deles tem potencial de distinguir pacientes com câncer, de indivíduos saudáveis (170). A biogênese dos miRNAs tem sido descrita nos últimos anos, entretanto o mecanismo que leva a sua incorporação em corpos multivesiculares, como exossomos, não é bem conhecido. Um desses possíveis processos, seguido de sua secreção no sangue, foi descrito por Fatima & Nawaz, (171) pelo experimento conhecido como *Dicer-knockout*. Além dos miRNAs encapsulados, existem outros tipos de miRNAs livres na circulação, como: miRNAs secretados passivamente estabilizados às proteínas de ligação ao RNA, ou aos complexos lipoprotéicos de alta densidade (172). A evidência de RNA livre no plasma/soro associado ao tumor, não apenas fornece novos alvos para monitoramento e detecção do câncer, mas abre a oportunidade de avaliação da expressão gênica não invasiva para o prognóstico de cânceres (173,174).

Exossomos

O transporte de materiais biológicos através de membranas é um processo crítico para manter a ordem da homeostase celular. Consiste de uma exportação ativa e passiva através de micropartículas, tais como exossomos, que mantêm uma adequada variedade de micro e macromoléculas interiorizadas relevantes. Ao cultivar reticulócitos de ovelhas, os pesquisadores Pan e John Stone relataram pela primeira vez os exossomos em 1983 (175).

Os exossomos são estruturas definidas como pequenas vesículas com membrana que são eliminadas das células com a função de troca de informações entre as células, além de atuarem como veículos de transporte para várias biomoléculas, como acidos nucléicos e proteínas (176,177); consequentemente são capazes de modificar a atividade gênica das células receptoras (126).

Os exossomos variam de tamanho, possuindo de dezenas a centenas de nanômetros (30–100nm) e, por causa da proteção por sua bicamada lipídica, têm uma notável vantagem sobre outras biomoléculas (178). Além disso, sua concentração sanguínea é geralmente maior que 10 bilhões por mL de sangue, dependendo da massa tumoral e estágio do tumor (176,179).

Exossomos, em comparação com CTCs e ctDNA, têm vantagens em vários aspectos. Devido ao seu tamanho homogêneo, e a sua forma particular, eles podem ser distinguíveis usando a microscopia eletrônica (180,181) e expressam proteínas de superfície específicas, que podem ser alvos terapêuticos. Além disso, levam informações das células tumorais, promovendo rotas e trocas de informações entre células que irão favorecer o processo da carcinogênese (182,183). Ainda neste contexto, o material genético encapsulado, como exemplo, RNAs e microRNAs podem descrever o fenótipo do tumor a ser formado (183).

Mais além, os exossomos são bastante inertes, mas a sua fusão entre a membrana celular podem alterar o mecanismo de expressão gênica da célula receptora. Este recurso torna-se potencialmente aplicável como perfeito carreador de drogas em nanoescala ou vetores de terapia gênica (126).

Marcadores bioquímicos e proteínas envolvidas no câncer de mama

Além dos fatores patológicos tradicionais, como tamanho, grau do tumor, status linfonodal, positividade dos receptores hormonais (RE, RP e HER2); os marcadores tumorais séricos têm assumido papeis coadjuvantes para sinalização da presença e resposta ao tratamento de muitas neoplasias malignas (184).

Segundo a Sociedade Americana de Oncologia Clínica (ASCO), os antígenos: carcinoembrionário (CEA), 15-3 (CA15-3) e 27-29 (CA27-29) devem ser usados com cautela devido a baixa sensibilidade e especificidade, e controvérsias de resultados (185). É preconizado que nunca devem ser usados como definidores de terapias, ou sinalizadores de resposta ao tratamento e recidivas. As recomendações para o uso destes marcadores são diretamente dependentes do histórico clínico de cada paciente, por exemplo, CA15-3 pode ser apresentado em alta concentração em casos de câncer de mama estágio 3 e 4, e em baixa concentração nos casos iniciais, estágio 1 e 2 (186). Mais além, níveis elevados podem ser encontrados em controles, quando comparados ao estágio 1 do câncer (186).

A partir da última década, a proteômica tem sido a principal ferramenta para descoberta de potenciais biomarcadores para o câncer, utilizando de diferentes fluidos, em especial o soro/plasma. Entretanto, entre milhares de proteínas ja identificadas até então, não há nenhum biomarcador capaz de ser usado na rotina para detecção precoce do câncer de mama, prognóstico e previsão da resposta ao tratamento (187). Apesar dos esforços notáveis realizados, existe uma discrepância entre a descoberta dos biomarcadores e o número real daqueles traduzidos a clínica (188). Isso pode ser explicado pelo número pequeno de casos incluídos em cada estudo, variabilidade e heterogeneidade populacional e dos procedimentos experimentais, como manuseio da amostra, limitação na metodologia e ausência de validação (188,189).

Neste contexto, as proteínas descritas para cada subtipo molecular de câncer de mama demonstraram ser comuns a outros tumores, porém fornecem um panorama das vias moleculares envolvidas em cada subtipo tumoral (190,191). Por exemplo, nos tumores luminais (classificação comum para as lesões impalpáveis) as proteínas envolvidas na arquitetura do citoesqueleto e transição epitélio-mesênquima (exemplo a vimentina) estão em super expressão, bem como todas as proteínas envolvidas na cascata imune do sistema complemento: C1s, C3, C3a, C4, C4a, C5 convertase, e C8 alpha (190,191).

Curiosamente, múltiplas proteínas ligadas as vias metabólicas associadas ao câncer, tais como glicólise, síntese de serina (PHGDH), consumo de glutamina (GLS), apresentam baixa expressão nos tumores luminais (191). As proteínas mais elevadas (FBP2 e FBP1) são enzimas chave no processo de gliconeogênese, que se opõem ao fluxo glicolítico. A rede do metabolismo energético revela diferenças acentuadas entre grupos moleculares e apontam metabólitos diferentes no crescimento e sobrevivência de fenótipo molecular. Neste caminho, muitos são os alvos possiveis de proteínas para rastreamento e detecção das lesões impalpáveis.

1. OBJETIVOS

1.1 Geral

Detectar potenciais marcadores circulantes envolvidos no desenvolvimento de lesões impalpáveis mamárias BIRADS 3 e 4.

1.2 Específicos

- Avaliar o perfil de metilação dos promotores dos genes *CDKN2A* e *ATM* no DNA de tecido tumoral e cfDNA de pacientes com lesões impalpáveis da mam;

- Identificar proteínas diferencialmente expressas na saliva e no plasma das pacientes com lesões impalpáveis da mama;

- Dosar e comparar a concentração da vitamina D do plasma das pacientes com lesões impalpáveis da mama *versus* grupo controle;

- Identificar mutações somáticas nos genes *TP53* (éxons 4 ao 9), *CDKN2A* (éxons 1 ao 3), *PIK3CA* (éxons 9 e 20) em DNA de tecido tumoral e cfDNA de pacientes com lesões impalpáveis da mama.

2. ARTIGO 1

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CDKN2A (*p14*^{ARF}/*p16*^{INK4a}) and *ATM* promoter methylation in patients with impalpable breast lesions

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Running head: DNA methylation in patients with impalpable breast lesions

Authors' disclosure of potential conflicts of interest
The authors declare no potential conflicts of interest

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Summary

Early detection of breast cancer increases the chances of cure, but the reliable identification of impalpable lesions is still a challenge. In spite of the advances in breast cancer detection, the molecular basis of impalpable lesions and the corresponding circulating biomarkers is not well understood. Impalpable lesions, classified by radiologists according to the Breast Imaging Reporting and Data System (BIRADS) in the category 3 and 4, can be either benign or malignant (slow growing or aggressive). In this paper, we report the DNA methylation pattern in *CDKN2A* ($p14^{ARF}$/$p16^{INK4a}$) and in *ATM* gene promoters from 62 impalpable lesions, 39 peripheral blood samples, and 39 saliva samples, assessed by Methylation Specific - Polymerase Chain Reaction (MSP-PCR) method. *ATM* showed the greatest percentage of methylation in DNA from lesions (benign and malignant), blood (even with $p16^{INK4a}$), and saliva, followed by $p16^{INK4a}$ and $p14^{ARF}$. Among the malignant cases, *ATM* promoter was the most hypermethylated in lesion DNA and in blood and saliva DNAs, and $p14^{ARF}$ the least. The highest percentage of $p16^{INK4a}$ methylation was found in the blood. Finally, our data are relevant because they were obtained using impalpable breast lesions from patients who were carefully recruited in 2 public hospitals of Rio de Janeiro.

1. Introduction

When imaging exams detect the presence of an impalpable lesion in the breast, women undergo invasive procedures to reveal the nature of the lesion (benign or malignant), and despite all the advances in imaging, one in two women receiving annual mammographic diagnosis must have at least one false-positive result during her lifetime [1]. The mapping of these lesions and their removal, considering the removal of the tumor free margin, as well as the preservation of the breast esthetics, has become a challenge for surgeons, mainly because these lesions are very small. In spite of the careful procedures, there remains a 50% chance of tumor progression [2,3].

The development of minimal invasive methods may revolutionize the detection of breast impalpable lesions, eliminating inconclusive repetitive biopsies, and avoiding physical and psychological discomfort of the patient. In recent years the introduction of tumor investigation known as "liquid biopsy" has allowed the detection of circulating tumor cells (CTC) and circulating tumoral free DNA (ctDNA) in the bloodstream [4], confirming that the tumors are systemic diseases.

DNA from white blood cells (WBC) are constantly suffering from molecular changes in the minimal presence of these circulating cells, even in response to distant lesions with atypical hyperplasia, benign lesions with the risk of breast carcinoma. Additionally, saliva DNA has demonstrated methylation rates similar to those found in the blood [5,6]. Therefore, the evaluations of methylation changes of target genes assessed in these fluids could contribute to the detection and/or monitoring of impalpable breast lesion evolution.

CDKN2A (*p14*^{ARF}/*p16*^{INK4a}) [7] is a tumor suppressor gene encoding the p16 protein, which is responsible for inhibiting the complex of cyclin-dependent kinases, at the G1/S phase of the cell cycle, resulting in cell cycle arrest [8]. The *CDKN2A* alternative protein is p14^{ARF}, which interacts with MDM2, responsible for the degradation of p53 [9]. The inactivation of the *CDKN2A* in breast tumors has been reported, and the methylation of the promoter region causes down expression [10]. The *ATM* gene [11] is responsible for encoding a protein (ATM) that is a member of the 3-kinase phosphatidylinositol family, which play a role in DNA repair pathways and in cell cycle control [12]. The *ATM* gene acts specifically in controlling double breaks in DNA and its [13] low expression is a result of the methylation of the promoter region in sporadic breast cancers [11]. In breast cancer patients, the *ATM* low expression is associated with shorter disease-free survival [14] and triple-negative tumors [15].

To contribute to understanding the nature and evolution of impalpable lesions, we assessed the methylation pattern in the promoters of the *p14*^{ARF}, *p16*^{INK4a}, and *ATM* genes in DNAs from 62 impalpable breast lesions, 39 blood samples, and 39 saliva samples. The impalpable lesions were classified according to the Breast Imaging Reporting and Data System as BIRADS 3 and 4, which could be either benign or malignant (slow growing or aggressive).

2. Materials and methods

Study population

Patients were recruited from 2008 to 2014, at the Instituto Nacional de Câncer (INCA) and at Hospital Universitário Gafrée-Guinle (HUGG), in the city of Rio de Janeiro, Brazil. All patients were diagnosed with impalpable lesions BIRADS 3 or 4. This study was approved by the Ethics Committee of both institutions (INCA-109/07; HUGG-07/2007 - 80/2012). Data on age, breast cancer family history, tumor classification, grade, size, nodal involvement, and immunohistochemical profile were obtained from hospital records.

All breast lesions obtained in this work by core biopsies, large excisions, and partial or total mastectomies were formalin-fixed and sections of 5 μm were stained with hematoxylin and eosin (HE). Histological classification was graded according to current (2012) World Health Organization (WHO) criteria [16] and nuclear grade was defined as grades I-III according to Elston and Ellis [17] (Figure 1). The histological classification and the nuclear grading were performed by a medical pathologist.

DNA from breast lesions were obtained from 62 patients; blood and saliva DNAs were obtained from the last 39 patients, of which 16 were from patients with benign lesions and 23 with malignant lesions.

2.1. DNA preparation

From Breast lesion – The tissue was collected during biopsy or surgery and washed with saline solution (0.9% NaCl). The DNA extraction was performed with proteinase K digestion and phenol-chloroform, according to standard protocols [18]. In the presence of microcalcifications, the tissue was immersed in the EDTA solution (0.5 M) and incubated at 55°C until the particles of calcium were completely dissolved before the steps for DNA extraction.

From Blood – 4 mL of blood was collected in EDTA and transferred to a 15 mL tube and then centrifuged at room temperature for 10 minutes at 2,000 g. The plasma was discarded and 10

mL of erythrocyte lysing solution (4ºC) was added to the cells (10 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM NaCl). The DNA extraction was performed according to standard protocols [18].

From Saliva - Approximately 1 ml of saliva was collected and centrifugated at 14,000 g for 15 minutes. The pellet was resuspended in 600 uL of lysis solution (10 mM TRIS, 2 mM EDTA, 400 mM NaCl) and 15 uL SDS 20%, and the DNA extraction was performed according to standard protocols [18,19].

DNA was quantified on the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.2. Evaluation of *p14^{ARF}*, *p16^{INK4a}*, and *ATM* promoter methylation

For DNA modification reaction, an EPITECT Kit (QIAGEN) was used according to the manufacturer's protocol. PCR amplification was performed in a 50 µL reaction mixture containing 50 ng of modified genomic DNA, STR 1X buffer (Invitrogen, Carlsbad, USA); 200 mM dNTPs (Invitrogen, Carlsbad, USA); 3 mM of $MgCl_2$ (50 mM) (Invitrogen, Carlsbad, USA); primers for each promoter (10 pmol/uL each); and 0.5 U *Platinum® Taq DNA Polymerase* (Invitrogen, Carlsbad, USA) in a final volume of 50 µL. The primers used for MSP-PCR (Methylation Specific Polymerase Chain Reaction) have been previously described [11,20,21]. *DLD-1* cell line was used as positive control for *p14^{ARF}* and *p16^{INK4a}*; for the *ATM* gene, DNA from peripheral blood of a healthy volunteer was treated *in vitro* with *SssI*® methyltransferase enzyme (New England Biolabs, Beverly, USA) as positive control.

PCR assays were performed in the thermal cycler PTC-100™ Programmable Thermal Controller (MJ Research, Massachusetts, USA). The PCR program consisted of a pre-denaturation at 94°C for the first 10 minutes, followed by 35 cycles at 94°C for 45 seconds, 60°C (methylated and non-methylated primers) for 45 seconds, and 72 for 1 minute. The final extension was performed at 72°C for 7 minutes.

MSP products were resolved in 10% polyacrylamide gels at 180 V in 1 X TBE buffer (Tris-Borate-EDTA), stained by silver [22].

2.3. Statistics

Contingency tables were used to associate hypermethylation (in lesion, blood, saliva and blood, or saliva) with age, menopausal age, malignancy, alcoholism, smoking, and family history of cancer. The chi-square test of Fisher was adopted to test the statistical significance of the association between these variables.

The survey data were processed in SPSS, version 17. In all statistical tests, a 5% significance level was considered. Thus, statistically significant associations were considered as those whose *p* value was less than 0.05.

3. Results

3.1 Clinical data

Sixty-two patients with BIRADS 3 and 4 were included in the study. The patients ranged in age from 18 to 80 years (mean 53.17 y, SD14.64), while the age range of the majority of the patients was 45 to 60 years, corresponding with the average age of the onset of menopause and breast changes. The social profile and clinical data of the cases are shown in Table 1. Statistical analysis showed no significant association between methylation findings and correlated clinic pathological variables. The significant *p* found was above 0.05 (p values for each variable are not shown). The malignancy of the lesions was statistically significant in patients older than 40 years ($p = 0.038$) and in those without family history of breast cancer ($p = 0.019$).

Among the 62 cases evaluated, the histopathological diagnosis revealed 41⁄62 (66%) malignant lesions (Figure 1, Table 2) and 21⁄62 (44%) benign lesions (Figure 2). The immunohistochemical evaluation of malignant tumors revealed that 22/41 were luminal, 5/41 cases were triple negative (ER/HER2 negative), and in 19/41 the expression of ER/HER2 was not determined, Table 3. Metastasis was present in 3/41 cases, being 2 luminal tumors (ER+) and 1 triple-negative (ER-/HER2-), Table 4. The luminal cases were diagnosed in 2008, developing metastasis 1 year later; then they were treated and are still alive as of now (2014). Unfortunately, one of the triple-negative cases, diagnosed in 2008, lymph node negative, generated metastasis 2 years later with the clinical outcome of death (Table 4). As of this writing (2014), none of the benign cases has progressed to malignancy.

3.2. Circulating $p14^{ARF}$/$p16^{INK4a}$ and *ATM* promoter methylation analysis

The DNA methylation pattern was assessed in promoters of $p14^{ARF}$, $p16^{INK4a}$, and *ATM* in 62 impalpable breast lesions and 39 blood and saliva samples. The overall results are summarized in Table 5. Among the 3 genes assayed, *ATM* showed the greatest percentage of methylation in promoter DNA from lesion (benign and malignant), blood (even with $p16^{INK4a}$), and saliva, followed by $p16^{INK4a}$ and $p14^{ARF}$ promoters. Among the malignant cases, *ATM* promoter was the most hypermethylated in lesion DNA and in blood and saliva DNAs, and $p14^{ARF}$ the least (Table 6). It is worth noting that the highest percentage of $p16^{INK4a}$

methylation was found in the blood. An example of the methylation results is found in Figure 3. For details of the histological types of hypermethylated cases shown in Table 6, please see supplementary Table 1.

4. Discussion

Advances in molecular characterization of breast cancer have revealed the molecular classification involved in the disease [23,24]. However, the phenotype, the molecular characteristics and the evolution of the impalpable lesions are still a matter of research. We believe that research on circulating biomarkers is especially important to breast tumor lesions that are heterogeneous [23,24,25].

The new data presented here on the methylation pattern of $p14^{ARF}/p16^{INK4a}$ and *ATM* promoters require careful interpretation. Overall, among the three genes tested, *ATM* was the one showing the greatest percentage of hypermethylation in lesion, blood, and saliva, in either benign or malignant cases (with one exception, see results). Considering just *ATM*, the greatest percentage was observed in breast lesion DNAs, followed by blood and saliva. To our knowledge this is the first study evaluating the profile of DNA methylation from saliva and its relation to the development of breast tumors, targeting the promoters of $p14^{ARF}/p16^{INK4a}$ and *ATM*. We believe that assessment of methylation could be performed as a screening tool for early breast cancer, complementing imaging tests and biopsies. Even though the methylation levels in WBC shall correspond to a significant risk factor for the development of breast cancer [26], more studies should be conducted to ensure the safety for clinical application.

In this study the hypermethylation of the evaluated promoters did not statistically differentiate between benign and malignant breast lesions. However, the data confirmed that, in special, WBC DNA undergo constant methylation modifications in the presence of cellular breast changes. Thus, hypermethylation should be applied to evaluation in a larger and different cohort for validation. The same analysis in additional populations is necessary to exclude the influence of social demographic factors and aging. Further, we have found that saliva DNA methylation rates were equivalent to those found in the blood, suggesting less invasive tests and more chances for success for tumor heterogeneity exploration.

The finding of circulating hypermethylated promoter of $p14^{ARF}/p16^{INK4a}$ and *ATM* (as well as *ATM* in breast lesion) in patients with benign lesions is not simply explained, and could be related with other unknown pathology or stress conditions. Hypothetically, among the malignant tumors it is possible that methylation levels in WBC DNA could be a result of

the presence of circulating tumor cells. So, more research about the methylation patterns of these genes and other genes may clarify the scenario.

The investigation of the $p14^{ARF}$ methylation pattern in breast cancer tissue has revealed hypermethylation in 47% of cases [27]. In our study this percentage was much lower, 1.6%. In another study with the blood of breast cancer patients, the percentage was from 9 up to 16% (grade dependent); in our study this percentage was greater, 26% [28].

The hypermethylation of $p16^{INK4a}$ (together with 21 other genes) was assayed in breast cancer tissues using high-throughput mass spectrometry on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) silico-chips in paraffin-embedded tissue samples (48 breast cancerous tissues and 48 paired normal tissues) [29]. The authors concluded that among the 10 most hypermethylated genes, $p16^{INK4a}$ showed 60% of hypermethylation. With the impalpable lesions we detected a much lower percentage (4.8%). One year later, the same group of authors published a work on the correlation of telomere length shortening with a promoter methylation profile of $p16^{INK4a}$ in the same cohort. They demonstrated that the shortening of the telomere length is correlated with the hypermethylation in $p16^{INK4a}$, leading to genomic instability [30].

The hypermethylation of $p16^{INK4a}$ in the blood was detected in 22%, compared with 1.3% in controls [28]. In the impalpaple lesions we detected 41%. Another publication reported the hypermethylation of $p16^{INK4a}$ (and 11 other genes) in serum DNA of breast cancer cases and benign breast disease controls by pyrosequencing [31]. The cases were separated: with lymph node-positive disease and lymph node-negative disease. There was also a benign breast disease control group, including women with non-proliferative benign changes (e.g. atrophic lobules, apocrine metaplasia) and women who had low-risk proliferative conditions (e.g. cysts, fibroadenoma and mild hyperplasia). Overall, median levels of promoter methylation were low, typically below 5%, for all genes analyzed in all study groups. Considering our blood results with $p16^{INK4a}$ promoter, the overall hypermethylation result was much higher, 41%. Considering only the benign impalpable lesions, the percentage was 25% and for the malignant lesions, 52.1%.

In advanced breast cases (stage II or greater), the methylation test MSP-PCR for *ATM* in the tumor tissues demonstrated hypermethylation in 78% of the cases [11]. Applying another method (high-throughput quantitative DNA methylation analysis technology), the methylation pattern in tumor tissues and adjacent normal tissues from breast cancer, diagnosed with ductal carcinoma (stages I - IV), was measured in 17 genes including *ATM*

[32]. The hypermethylation of *ATM* was shown in 36% of T1-T2 tumors and 46% in T3-T4. The percentage we determined in the impalpable lesions was 53.2%.

The *ATM* methylation pattern was measured in 2 *ATM* intragenic loci (ATMmvp2a and ATMmvp2b) from blood DNA from cases of invasive breast cancer and controls by pyrosequencing [26]. The combined analysis associating the methylation levels in ATMmvp2a and the breast cancer risk in 3 different breast cancer patient groups indicated that the ATM DMR (intragenic differentially methylated region) might be a biomarker of breast cancer risk.

Unfortunately, the comparison of our data on the hypermethylation in the promoters of $p14^{ARF}$, $p16^{INK4a}$, and *ATM* from the impalpable lesions and the corresponding fluids (blood and saliva) with the data from other publications [11, 26-32] is not possible due to differences in the applied methodology. Besides, most of our patients are exposed to social and economic conditions of stress and other variables intrinsic to the environment contributing to tumor development, which may explain in part the rates presented here.

The understanding of molecular biology and development of impalpable lesions is still a challenge for the medical field. In Brazil, 57,000 new female breast malignant cases are expected for 2015 [33] and, in spite of this huge number, the diagnosis of the cancer is rarely performed in the Brazilian public health system. There are often delays in the imaging exams, in the diagnosis, and in the treatment, resulting in a smaller survival rate for patients of the public service when compared to patients of the private health services [34,35]. Therefore, we believe our data are relevant because they were from impalpable breast lesions from patients who were carefully recruited in 2 public hospitals of Rio de Janeiro.

Further, molecular determination and development of impalpable lesions are still in characterization and open to questions. This study is original and is in extension. New analyses in monitoring and evaluation of methylation levels presented here show only a link between diversity and the response of the system to the tumor.

5. Conclusion

The methylation levels described herein in biological fluids (blood, saliva) of women with benign and malignant breast lesions confirm that these lesions generate systemically epigenetic responses. Assessment of methylation status of these fluids can increase knowledge of the individual heterogeneity of every woman confronted with breast lesions, minimizing unnecessary biopsies and imaging tests that are often noninformative or slow to reflect progression.

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Table 1 Clinical data of the 62 women who participated in the study

Characteristics	N=62	%
Age		
≤49	29	47%
50-59	10	16%
≥59	20	32%
NI	3	5%
Race [a]		
White	25	40.3%
Black / Mixed	25	40.3%
NI	12	19.4
Menarche		
≤12	22	35%
>12	31	50%
NI	9	15%
Menopause		
≤40	3	5%
40-45	3	5%
≥45	22	35%
NM	26	42%
NI	8	13%
Children		
No child	9	14%
1	8	13%
2	16	26%
3	9	14%
>4	8	13%
NI	12	20%
Oral contraceptive use		
Yes	32	52%
No	18	29%
NI	12	19%
History of breast / ovarian cancer		
Yes	25	40%
No	26	42%
NI	11	18%
Alcoholism		
Yes	14	23%
No	39	63%
NI	9	14%
Smoking		
Yes	15	24%
No	38	62%
NI	9	14%

Abbreviations: NI, not informed; NM, not menopausal.

[a] race was self-declared.

Table 2 Histological data

Histological type	n = 62	%
IDC	24	39%
DCIS	3	5%
IDC -DCIS	7	11%
ILC	1	2%
Other malignant lesions [a]	6	9%
Fibroadenoma	11	18%
Other benign lesions [b]	10	16%

Abbreviations: IDC, Invasive ductal carcinoma; DCIS, Ductal carcinoma *in situ*; ILC, Invasive lobular carcinoma; MC

[a] mucinous carcinoma, mucinous carcinoma associated with DCIS, micropapillary carcinoma and etc.

[b] ductal ectasia, atypical hyperplasia, pappiloma and etc.

Table 3 Immunohistochemical profile and tumor evolution data

Characteristics		%
ER status (n=41)		
Positive	15	37%
Negative	7	17%
Unknown	19	46%
PR status (n=41)		
Positive	16	39%
Negative	7	17%
Unknown	18	44%
HER2 status (n=41)		
Positive	4	10%
Negative	12	29%
Unknown	25	61%
Microcalcification (n=62)		
Yes	24	39%
No	19	30.5%
Unknown	19	30.5%
Lymphovascular invasion (n=41)		
Present	12	29%
Not present	18	44%
Unknown	11	27%
Metastic progression (n=41)		
Yes	3	7%
No	38	93%

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, Human Epidermal growth factor Receptor 2.

Table 4 Triple-negative cases

Cases	Age	Menarche	Menopause	Histopatologic	Grade	Linfonode	Metastatic	Death
1	41	12	No	IDC – DCIS	3	Free	Yes	Yes
2	51	13	49	IDC – DCIS	3	NI	No	No
3	42	9	No	IDC	3	NI	No	No
4	46	12	No	IDC	3	12	No	No
5 [a]	43	NI	NI	IDC	3	Free	No	No

Abbreviation: IDC, Invasive ductal carcinoma; DCIS, Ductal carcinoma *in situ*; NI, not informed.

[a] hereditary breast cancer not tested for *BRCA1* and *BRCA2*, confirmed cases in the family.

Table 5 DNA methylation pattern in $p14^{ARF}$, $p16^{INK4a}$ and *ATM* promoters

Tissue/Fluid	Promoter hypermethylation		
	$p14^{ARF}$	$p16^{INK4a}$	*ATM*
Lesion	1/62 (1.6%)	3/62 (4.8%)	33/62 (53.2%)
Blood	10/39 (26%)	16/39 (41%)	16/39 (41%)
Saliva	1/39 (26%)	6/39 (15.4%)	9/39 (23%)

Table 6 Lesion profile of malignancy and hypermethylation of *p14*^{ARF}, *p16*^{INK4a} and *ATM* promoters

	Benign			Malignant		
	p14^{ARF}	*p16*^{INK4a}	*ATM*	*p14*^{ARF}	*p16*^{INK4a}	*ATM*
Lesion	0/21	0/21	7/21 (33.3%)	1/41 (2.4%)	3/41 (7.3%)	26/41 (63.4%)
Blood	2/16 (12.5%)	4/16 (25%)	6/16 (37.5%)	8/23 (34.7%)	12/23 (52.1%)	10/23 (43.4%)
Saliva	0/16 (0%)	1/16 (6.25%)	4/16 (25%)	1/23 (4.3%)	5/23 (21.7%)	4/23 (17.4%)

Supplementary Table 1 Histological types of hypermethylated cases

	Benign (%)				Malignant n=41 (%)		
	p14^{ARF}	*p16^{INK4a}*	*ATM*		*p14^{ARF}*	*p16^{INK4a}*	*ATM*
Lesion (n=21)	0	0	1 Adenose 2 AH 1 Ductal Ectasia 3 Fibroadenomas	Lesion (n=41)	1 IDC	1 IC 1 IDC 1 DCIS-CM	1 DCIS 1 DCIS-CM 1 IC 12 IDC 5 IDC-DCIS 1 IDC-IC 1 ILC 2 MC 1 MC-DCIS 1 MPL
Blood (n=16)	1 AH 1 Fibroadenoma	3 AH 1 Fibroadenoma	3 AH 3 Fibroadenomas	Blood (n=23)	4 IDC 3 IDC-DCIS 1 DCIS	1 IC 3 IDC 1 DCIS 1 IDC-IC 4 IDC-DCIS 1 IDC-MC 1 MLP	1 IC 4 IDC 1 DCIS 2 IDC-DCIS 1 IDC-MC 1 IDC-IC
Saliva (n=16)	0 (0%)	1 AH	1 AH 2 Fibroadenomas 1 HWA	Saliva (n=23)	1 IDC	1 IC 1 IDC 1 IDC-DCIS 1 DCIS-MC 1 MPL	1 IDC 1 DCIS 1 IDC-DCIS 1 DCIS-MC

Abbreviations: AH, atypical hyperplasia, HWA, Hyperplasia without atypia; IDC, Invasive ductal carcinoma; DCIS, Ductal carcinoma *in situ*; IC, Intraductal carcinoma; MC, mucinous carcinoma; MLP, malignant papillary lesion; ILC, Invasive lobular carcinoma; MP, micropapillary carcinoma.

Figure 1. Histopathology of an IDC case

Legend

IDC [A-C] histological grade 3 (core 3, mitotic index 3, tubular formation 3) and MC [D] nuclear grade 2. Hematoxylin-eosin staining [A-D], original magnification × 4 [A], × 10 [B], and × 40 [C-D]. Abbreviations: IDC, infiltrating ductal carcinoma; MC, mucinous carcinoma.

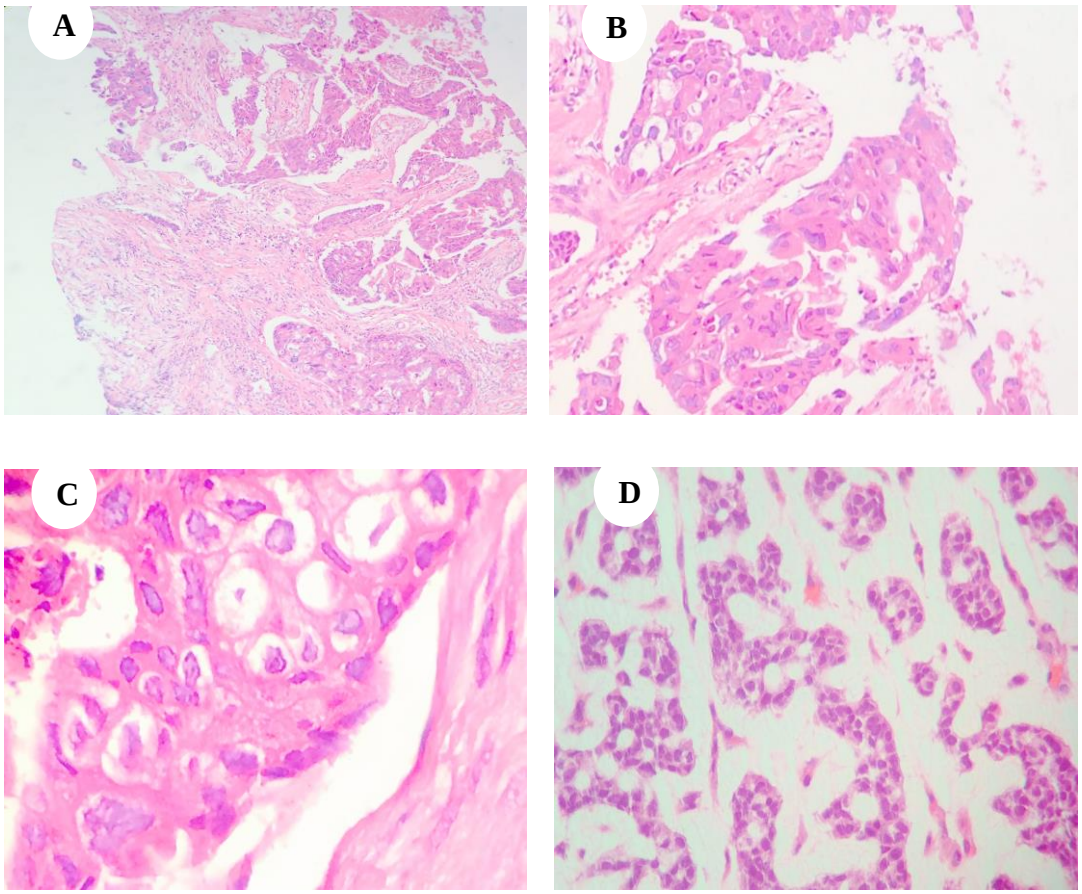


Figure 2. Histopathology of two benign lesion cases

Legend

Case of fibroadenoma [A-B] and case of hyperplasia without atypia [C-D] stained with hematoxylin-eosin. In the pictures A (× 10) and B (× 20) it is possible to observe the intralobular stromal proliferation of the glands with expanding edges (star, B). In pictures C-D (10 ×, 40 ×) the changes in columnar cells without atypia are highlighted by arrows. The ducts are enlarged and are covered by columnar cells arranged perpendicularly to membrama basal and luminal beheading.

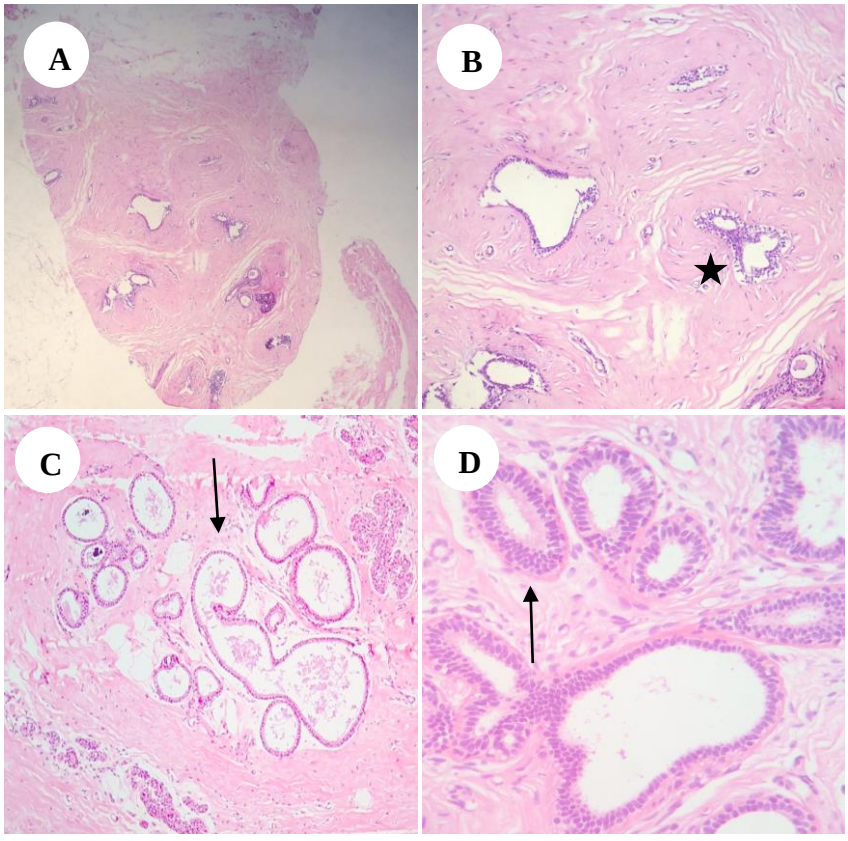
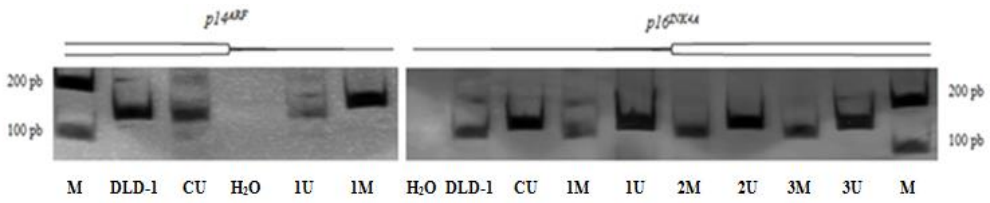


Figure 3: Methylation profile of breast lesions for *CDKN2A* (*p14ARF/p16INK4a*) in nondenaturing polyacrylamide gel stained with silver

Legend

p14ARF - M – molecular weight marker; *DLD-1* – positive control for methylation (122 pb); UC – unmethylated control (12 bp); N – H2O; U – DNA breast lesion unmethylated; M1 – DNA breast lesion methylated. *p16INK4a* – N – H_2O; *DLD-1* – positive control for methylation (150 bp); UC – unmethylated control (151 bp); M1, M2, M3 – DNA breast lesions methylated; U1, U2, U3 – DNA breast lesion unmethylated; M – molecular weight marker.



3. ARTIGO 2

Este artigo está aprovado para publicação na revista Oncology Letters, *in press*

Methylation profiling in promoter sequences of *ATM* and *CDKN2A* (*p14^{ARF}/p16^{INK4a}*) genes in blood and cfDNA from women with impalpable breast lesions

Methylation in liquid biopsy of women with impalpable breast lesions

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Abstract

The objective of this study was to evaluate the epigenetic changes occurring in early stages of breast cancer. We investigated the methylation profile of the *ATM*, $p14^{ARF}$, and $p16^{INK4a}$ promoters in total blood and plasma cell-free DNA (cfDNA) from women with impalpable breast lesions in comparison to total blood of a control cohort of women without breast lesions. The samples were evaluated by methylation-specific polymerase chain reaction 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, 56 women with impalpable breast cancer (39 out of 56 also had paired plasma cfDNA) and 55 women in the control cohort (55 blood DNA). As for blood DNA from women with malignant impalpable breast lesions, $p16^{INK4a}$ showed the greatest percentage of methylation (48%), followed by *ATM* (37.5%) and $p14^{ARF}$ (27%) promoters, regardless of age variation. For plasma cfDNA, the methylation rates for *ATM*, $p14^{ARF}$, *and* $p16^{INK4a}$ were 26%, 26%, and 10% respectively. The methylation rates for the blood DNA of controls were the lowest for *ATM* (9%), $p14^{ARF}$ (7%), *and* $p16^{INK4a}$ (7%). The women with impalpable breast lesions (benign and malignant lesions) had the highest methylation rate, regardless of age, compared to the paired plasma cfDNA and controls. This epigenetic change was statistically significant for the promoters of *ATM* (P=0.009) and $p16^{INK4a}$ (P=0.001) (impalpable breast lesions versus control). This study shows that epigenetic changes occurring in the *ATM* and *CDKN2A* genes detectable in liquid biopsy correlates the development of impalpable breast lesions.

Key words: Impalpable breast lesions, Methylation, White Blood Cells, Nipple Aspirate Fluid, cfDNA, *ATM*, $p14^{ARF}$, $p16^{INK4a}$

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 of 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 *CDKN2A* ($p14^{ARF}$/$p16^{INK4a}$) 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 Gafrée-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, number 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 (BIRADS) 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 g. The plasma was discarded and 10 mL of erythrocyte lysing solution (4ºC) was added to the cells (10 mM Tris-HCl, 5 mM $MgCl_2$, 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 g for 10 min at 20ºC to remove debris. Plasma was harvested and stored at -80ºC. When DNA was to be analyzed, 2 mL was used to obtain cfDNA using the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The blood DNA and cfDNA samples were quantified with the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

Evaluation of ATM and CDKN2A promoter methylation. For DNA modification reaction, the EpiTect Bissulfite (Qiagen, Hilden, Germany) and EZ DNA Methylation (Zymo Research, Irvine, CA, USA) 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 (Invitrogen, Carlsbad, CA, USA), 200 mM dNTPs (Invitrogen, Carlsbad, CA, USA); 3 mM of $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), primers for each promoter (10 pmol/uL each), and 0.2 U *Platinum® Taq DNA Polymerase* (Invitrogen, Carlsbad, USA) in a final volume of 25 µL. The primers used for MSP-PCR (Methylation Specific Polymerase Chain Reaction) have been previously described (39-41). Universal Methylated DNA Standard (Zymo Reseach, Irvine, CA, USA) 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 Scientific, Foster City, CA, USA). 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 gels at 160 V in 1 X TBE buffer (Tris-Borate-EDTA), stained by silver.

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 less than 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 y, *SD* = 7.12), while the patients with malignant breast lesions ranged in age from 33 to 90 (*M* = 61 y, *SD* = 11.62). The NAF patients ranged in age from 30 to 82 years (*M* = 50 y, *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 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, $p16^{INK4a}$ showed the greatest percentage of methylation, regardless of age variation, followed by *ATM* and $p14^{ARF}$ promoters. However, the $p14^{ARF}$ 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 $p14^{ARF}$ in cases older than 50 years old (Table III). Regarding the control subjects, there was no difference in the methylation positivity for $p14^{ARF}$ and $p16^{INK4a}$ (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, please see Table 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 $p16^{INK4a}$ 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 $p16^{INK4a}$ 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*, $p14^{ARF}$, $p14^{ARF}$/*ATM*, and $p16^{INK4a}$, respectively. For clinical and histopathological details, see 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*, $p14^{ARF}$, and $p16^{INK4a}$) and age (ROC curve) did not reveal statistically significant values. For all analyses, *P* value was greater than 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 $p16^{INK4a}$ (*P*=0.001) (*P*=0.003) (impalpable breast lesions versus control and cfDNA) (Tables IV and V).

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*, $p14^{ARF}$, and $p16^{INK4a}$ genes in the previous study were 41%, 26%, and 41%, respectively, whereas in the current study frequencies were 37.5%, 27%, and 48% (Table II), respectively. Both studies show the *ATM* and $p16^{INK4a}$ 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 (ATMmvp2a and ATMmvp2b) 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) $p14^{ARF}$ and $p16^{INK4a}$ hypermethylation in blood from women with breast cancer was 11% and 22%, respectively. Further, the authors found a significant association between hypermethylation for $p14^{ARF}$ (P=0.004) and $p16^{INK4a}$ (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 $p14^{ARF}$ and $p16^{INK4a}$, respectively. In addition, the significant association between hypermethylation of $p16^{INK4a}$ and *ATM* was revealed in women over 50 years old (Tables IV and V). These data show that hypermethylation of $p14^{ARF}$ and $p16^{INK4a}$ promoters demonstrate 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 S2). In this context, it is not possible to affirm that the hypermethylation found in these cases originated from the CTCs.

The $p16^{INK4a}$ 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 $p16^{INK4A}$ 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 the data supporting the results and conclusions are included within this article.

Author contributions

LD, MHFO and GA conceived and designed the study. LD, RJG, POV, ABPS conducted the experiments. LD, MASMC, MVF, CMA and GA recruited the cases, acquired pathological and radiological reports, analyzed and interpreted the data. LD, LRS, MVF and GA obtained statistical data, analyzed and interpreted. 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

This study was approved by the Institutional Ethics Committee of the Hospital Universitário Gafrée-Guinle (HUGG) and Rio de Janeiro State University Hospital (approval numbers are HUGG-07/2007 - 80/2012, and CAAE:43560115.5.0000.5259, respectively). The subjects enrolled in this study signed the informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Supplementary material

Table SI – Description of the demographic and clinical data of the evaluated cases.

Table SII - Socio-demographic and clinical data from cases that had coincident methylation results between blood DNA and cfDNA.

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Table I. Social demographic and clinical data of the cases available.

	Patients
	N=111 (%)
Characteristic	
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 (serupurulent)	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 IIa)	6 (13)
T2N1M0 (stage IIa)	1 (2)
TisN0M0 (stage IIa)	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)
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)

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. *Malignant lesions.

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

Blood DNA from women with impalpable breast lesions		
Gene	Benign lesions (N=8) (%)	Malignant lesions (N=48) (%)
ATM	0	18/48 (37.5)
$p14^{ARF}$	4/8 (50)	13/48 (27)
$p16^{INK4a}$	3/8 (37.5)	23/48 (48)
cfDNA from women wih impalpable breast lesions		
	Benign lesions (N=6) (%)	Malignant lesions (N=39) (%)
ATM	2/6 (33.3)	10/39 (26)
$p14^{ARF}$	0	10/39 (26)
$p16^{INK4a}$	0	4/39 (10)
Blood DNA from women with NAF		
	Cases (N=55) (%)	
ATM	5/55 (9)	
$p14^{ARF}$	4/55 (7)	
$p16^{INK4a}$	4/55 (7)	

cfDNA = circulating free DNA; NAF = Nipple aspirate fluid.

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

Blood DNA from women with impalpable breast lesions			
Gene	≤ 50 years Benign (N=8) (%)	≤ 50 years Malignant (N=9) (%)	> 50 years Malignant (N=39) (%)
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)
cfDNA from women wih impalpable breast lesions			
	≤ 50 years Benign (N=6) (%)	≤ 50 years Malignant (N=6) (%)	> 50 years Malignant (N=27) (%)
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 from women with NAF			
	≤ 50 years (N=28) (%)	> 50 years (N=27) (%)	
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.

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

Genes/Methytation	Groups (> 50 years old)		*P*-values
	Cases (%)	NAF (%)	
ATM			
No	25 (64.1)	25 (92.6)	0.009
Yes	14 (35.9)	2 (7.4)	
$p14^{ARF}$			
No	29 (74.3)	25 (92.6)	0.102
Yes	10 (25.7)	2 (7.4)	
$p16^{INK4a}$			
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*^{ARF} and *p16*^{INK4a} genes among the groups of women with impalpable breast lesions (blood and cfDNA).

Genes/Methylation	Group (> 50 years old)		*P*-value
	IBL (N=39) (%)	cfDNA (N=27) (%)	
ATM			
No	25 (64.1)	18 (66.7)	1.000
Yes	14 (35.9)	9 (33.3)	
p14^{ARF}			
No	29 (74.3)	18 (66.7)	0.584
Yes	10 (25.7)	9 (33.3)	
p16^{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.

Table SI. Description of the demographic and clinical data of the evaluated cases.

		Cases			cfDNA			Histopathological diagnosis	TNM	Lymph node infiltration	IHC ER/PR/HER	Ki67
	Age (years)	*ATM*	*p14^{ARF}*	*p16^{INK4a}*	*ATM*	*p14^{ARF}*	*p16^{INK4a}*					
1	49		X	X				Ductal ectasia and aprocrine metaplasia				
2	48			X	No evaluated			Ductal ectasia and aprocrine metaplasia				
3	46		X					Hyperplasia of columnar cells with and without atypia				
4	35		X		X			Ductal ectasia and aprocrine metaplasia				
5	45		X					Hyperplasia of columnar cells with and without atypia				
6	27							Fibroadenoma				
7	46			X	X			Hyperplasia of columnar cells with				

								and without atypia				
8	45				No evaluated			Ductal ectasia and aprocrine metaplasia				
9	47			X				IDC grade 2	T1N0M0	no	+/+/-	10%
10	41	X	X					IDC grade 3	T1N1M0	yes	-/-/-	95%
11	50						X	IDC grade 2	T1N0M0	no	+/+/+	20%
12	45				No evaluated			IDC grade 1	T1N0M0	no	+/+/-	7%
13	42	X	X					IDC + Dcis grade 1	T1N0M0	no	+/+/-	10%
14	47	X		X				IDC grade 3	T1N0M0	no	-/-/-	80%
15	39	X	X	X	X	X		IDC grade 3 + DCIS	T1N0M0	no	+/-/+	40%
16	39			X	No evaluated			IDC grade 2 + DCIS	T1N0M0	no	+/+/-	NI
17	33			X				IDC grade 2	T1N0M0	no	+/+/-	NI
18	62			X				IDC grade 2	T1N1M0	yes	NI	NI
19	90		X	X				IDC grade 2 + DCIS	T1N0M0	no	+/+/-	10%
20	70			X	X			DCIS grade 3	T1N0M0	no	+/-/-	25%
21	62	X		X	X		X	DCIS grade 2	TisN0M0	no	+/+/NI	NI
22	61	X		X				DCIS and IDC grade 2	T1N0M0	no	+/+/-	NI
23	81	X	X					IDC grade 2	T1N1M0	yes	+/+/-	10%
24	63	X						DCIS grade 1	T1N0M0	no	+/+/NI	NI
25	64							IDC grade 1 + DCIS	T1N1M0	yes	+/+/-	5%

								grade 2				
26	86				No evaluated			Micropapillar carcinoma grade 2 + DCIS grade 2	T1N0M0	no	+/+/+	25%
27	70	X					X	IDC grade 1	T1N0M0	no	+/-/-	5%
28	63					X		DCIS grade 2	T1N0M0	no	NI	NI
29	69			X				IDC grade 2 + DCIS grade 3	T1N0M0	no	+/-/-	20%
30	57	X					X	IDC grade 2 + DCIS grade 2	T1N1M0	yes	+/+/-	10%
31	56				No evaluated			IDC grade 1	T1N0M0	no	+/+/-	10%
32	65			X				IDC grade 2 + DCIS grade 1	T1N0M0	no	+/+/-	5%
33	60							IDC grade 2 + DCIS grade 2	T1N0M0	no	+/+/-	10%
34	64							IDC grade 3	T1N0M0	no	+/+/+	NI
35	59	X			X			ILC grade 2 + LCIS grade 2	T2N1M0	yes	+/+/-	7%
36	55					X		IDC grade 2	T1N0M0	no	+/+/+	10%
37	51			X	X	X		ILC grade 2 + LCIS grade 2	T1N0M0	no	+/+/-	5%

38	58			X		X		IDC grade 3 + DCIS grade 3	T1N0M0	no	+/+/-	40%
39	69			X		X		IDC grade 2 + DCIS grade 2	T1N0M0	no	+/+/-	5%
40	62		X	X		X		DCIS grade 3	T1N0M0	no	NI	NI
41	59	X	X		X			IDC grade 2 + DCIS grade 2	T1N0M0	no	NI	NI
42	71		X					IDC	T1N0M0	no	+/+/-	15%
43	70	X	X		X			IDC grade 3	T1N0M0	no	+/+/-	15%
44	55	X	X	X				IDC grade 1	T1N0M0	no	NI	NI
45	64							IDC grade 1 + DCIS grade 1	T1N0M0	no	+/+/-	7%
46	77		X			X		IDC grade 2	T1N0M0	no	NI	NI
47	61	X	X		X	X		ILC grade 2 + LCIS grade 2	T1N0M0	no	+/+/-	5%
48	58			X				IDC grade 3 + DCIS grade 3	T1N0M0	no	+/+/-	25%
49	55	X		X	X			IDC grade 1	T1N0M0	no	+/+/-	5%
50	54			X	X			LCIS grade 2	T1N0M0	no	+/-/-	NI
51	70					X		IDC grade 2	T1N0M0	no	+/+/-	15%
52	65				No evaluated			ILC + LCIS grade 1	T1N0M0	no	+/+/-	NI

53	59							IDC grade 1	T1N0M0	no	+/+/-	NI
54	72			X				IDC grade 2 + DCIS grade 3	T1N0M0	no	+/+/-	NI
55	55	X		X				IDC grade 2 + DCIS grade 2	T1N0M0	no	+/-/+	NI
56	63	X	X	X				Micropapillar carcinoma grade 3	T1N1M0	yes	+/-/+	20%

IDC = infiltrative ductal carcinoma; DCIS = ductal carcinoma in situ; ILC = infiltrative lobular carcinoma; ILCS = lobular carcinoma in situ; IHC = Immunohistochemistry; NI = not informative; ER = estrogen receptor; PR = progesterone receptor; HER = Human Epidermal Receptor.

Table SII. Socio-demographic and clinical data from cases that had coincident methylation results between blood DNA and cfDNA.

Cases Number	Blood DNA			cfDNA			Age (years)	Histologic classification	Lymph node infiltration	IHC ER/PR/HER2	Ki67
	ATM	$p14^{AR}_{F}$	$p16^{INK4}_{a}$	*ATM*	$p14^{AR}_{F}$	$p16^{INK4}_{a}$					
1	M	M	M	M	M		39	IDC grade 3/DCIS	No	+/-/+	40%
2	M		M	M		M	62	DCIS grade 2	No	+/+/NI	NI
3	M			M			59	ILC grade 2/LCIS grade 2	Yes	+/+/-	7%
4		M	M		M		62	DCIS grade 3	No	NI	NI
5	M	M		M			59	IDC grade 2/DCIS grade 2	No	NI	NI
6	M	M		M			70	IDC grade 3	No	+/+/-	15%
7		M			M		77	IDC grade 2	No	NI	NI
8	M	M		M	M		61	ILC grade 2/LCIS grade 2	No	+/+/-	5%
9	M		M	M			55	IDC grade 1	No	+/+/-	5%

M, methylated cases; IDC, infiltrative ductal carcinoma; DCIS, ductal carcinoma *in situ*; ILC, infiltrative lobular carcinoma; LCIS, lobular carcinoma *in situ*; IHC, Immunohistochemistry; NI, not informative; ER, estrogen receptor; PR, progesterone receptor; HER2, Human Epidermal Receptor 2.

4. DISCUSSÃO ARTIGOS 1 e 2

Os dados aqui apresentados apontam que as lesões impalpáveis da mama podem ser capazes de gerar alterações epigenéticas de forma sistêmica. A metilação do DNA é uma alteração genética modificável e possui a capacidade de regular a expressão gênica sem modificar a sequência do DNA. Alguns estudos prévios a este tem demonstrado que a hipermetilação em genes específicos nas células brancas do sangue (WBC) fornecem uma alternativa promissora para o rastreamento do câncer de mama (19,118-121,192). Dentre este contexto, os genes *ATM* e *CDKN2A* ($p14^{ARF}$ e $p16^{INK4A}$) tem sido descritos, não apenas hipermetilados no tecido tumoral, mas também no DNA do sangue total e cfDNA de casos com câncer de mama (4,119,121,192,193).

A avaliação da presença da metilação nos promotores de *ATM* e *CDKN2A* ($p14^{ARF}$ e $p16^{INK4A}$) foi realizada em duas coortes de mulheres com lesões impalpáveis da mama com resultados sócio-demográficos e clínico-patológicos parecidos (Tabelas 1, 2 e 3 artigo 1 e Tabela 1, artigo 2). Sessenta e duas mulheres foram incluídas no primeiro estudo, das quais 24/62 (39%), 7/62 (11%) e 21/62 (34%) tiveram diagnóstico de lesões mamárias ductais infiltrativas, lesões mistas (*in situ* e infiltrativas) e benignas, respectivamente. No segundo estudo, das 56 mulheres avaliadas, 19/56 (40%), 17/56 (35%) e 8/56 (14%) tiveram diagnóstico de lesões mamárias ductais infiltrativas, lesões mistas (*in situ* e infiltrativas) e benignas, respectivamente. Estes dados demonstram a característica da carcinogênese inicial, com o microambiente tumoral compartilhado entre lesões *in situ* e infiltrativas. Mais além, poucos casos apresentaram infiltração de linfonodo (3/41 (7%) e 7/48 (15%), para primeiro e segundo estudo, respectivamente).

Os receptores hormonais são cruciais para determinação do fenótipo e tratamento do câncer de mama, sendo assim, cabe ressaltar a disparidade entre os resultados imunohistoquímicos das duas coortes avaliadas. Dos 41 casos avaliados, 19/41 (46%), 18/41 (44%) e 25/41 (61%) não tiveram laudo de determinação do status dos receptores hormonais (RE, RP e HER2) para o primeiro estudo (rede pública). Para o segundo estudo (rede privada), todos os valores foram inferiores a 20%. Estes dados são alarmantes e chamam atenção pela deficiência na rede pública de saúde. No Brasil, infelizmente o tempo e as taxas de diagnóstico e sobrevida para os casos atendidos na rede pública são inferiores aquelas de casos atendidos na rede particular (194,195).

Em relação às frequências de metilação encontradas no sangue e nos tumores mamários, como amplamente discutido nos artigos, os dados estão em concordância com os da literatura (92,119,121,123,193,197,198).

As frequências de metilação encontradas no DNA do sangue foram de 41%-37,5%, 26%-27% e 41%-48% para os genes *ATM*, $p14^{ARF}$ e $p16^{INK4a}$, artigo 1 artigo 2, respectivamente. Ambos os estudos revelam frequências próximas, entretanto os genes *ATM* e $p16^{INK4a}$ apresentam alta taxa de hipermetilação nos casos malignos, demonstrando o silenciamento e o envolvimento das vias de reparo, senescência e controle do ciclo celular no estabelecimento das lesões mamárias impalpáveis.

A frequência de metilação encontrada nos tumores avaliados (N=62) foram de 1/62 (1,6%), 3/62 (4,8%) e 33/62 (53,2%) para os genes $p14^{ARF}$ e $p16^{INK4a}$ e *ATM*. Esses achados, variam, especialmente pelo estadiamento e agressividade do tumor (76-80,82,83,92,94,197). Por exemplo, em três estudos, nos quais os tumores mamários avaliados eram avançados, as taxas de metilação foram superiores (92,197,199) as encontradas em nosso estudo. No estudo de Sharma *et al.* (199) a hipermetilação do gene $p14^{ARF}$ foi detectada em 47% dos tumores mamários, em contradição, nós encontramos em 1,6% dos casos. Da mesma forma, no estudo de Radpour *et al.* 2009 (200), 60% dos tumores estavam metilados para $p16^{INK4A}$, enquanto no nosso estudo apenas 4,8%. No estudo de Vo *et al*. (92), 78% dos tumores de mama apresentaram hipermetilação para o gene *ATM*. Já para os tumores iniciais, como os aqui avaliados (T1-T2), essa taxa foi de 36% (197).

As análises de metilação realizadas na saliva foram exploratórias, entretanto trazem um perfil das modificações sistêmicas ocorridas na instalação do câncer mamário. Dos 39 casos avaliados, 1/39 (2,6%), 6/39 (15,4%) e 9/39 (23%) estavam metilados para $p14^{ARF}$, $p16^{INK4a}$ e *ATM*. Não há dados na literatura para comparação, entretanto as avaliações a saliva tem mostrado ser um fluido promissor para descoberta de novos marcadores (201,202). Os trabalhos recentes detalham dificuldades, porém enaltecem a possibilidade de novas descobertas, em especial para as proteínas que possam estar alteradas nas neoplasias da mama (203,204). O trabalho de Zhang *et al.,* (205) é considerado o primeiro estudo de desenho experimental detalhado da validação de oito mRNAs e a proteína a anidrase carbônica VI, em pacientes com câncer de mama. Os achados demonstraram sensibilidade de 83% e especificidade de 97%. Entretanto, os autores enfatizam a validação em coortes ampliadas, e em difereres tipo de tumores.

Vale ressaltar que várias técnicas são utilizadas para detecção da metilação, e estas consequentemente refletem as análises e resultados encontrados. A técnica mais comum e

utilizada é a *MSP*, entretanto, devido a sua limitação qualitativa, os ensaios multigênicos (Pirosequenciamento, *Infinium HumanMethylation* 27K e 450K B*eadchip*) têm ampliado e refinado o número de possíveis marcadores circulantes para detecção de patologias mamárias (19,118-121,206-208).

Nós ressaltamos a necessidade de expansão do estudo, em especial para caracterização das modificações ocorridas nas células imunes presentes no sangue. Os achados aqui revelados representam a amplitude e a diversidade dos mecanismos envolvidos na carcinogênese e evolução das lesões impalpáveis da mama.

Como apêndice, nós destacamos que a segundo coorte de mulheres teve o seu tumor e cfDNA sequenciados para os genes *PIK3CA* (éxons 9 e 20), *TP53* (éxons 4-9) e *CDKN2A* (éxons 1-3). Um total de 37 mutações foram encontradas, sendo 8/58 (14%), 18/58 (31%) e 11/58 (19%) para os genes *PIK3CA*, *TP53* e *CDKN2A*, respectivamente (Apêndice A). Das 37 mutações detectadas, 10 (27%) e 16 (43%) mutações foram detectadas em lesões de mama benignas e malignas, respectivamente, enquanto 2 (5%) e 9 (24%) foram encontradas em cfDNA de mulheres com lesões benignas e malignas, respectivamente. O comprometimento linfonodal com mutações no *PIK3CA* em lesões malignas (P=0,001), e a relação entre mutações no *PIK3CA*, comparando tumores ductais com lesões benignas (P=0,05), foram estatisticamente significantes. Deste estudo, nós detectamos mutações diversificadas nas lesões impalpáveis e no correspondente cfDNA. Estes dados demonstram que os testes não invasivos são provavelmente bem sucedidos na avaliação da heterogeneidade do tumor. Embora tenhamos encontrado taxas representativas de mutação nas lesões benignas, existem fatores intrínsecos para cada microambiente tumoral que podem ser influenciados por variáveis ambientais e comportamentais que merecem ser estudadas individualmente.

5. ARTIGO 3

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Proteomic profile of saliva and plasma from women with impalpable breast lesions

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Running head: Circulating proteins from women with impalpable breast lesions

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The authors declare no potential conflicts of interest

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Abstract

This study evaluates the proteomic profile of saliva and plasma from women with impalpable breast lesions using the nLC-Q-TOF technology. Plasma and saliva from patients with fibroadenoma (n = 10), infiltrating ductal carcinoma (n = 10) were analyzed by combinations of inter/intra- and control groups (n = 8), revealing significant quantitative and qualitative differences. The major differentially expressed proteins in saliva compared to the control were alpha 2-macroglobulin and ceruloplasmin, but the proteins with fold change and p-value cut-off were leukocyte elastase inhibitor and alpha-enolase, and deleted in malignant brain tumors 1. Concerning plasma, alpha-2-macroglobulin and ceruplasmin were overexpressed while other proteins such as haptoglobin, hemopexin, and vitamin D-binding protein were down-expressed. The changes in immune, molecular transport and signaling pathways were the most representative in the proteomic profile of the saliva and plasma. This is the first study that describes the proteome of saliva and plasma from the same women with impalpable breast lesions.

Introduction

The detection of impalpable breast lesions by mammography has become more common, accounting for about 25% of diagnosed breast pathologies (1). The detection of impalpable breast lesions generates questions related to the nature of the lesion (benign or malignant) and, consequently, to the conduct of the investigation to be executed. Even with the advances in imaging, the need for biopsies to determine the origin of the lesion has not been eliminated. Furthermore, the biopsies do not represent the best method, as the tumor microenvironment can be heterogeneous and unpredictable (2).

The development of non-invasive techniques would revolutionize the detection of early breast cancer, avoiding the physical and psychological discomfort of patients who are submitted to biopsies (3). Currently, the growing research field of circulating biomarkers present in the body fluids or "liquid biopsy," promises to revolutionize the undetected malignant cells or their molecular biomarkers (2,4-6).

Until a few years ago, saliva was considered only a fluid supporting the digestive system and a stimulating factor for release of endogenous enzymes. More recently, saliva has become a new alternative source for biomarkers that can reveal local and systemic diseases (7-9). Saliva is a liquid less complex than blood, but still able to contain the same proteins and represent the pathological condition of the individual (6,10).

On the other hand, blood has been the most studied fluid for biomarker research. The disadvantage of using plasma (blood liquid component) is that it is rich in albumin and other high abundance proteins. However, methods for enrichment of low abundance proteins found in plasma have been developed (11).

As a matter of scientific interest, the proteomic profiles of saliva and albumin-depleted plasma were herein studied, comparing specimens obtained from patients with impalpable breast lesions and a control group. One-dimensional gel electrophoresis and liquid chromatography, followed by mass spectrometry, were applied.

Materials and Methods

Patient selection. Patients were recruited between 2008 and 2014, at two public hospitals in Rio de Janeiro, Brazil: Instituto Nacional de Câncer (INCA) and Hospital Universitário Gafrée-Guinle (HUGG). Controls were recruited in 2008-2009 in HUGG. Ethics committee approval numbers are INCA-109/07 and HUGG-07/2007 - 80/2012, respectively. The subjects enrolled in this study signed the informed consent. Patients with breast lesions

classified as 3 or 4 by an experienced radiologist using the Breast Imaging Reporting and Data System (BIRADS) were included in this study as cases. On the other hand, the subjects who were evaluated and considered systemically healthy were included as controls. Subjects were excluded from the study if they showed immunodeficiency syndromes or genetic syndromes, or were previously diagnosed cancer patients and in treatment. The patients clinical data were obtained from hospital records, Table I. Histological classification was graded according to current (2012) World Health Organization (WHO) criteria (12), and nuclear grade was defined as grades I-III according to Elston and Ellis (13). The number of individuals enrolled and their histopathological types are shown in Table II.

Study Design: For the investigation, the specimens of saliva and plasma were pooled (Table II). For all analyses of saliva and plasma, we evaluated two pools from each tumor group (biological replicates).

Saliva and plasma collection: Saliva was collected as previously described (8). Approximately 1 mL of saliva was collected and homogenized with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA) and 1 mM ethylenediamine tetraacetic acid (EDTA) (Sigma, St. Louis, MO, USA). Samples were kept on ice and, as soon as possible, were centrifuged at 14.000 g for 15 minutes at 4ºC. The supernatant was transferred to another microtube and homogenized with 1 mM Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA). The tube was hand shaken and stored at -80°C.

Blood samples (5 mL) were obtained from each subject by venipuncture and collected into evacuated tubes containing EDTA as an anticoagulant, and plasma samples were separated by centrifugation (1.000 g). The plasma samples were transferred to 1.5 mL tubes and homogenized with 1 mM Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA) and preserved at -80°C.

Sample preparation and SDS PAGE gel electrophoresis: Plasma specimens were enriched by depletion of albumin using Cibracon blue collumn (Sigma, St. Louis, MO, USA), according to the manufacturer's recommended procedure. The enriched product was stored at -80°C until analyses. The saliva and plasma protein concentrations were measured using the bicinchoninic acid or Smith reagent methods (Pierce BCA Protein Assay kit; Pierce Biotechnology, Inc., Rockford, IL, USA) (14). Subsequently, specimens were pooled,

according to the histopathological classification (Table I), totalizing 20 µg, which was loaded in Amersham ECL high resolution gels (GE Healthcare Life Sciences, Chalfont, UK), concentration of 4-20%. After the run, the SDS-PAGE gels were stained with Coomassie Blue R-250, according to the manufacturer's instructions.

In gel digestion: Each lane of the gel was divided in slices with 2 – 5 mm, and washed with 25 mM ammonium bicarbonate in 50% acetonitrile (ACN), overnight, at room temperature to destain the proteins. They were then dehydrated in 100% ACN for 10 min and dried completely in a speed-vac centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). The gel fragments were immersed in 10 µL of the digestion buffer containing trypsin (Promega, modified sequencing grade) at a final concentration of 10 ng/µL in 25 mM ammonium bicarbonate.

The gel fragments were digested with trypsin for 16 h at 37°C. The resulting tryptic peptides were extracted from the gel pieces by incubating with 50 µL of 50% ACN in 5% *trifluoroacetic acid (*TFA), twice, for 15 min with agitation. Supernatants were transferred, pooled, and concentrated as previously described (15). Each sample was then diluted with 10 µL of water in 0.1% TFA to produce final volume of digested ultrafiltrate sample (DIUs).

Analysis of DIUs by nLC-Q-TOF: Prior to the *n*LC-Q-TOF analysis, the DIUs underwent manual desalination Zip Tip (Eppendorf, Hamburg, Germany). Each Zip Tip was activated with 10 µL ACN, was washed three times with 10 µL ultrapure sterile water, and a 10 µL sample was loaded by pipetting up and down 10 times within the tube. Each Zip Tip was then washed three times using water and ACN elution was performed. Subsequently, the samples were reduced to a final volume of 20 µL in a speed-vac centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) and were stored at -20˚C until mass spectrometry analysis (Q-TOF Ultima Global; Waters, Manchester, UK).

The resulting peptides were loaded into an electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer (Waters Corporation, Wilmslow, UK). The DIU samples were loaded onto the Waters nanoACQUITY UPLC® System (Waters Corporation, Milford, MA, USA) with a Waters Opti-Pak C18 trap column coupled to Q-Tof Ultima® (Waters Corporation, Milford, MA, USA). Subsequently, a 3.0 µL sample was injected into a nanoEase C18 150 mM x 75 µm column (Waters Corporation) at a flow rate of 0.6 µL/min, and eluted with ACN containing 0.1% formic acid.

The instrument control and data acquisition were performed using a MassLynx data system (Version 4.0, Waters Corporation). The experiments were performed by scanning from a mass-to-charge ratio (m/z) of between 200 and 2,000. The exact mass was automatically determined using the Q-Tof's LockSpray™ (Waters Corporation, Milford, MA, USA).

Database searching: The data generated by the MassLynx data system (Version 4.0, Waters, Milford, USA) were imported, and all MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.02). The following parameters were used to search the database: a) taxonomy; b) peptide by digestion with a trypsin cleavage site allowed; c) carbamidomethylation (C) as fixed modification; d) and oxidation (O) as varied modification. The proteins were identified by the correlation of tandem mass spectra to the NCBInr proteins and MSDB database, using MASCOT online software (www.matrixscience.com). For protein quantification, the data files were analyzed by Scaffold Q+ (version 4.4.3., Proteome Software, Inc., Portland, OR, USA). Protein probabilities were assigned by the Protein Prophet algorithm (16).

Criteria variables were tested to identify differentially expressed proteins. In the first analysis we compared the pools of benign and malignant cases (subgroups) with the control pool. The second time, we compared each individual tumor group with another tumor group. The Fisher´s Exact Test was used to account for the sample pairing. A fold change cut-off of 1.5 and P-value cut-off of 0.05, used by various quantitative proteomic studies (17-20), were used as minimum criteria for differential protein expression.

The proteins present in saliva and in plasma according to their functional class were determined by the PANTHER program version 10.0 (21) (release date May 15, 2015, containing 11,928 protein families, divided into 83,190 functionally distinct protein subfamilies, http://www.pantherdb.org/). The construction of the interaction pathways between the differentially expressed proteins was generated by Integrated System Interactome (ISS) (22).

Results

In the quantitative analysis of saliva, considering p<value of 0.05, the fibroadenoma (FBR) and the infiltrative ductal carcinoma (IDC) groups, revealed 8 and 9 proteins differentially expressed in relation to the control, respectively (Table III). The proteins, alpha-2-macroglobulin, and ceruloplasmin were down-expressed in all cases evaluated in

comparison to the control group, but both were overexpressed for FBR group when compared between the other case groups. The other proteins, leukocyte elastase inhibitor, deleted in malignant brain tumors 1 protein, and alpha enolase, were the only ones that had isolated cases of overexpression, but with the established p-value and fold change cut-off (Table III).

Concerning plasma, the analysis of groups of FBR and IDC revealed 6, 3, and proteins differentially expressed (p <value 0.05) in relation to the control, respectively. The list of these proteins is shown in Table IV. The alpha-2-macroglobulin protein showed overexpression in all cases in comparison to the control group, and ceruloplasmin showed overexpression in cases of benign lesions. In particular, the vitamin D-binding protein was down-expressed in all cases evaluated in relation to the control, and when compared between tumor groups was down-expressed for the IDC group.

For the quantitative analysis (considering p-value <0.05 and fold change > 1.5), only the alpha-2-macroglobulin, chain B quaternary R co-liganded hemoglobin structure in complex with the thiol containing compound, ceruloplasmin, hemopexin and vitamin D-binding proteins showed significant qualitative changes in plasma (Table IV).

Discussion

This work revealed proteins of women with impalpable breast lesions, reinforcing the idea that saliva is a fluid containing biomarkers that are able to promote early detection of breast cancer, or even act as a complementary test for the cancer prognosis.

Some proteins herein identified were previously described in the saliva pool from the cases of FBR described by Streckfus *et al*. (23). According to the authors, the alpha-enolase protein was overexpressed in the saliva pool of 10 cases of FBR (23). In contrast, in our pool of saliva from the FBR cases, the alpha-enolase was found to be down-regulated. This difference in expression probably can be explained by the individual/population profiles; for example, tobacco users were included in our study, while Streckfus *et al*. (23) excluded tobacco smokers. However, the same group (24,25) found the alpha-enolase protein overexpressed in the saliva pool of IDC cases (histological grade 2, HER2 positive or negative, and positive sentinel lymph node). Our results for the IDC tumors (luminal A and B) were in agreement. Therefore, we suggest that alpha-enolase protein may be associated with early breast carcinogenesis tumors in most of the breast cancer subtypes (luminal A, luminal B, and HER2 positive).

Our results on proteins, such as deleted in malignant brain tumors 1 and leukocyte elastase proteins, also showed contradictory levels of expression in relation to the studies of

Streckfus *et al.* (24,25). According to the authors, these proteins showed down-expression in the pool of saliva of women affected with IDC (histological grade 2, negative HER2 receptors, and positive sentinel lymph node). On the other hand, in our study, these same proteins were overexpressed in the saliva pool of cases with IDCs (luminal A and B). Thus, we believe that the results are incompatible, due to differences in the populations studied.

The other proteins, actin cytoplasmatic 2, alpha-2-macroglobulin, ceruloplasmin, and chain A and B quaternary R co-liganded hemoglobin structure, present in the saliva pool of FRB and IDC cases, exhibited variability in their expression in the intra-group analysis (Table III). The same variability was observed in other studies (23-27). For example, the alpha-2-macroglobulin described in the saliva pool of cases with IDC (histological grade 2, HER2 positive or negative, and positive sentinel lymph node) was overexpressed (25), while in our saliva pools (IDC, FBR) alpha-2-macroglobulin was down-expressed.

The proteins present in saliva and in plasma according to their functional class, determined by the PANTHER program version 10.0, revealed immune system proteins, binding to nucleic acids, and transportation proteins, which are common among tumors (data not show) (4,23-29). When observing the interactome, the highlighted proteins were involved in the initial process of carcinogenesis, for example, metalloproteinases (*MMP2* gene) and epidermal growth factor receptor (*EGFR* gene) (data not show) (30).

Plasma is the main fluid in the discovery of predictive and prognostic biomarkers for diseases in general. The proteins identified in plasma have contributed to enhancing the molecular classification of breast cancer (31). We chose to deplete albumin plasma protein, probably causing fewer proteins to be identified in the plasma specimens as compared to the saliva specimens from the same patients. Thus, we believe that this depletion has influenced our results. Other authors have also described that depletion reduced the number of final proteins identified (32-34).

We observed the alpha-2-macroglobulin protein overexpressed in the plasma pool from all groups (FBR, IDC), and haptoglobin and vitamin D-binding proteins down-expressed, in comparison to controls. Other proteins such as hornerin, haptoglobin, ceruloplasmin, and hemopexin exhibited contradictions in the analysis and differences among groups, but seemed to be overexpressed in the pool of plasma of FBR cases (See Table IV).

Alpha-2-macroglobulin and ceruloplasmin proteins have already been identified as down-expressed in the plasma of women, 21 months before the diagnosis of breast cancer (35), as well as in the pool of serum of women with IDC (positive HER) who were treated with neoadjuvant chemotherapy (36). Moreover, the level of plasma concentration of alpha-2-

macroglobulin and ceruplasmin proteins appears to be overexpressed in the plasma pool of the IDC group in our study.

Finally, a protein associated with the vitamin D transportation was down-expressed in all tumor groups that were evaluated herein (FBR, IDC). Vitamin D is very important in cancer prevention, acting as an anti-apoptotic, anti-proliferative, and anti-tissue invasion agent. In recent studies, the reduction in vitamin levels of D-binding protein has enhanced the risk for developing breast cancer (37), as well as for bladder (38) and pancreatic (39) cancer and prostate tumors (40).

It is known that in the down-regulation of vitamin D receptors in breast epithelial tissue, or even in reducing its active form, the breast tissue is able to start the process of ductal and branch lengthening, becoming a relative risk for development of breast cancer (41,42). As determined in the results, vitamin D-binding protein was down-expressed in all breast lesion groups, suggesting that complementation of vitamin D is important for preventing the evolution of and improving the prognosis of breast cancer. However, we believe that supplementation of vitamin D should be reviewed, as the defect may lie in the transportation of vitamin D, as observed here.

There are no well-defined data on the intersection of proteins that have been revealed between the saliva and plasma. For example, the alpha-2-macroglobulin protein was down-expressed in the saliva pool, while in the plasma pool displayed overexpression for all groups evaluated (FBR, IDC). The deleted malignant brain tumors 1 protein showed overexpression only in the saliva pool. Thus, we believe that the concentration of many of these proteins may be fluid dependent (26).

To the best of our knowledge, this is the first study assessing the proteomics of pools of saliva and plasma from the same cases characterized by impalpable breast lesions. We believe that the study should be continued, comparing our results with other breast cancer subtypes and increasing the number of subjects to validate the proteins here described in saliva and plasma.

Conclusion

It is expected that with the advances in the imaging technologies, the detection of impalpable breast lesions has become increasingly common, and the development of less invasive testing is a real necessity. Some proteins found herein give us evidence of the molecular changes that are related to early breast lesions. The cause of down-regulation of

vitamin-D, as an additional risk factor for breast cancer, should be tracked considering that the cause may possibly lie in its transportation pathway.

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Table I. Clinical data of the cases evaluated in this study

Characteristics	FBR (n=10)	IDC (n=10)	Control (n=8)
Age (y), SD			
	41.3, 16.3	49.7, 14.7	47.37, 8.01
Race [a]			
White	7⁄10 (70%)	3⁄10 (30%)	7⁄8 (87.5%)
Black⁄Mixed	1⁄10 (10%)	3⁄10 (40%)	1⁄8 (12.5%)
NI	2⁄10 (20%)	4⁄10 (40%)	0
Menarche (y)			
≤12	4⁄10 (40%)	3⁄10 (30%)	5⁄8 (62.5%)
>12	4⁄10 (40%)	4⁄10 (40%)	3⁄8 (37.5%)
NI	2⁄10 (20%)	3⁄10 (30%)	0
Menopause (y)			
<45	0	1⁄10 (10%)	2⁄8 (25%)
≥45	2⁄10 (20%)	3⁄10 (30%)	3⁄8 (37.5%)
NI	1⁄10 (10%)	2⁄10 (20%)	0
NM	7⁄10 (70%)	4⁄10 (40%)	3⁄8 (37.5%)
Children			
No child	5⁄10 (50%)	3⁄10 (30%)	2⁄8 (25%)
1	1⁄10 (10%)	2⁄10 (20%)	2⁄8 (25%)
2	2⁄10 (20%)	1⁄10 (10%)	2⁄8 (25%)
≥3	1⁄10 (10%)	3⁄10 (30%)	1⁄8 (12.5%)
NI	1⁄10 (10%)	1⁄10 (10%)	1⁄8 (12.5%)

Oral contraceptive use			
Yes	7/10 (70%)	3/10 (30%)	7/8 (87.5%)
No	1/10 (10%)	5/10 (50%)	1/8 (12.5%)
NI	2/10 (20%)	2/10 (20%)	0
History of breast/ovarian cancer			
Yes	7/10 (70%)	2/10 (20%)	4/8 (50%)
No	1/10 (10%)	5/10 (50%)	4/8 (50%)
NI	2/10 (20%)	3/10 (30%)	0
Alcoholism			
Yes	4/10 (40%)	1/10 (10%)	2/8 (25%)
No	4/10 (40%)	7/10 (70%)	6/8 (75%)
NI	2/10 (20%)	2/10 (20%)	0
Smoking			
Yes	1/10 (10%)	3/10 (30%)	4/8 (50%)
No	7/10 (70%)	5/10 (50%)	4/8 (50%)
NI	2/10 (20%)	2/10 (20%)	0
Lymph node infiltration [b]			
Yes	-	5/10 (50%)	-
No	-	2/10 (20%)	-
NI	-	3/10 (30%)	-

Abreviattions: y, years; SD standard deviation; FBR, fibroadenoma; IDC, infiltrative ductal carcinoma; NI, not informed; NM, not menopausal.

[a] Race was self-declared.

[b] Sentinel lymph node infiltration

Table II. Number of specimens that were included in this studied

Fluid	FBR	IDC	Controls
Saliva	10	10	8
Plasma	10	10	8

Abreviattions: FBR, Fibroadenoma; IDC, infiltrative ductal carcinoma (luminal A and B).

Table III. Saliva proteins up-expressed and down-expressed identified in the cases available

Protein name [a]	NCBI number	MW (kDa)	Sequence coverage (%) [a]	Sequence	Score [b]	*p*-value (cut-off 0.05)	Up-expressed /down-expressed
FBR *vs* healthy control							
Actin, cytoplasmic 2	gi|578831328	51	6% (27/468)	VQTLEAWVIHGGREDSR	53.2	<0,00010	Control high, FBR low
Alpha-2-macroglobulin	gi|578822814	167	32% (487/1512)	AFQPFFVELTMPYSVIR	53.2	<0,00010	Control high, FBR low
IDC *vs* healthy control							
Alpha-2-macroglobulin	gi|578822814	167	32% (487/1512)	LLLQQVSLPELPGEYSMK	145.2	<0,00010	Control high, IDC low
Ceruloplasmin	gi|578807061	125	19% (205/1090)	EYTDASFTNRK	80.4	<0,00010	Control high, IDC low
Chain A, Quaternary R Co-liganded Hemoglobin Structure In Complex With A Thiol Containing Compound	gi|685425643	15	31% (44/141)	LRVDPVNFK	43	0,0001	Control high, IDC low
Chain B, Quaternary R Co-liganded Hemoglobin Structure In Complex With A Thiol Containing Compound	gi|685425644	16	47% (68/146)	FFESFGDLSTPDAVMGNPK	54.1	0,0007	Control high, IDC low
Complement factor H	gi|578800846	133	8% (92/1173)	EIMENYNIALR	64.2	<0,00010	Control high, IDC low
Leukocyte elastase inhibitor (fold change 4.0)	gi|578811455	43	16% (59/379)	EATTNAPFR	56.3	0,00076	Control low, IDC high

FBR *vs* IDC							
Alpha-enolase (fold change 3.4)	gi|578798587	47	23% (101/434)	AAVPSGASTGIYEALELR	117.4	< 0,00010	FBR low, IDC high
Alpha-2-macroglobulin	gi|578822814	167	2% (29/1512)	LLLQQVSLPELPGEYSMK	72.5	< 0,00010	FBR high, IDC low
Ceruloplasmin	gi|578807061	125	14% (152/1090)	EYTDASFTNRK	70.1	< 0,00010	FBR high, IDC low

Abreviations: MW, molecular weight; FBR, fibroadenoma; IDC, infiltrative ductal carcinoma.

[a] The proteins in bold represent the only ones that showed above the fold change cut-off of 1.5[a] Peptides identified by the total protein.

[b] Ion mascot score ≥40.

Table IV. Plasma proteins up-expressed and down-expressed identified in the cases available

Protein name [a]	NCBI number	MW (kDa)	Sequence coverage (%)	Sequence	Score [b]	*p*-value	Up-expressed /down-expressed
FBR *vs* healthy control							
Alpha-2-macroglobulin (fold change 2.3)	gi|578822814	167 kDa	2% (25/1512)	AIGYLNTGYQR	57.8	< 0.00010	Control low, FBR high
Chain B, Quaternary R Co-liganded Hemoglobin Structure In Complex With A Thiol Containing Compound (fold change 3.7)	gi|685425644	16 kDa	42% (62/146)	FFESFGDLSTPD AVMGNPK	46.1	< 0.00010	Control low, FBR high
Ceruloplasmin (fold change 4.4)	gi|578807061	125 kDa	25% (269/1090)	KGSLHANGR	45.7	< 0.00010	Control low, FBR high
Haptoglobin	gi|530423845	167 kDa	2% (25/1512)	HYEGSTVPEKK	57.8	< 0.00010	Control low, FBR high
Hornerin	gi|578800920	213 kDa	3% (57/2146)	GEQHGSSSGSS SSYGQHGSGSR	73.3	0.0043	Control low, FBR high
Vitamin D-binding protein	gi|578809023	48 kDa	12% (52/425)	KFPSGTFEQVS QLVK	106.8	0.00022	Control high, FBR low
IDC *vs* healthy control							

Alpha-2-macroglobulin (fold change 13)	gi|578822814	167 kDa	6% (95/1512)	AIGYLNTGYQR	93.7	< 0,00010	Control low, IDC high
Chain B, Qauternary R Co-liganded Hemoglobin Structure In Complex With A Thiol Containing Compound (fold change 2.1)	gi|685425644	16 kDa	31% (45/146)	VNVDEVGGEALGR	54.5	0,00013	Control low, IDC high
Vitamin D-binding protein	gi|578809023	48 kDa	6% (24/425)	LAQKVPTADLEDVLPLAEDITNILSK	106.8	< 0,00010	Control high, IDC low
FBR *vs* IDC							
Alpha-2-macroglobulin (fold change 3.5)	gi|578822814	167 kDa	6% (95/1512)	AIGYLNTGYQR	93,7	0,0038	FBR low, IDC high
Ceruloplasmin	gi|578807061	125 kDa	1% (13/1090)	GAYPLSIEPIGVR	45.7	< 0.00010	FBR high, MC low
Hornerin	gi|578800920	213 kDa	3% (57/2146)	GEQHGSSSGSSSSYGQHGSGSR	73.3	0.0016	FBR high, MC low

Abreviations: MW, molecular weight; FBR, fibroadenoma; IDC, infiltrative ductal carcinoma.

[a] The proteins in bold represent the only ones that showed above the fold change cut-off of 1.5[a] Peptides identified by the total protein.

[b] Ion mascot score ≥40.

6. ARTIGO 4

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Low levels of Vitamin D in a cohort of women with impalpable breast lesions from Rio de Janeiro/Brazil

Low levels of Vitamin D in women

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Conflict of interest

None of the authors has any conflict of interests to declare.

Abstract

Background: Low levels of vitamin D have been described as a risk factor for the development of breast cancer. The aim of this study was to evaluate the serum levels of vitamin D (25OHD) in patients with impalpable breast lesions comparing with a control group. **Methods:** Vitamin D quantification (25OHD) was assessed in the plasma of 65 patients with impalpable breast lesions and from 20 health controls using a chemiluminescent microparticle immunoassay. Pearson's chi-square test and nonparametric t-Student were used to evaluate statistical significance between the clinical variables and the means of quantification of vitamin D. The receiver operating characteristic (ROC) curve was used to evaluate the correlation between age and vitamin sufficiency for the cases and the controls. **Results**: The prevalence of vitamin D deficiency and/or insufficiency in women with malignant lesions was 84% and 60% for the control group. Using the chi-square or Fisher's exact test, the relationship between vitamin D levels and age presented significant association only for the control group (P=0.002). Using ROC curve, the plot area (0.778) for the control group defined a cut-off value of 45 years to age, with specificity and sensitivity of 60% and 50%, respectively. Thus, the odds ratio for vitamin D insufficiency in women over 45 years was 1.37 (P=0.011). For the case group, clinical characteristics, histological grade, and lymph node involvement did not show any significant association. **Conclusion**: The prevalence of vitamin D deficiency/insufficiency is high in women with impalpable breast lesions, as well as in the control group, even in a tropical city. According to the results the age advancement may be involved with the decrease in vitamin D levels in plasma, but there was no statistical association between low levels of Vitamin D and breast cancer.

Keywords: impalpable breast lesions; 25OHD deficiency; hypovitaminosis D; aging

Introduction

Vitamin D is a pre-hormone synthesized mainly following the skin's exposure to solar UV radiation (UV-B), or alternatively found naturally in some foods and ingested from supplementation (Tagliabue et al., 2015). On the skin, it is synthetized as 7-dehydrocholesterol, so this compound is converted by UV into cholecalciferol, which is then transported to the liver associated with DBP (vitamin D carrier protein). The product of hydroxylation in the liver is termed 25-hydroxyvitamin D (25OHD), which is the most abundant form of the vitamin and is the circulating marker used to determine its sufficiency (Bikle, 2014). Circulating 25OHD is transported to the kidneys by vitamin D binding (DBP), where conversion to calcitriol or 1,25-dihydroxyvitamin D [1,25(OH)2D] occurs. This metabolite, due to its liposolubility, is associated with the vitamin D receptor (VDR - nuclear cell receptor group), which will consequently trigger and mediate genomic effects at the cellular level (Bandera et al., 2017). Due to its genomic role, in the last 10 years vitamin D has been identified as a possible therapeutic target for cancer, since it influences the transcription of genes involved in the process of cell growth and differentiation (Richards et al., 2015; Moukayed and Grant, 2017; Rosen et al., 2012).

More recently, low levels of circulating vitamin D in the population have been identified as a risk factor for tumors in general, especially breast cancer, but this association of risk is still controversial (Eliassen et al., 2016; Acevedo et al., 2016). On the other hand, high levels of vitamin D have contributed to better prognosis and overall survival for breast cancer cases (Yao et al., 2017; Villaseñor et al., 2013), and have even reduced the ability of metastasis in *in vitro* and *in vivo* assays (Wilmanski et al., 2016; Williams et al., 2016).

In Brazil, breast cancer is the most common tumor affecting women, comprising about 28% of all tumors diagnosed annually, excluding skin tumors (non-melanoma) (INCA, 2017). With the improvement of breast cancer screening, more impalpable breast lesions suspicious of cancer have been detected (Delmonico et al., 2015; Romeiro et al., 2016). The aim of this study was to evaluate the serum levels of vitamin D (25OHD) in patients with impalpable breast lesions comparing with a control group.

Materials and methods

Study population

Patients were recruited from 2008 to 2016 at Hospital Gafrée-Guinle University Hospital (HUGG) and at Americas Barra Medical City (Maurício Magalhães Costa Clinic), in the city of Rio de Janeiro, Brazil. All patients provided written informed consent. The study

was conducted according to the guidelines of the Declaration of Helsinki. This study was approved by the ethics committee of both institutions (HUGG-07/2007-80/2012 and Rio de Janeiro State University 43560115.5.0000.5259). All patients were diagnosed with impalpable lesions BIRADS (Breast imaging-reporting and data system) grade 4. Following biopsy or surgery, the lesion was classified as malignant (N =58) or benign (N=7). Data on age, tumor classification, grade, size, nodal involvement, and immunohistochemical profile were obtained from participating institutions. Histologic classification was graded according to current (2012) World Health Organization criteria (Lakhani et al., 2012), and nuclear grade was defined as grades I to III according to Elston and Ellis (Elston and Ellis, 1991). The histologic classification and the nuclear grading were performed by a medical pathologist. Peripheral blood of 20 eligible healthy women (controls) was collected in Rio de Janeiro.

Laboratory analysis

Four mL of blood was collected in EDTA and centrifuged at room temperature for 10 minutes at 2000 g and plasma was stored at -80℃. We measured serum 25-hydroxyvitamin D (25OHD) using the Roche Elecsys Vitamin D Total assay (Roche Diagnostics, Mannheim, Germany). The measurements were performed on the Modular Cobas E601 Analyzer according to the manufacturer's protocol. Serum concentrations below 20 ng/mL (50 nmol/L) were classified as deficiency, ≥20 ng/mL (50 to 74 nmol/L) as insufficiency, and ≥30 ng/mL (≥75 nmol/L) as sufficiency (Holick, 2006; Holick et al., 2011).

Statistical Analysis

Calculation of the sample size

The calculation of the sample size required to establish the chi-square test depends on the specification of the following parameters, described in the literature according to Cohen (1988):

a) Level of significance is 5% ($\alpha = 0.05$ two-sided); b) for the measurement of the effect, the value described in the literature, according to Cohen (1988), was considered the following reference value: medium effect = 0.30; c) test power (1-β): $\beta = 0.25$;

d) degree of freedom equal to 1, since each variable has two categories. These parameters showed that the sample size is 77 individuals.

Analysis of vitamin D levels

Initially, an exploratory analysis was carried out to characterize the patients' sample, using frequency distributions and descriptive measures for the clinical variables.

For a bivariate analysis, individuals with vitamin D deficiency and insufficiency were grouped into a single group (I/D) due to the number of cases evaluated here. The Fischer chi-square or Pearson test was used for the evaluation of differences between the benign/malignant lesions and control group regarding the vitamin D levels. It was calculated the odds ratio for significance chi-square test.

For the associations between deficient and insufficient in control group and cases, the parametric t-Student test was used to determine differences of 25(OH)D concentrations.

The optimal cut-off point for sensitivity and specificity to evaluate the correlation between vitamin sufficiency and the age was done using the receiver operating characteristic (ROC curve). A contingency table was used to associate the age variable with the presence of sufficient amount of vitamin D. The chi-square or Fisher's Exact test was used to test the statistical significance of the association between these variables. The odds ratio for the occurrence of clinical events was calculated by comparing the age groups. The 95% confidence interval was obtained for the odds ratio.

The data were processed in the statistical program Predictive Analytics Software (PASW 18). A level of significance of 5% was considered in all the statistical tests used. Statistically significant data were considered as those whose *P* value was less than 0.05.

Results

Our study quantified vitamin D in 58 women with malignant breast lesions, which included 45/58 (77%) Luminal A/B, 8/58 (14%) HER positive, 5/58 (9%) triple negative, and 7 cases with benign breast lesions. The mean age was 59 years (SD 12.66), 62 (SD 12.58), 43 (SD 3.07), and 46 (SD 6.96) for the Luminals A/B, HER2, triple negative, and benign lesions, respectively. The mean age of the control group was 37 years (SD 11.31). Clinical-pathological variables of cases are summarized in Table 1.

Regarding vitamin D dosage, 49/58 (84%) of patients with malignant tumors presented deficiency and/or insufficiency, of which 30/58 (65%) were Luminals A/B. All patients with HER2 (n=8) and triple negative (n=5) tumors had insufficiency and/or deficiency as well.

Using the chi-square test, the vitamin D levels were compared between groups, malignant and benign lesions versus control, and between subtypes of breast cancer versus control. These associations are summarized in Table 2.

The results of associations between deficient and insufficient 25(OH)D concentrations and case and control are described in table 3. There was no statistical association of deficiency and insufficiency between groups.

The relationship between vitamin D levels and age presented significant association only for the control group (*P*=0.002). For this reason, according to the ROC plot area (0.778) of the control group, a cut-off value of 45 years old for women was determined, with specificity and sensitivity of 60% and 50%, respectively (Fig. 1). Once the 45-year cutoff point was determined, the odds ratio for vitamin D insufficiency in women over 45 years of age was 1.37 (*P*=0.011).

The statistical relationship between sufficiency and deficiency/insufficiency of vitamin D for different types of histological grade (*P*=0.102), and lymph node involvement (*P*=1.00) did not show any significant association, according to the chi-square test.

Discussion

This study revealed low levels of vitamin D in a heterogeneous group of women with impalpable breast lesions and in the controls as well. Although vitamin D deficiency is a widespread health problem, it has been gaining prominence in regions where sunshine prevails all the year (Schoenmakers et al., 2008). Indoor lifestyles, urban living, and nutrition may explain this finding (Arabi et al., 2010). Nevertheless, low levels of vitamin D were positively associated with aging only in the control group.

Based on our previous study, the vitamin D-binding protein level was considered a candidate to differentiate patients with breast impalpable lesions from controls. A nano-liquid chromatography-quadrupole-time-of-flight (nLC-Q-TOF) technology was used, and vitamin D-binding protein was found down expressed in the plasma of cases with benign and malignant breast lesions in comparison with controls´ plasmas (Delmonico et al., 2016). In view of these findings, vitamin D-binding protein should be validated as a putative biomarker in a future study.

With regard to the Brazilian population, two reports evaluated breast cancer and vitamin D deficiency (de Lyra et al., 2006; Oliveira et al., 2016). Lyra (2006) studied the levels of $1,25(OH)_2D3$ and 25OHD in 88 women with breast cancer and 35 controls (submitted to mammoplasties or resection of benign lesions) in the city of São Paulo. These authors found no significant difference between the groups (*P*= 0.722). Further, when they evaluated the differences in vitamin D levels according to the histological grade of the tumor, no significant difference was found between grade tumors I/II (*P*=0.329) and III/IV

(*P*=0.256). These results were similar to those of our study, in which no significant difference was detected for the different degrees of tumors (*P*=0.102) and lymph node involvement (*P*=1.00). On the other hand, the second Brazilian study, conducted in Belo Horizonte city, evaluating 181 women with breast cancer and 197 controls, determined that the levels of vitamin D sufficiency (*P*=0.012) and the moderate practice of physical activity (*P*=0.037) were protective variables for breast cancer (Oliveira et al., 2016).

Considering population studies from other countries, Acevedo (2016) reported that 98/105 (93.3%) Chilean women with breast cancer and 88/93 (94.6%) of cases with Luminal A/B tumors had vitamin D insufficiency/deficiency (≤29.9 ng / mL). Further, 51/53 (96%) of histological grade 3 tumors showed levels of vitamin D in the same range of insufficiency. In our study, these values were lower, with 30/45 (67%) of the Luminal tumors A/B and 15/17 (88%) from grade 3 tumors (see Table 3). In the study by Bilinski and Boyages (2013), 217 Australian women with breast cancer and 852 controls had vitamin D insufficiency with rates of 39.7% (85/214) and 32.3% (275/852), respectively. We found a higher value, being 49/65 (75%) and 12/20 (60%) for malignant tumors and controls, respectively.

The low levels of Vitamin D herein presented for the control group, as well as for the other studies (Oliveira et al., 2016; Bilinski et al., 2013; Unger et al., 2010), are alarming. These values become more evident in countries whose sun incidence is low or that have periods of rigorous winter (Nouri et al., 2017; Shirazi et al., 2016). Consequently, we believe that a part of the global population may be experiencing hypovitaminosis D, increasing the susceptibility to development of the carcinogenic process and other diseases. In relation to the carcinogenic process, the studies with larger numbers of evaluated cases have highlighted low levels of vitamin D as a risk factor for breast cancer (Rose et al., 2013), colorectal cancer (Maalmi et al., 2014), and haematological tumors (Wang et al., 2015). In addition, low levels of vitamin D were associated with the lower overall survival rate (OS) and the lower rate of disease-free survival (RFS) in these tumors.

An equally important finding here was the relationship between the low levels of vitamin D and aging. One reason why vitamin D tends to decrease with aging is directly related to the decreasing capacity to produce vitamin D3, due to degeneration in the cutaneous levels of 7-dehydrocholesterol (Bolland et al., 2006; Bolland et al., 2007). Two other reasons associated with decreasing vitamin D in the elderly are the increase of fat mass and the decrease of sun exposure. The first is related to the number of adipocytes that sequester vitamin D naturally, ingested or produced in the skin, before being transported to the liver to be converted to 25(OH)D3 (Bolland et al., 2007); and the second that elderly tend to wear

more clothing and spend more time indoors due to reduced walking capacity (Unger et al., 2010).

This study is original in that it shows that vitamin D deficiency is prevalent in women with impalpable breast lesions and that vitamin levels are directly related to aging. We emphasize the need for monitoring vitamin D levels especially in the elderly, even in sunny countries, to prevent diseases such as cancer.

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Table 1. Clinical data of the cases with malignant impalpable lesion evaluated in this study

Age	Mean (years)	
Luminal A/B	59 (SD 12.66)	
HER2	62 (SD 12.58)	
Triple Negative	43 (SD 3.07)	
	Patients N=58	%
Histopathologic grade		
I	13	22%
II	28	49%
III	17	29%
Molecular classification		
Luminal A/B	45	77%
HER2	8	14%
Triple negative	5	9%
Histopathologic classification		
IDC	36	62%
DCIS	5	9%
IDC-DCIS	6	10%
ILC	2	3%
Other malignant lesions (LCIS, Mucinous carcinoma, micropapillary carcinoma, and others)	9	16%

IDC, infiltrative ductal carcinoma; DCIS, ductal carcinoma *in situ*; ILC, infiltrative lobular carcinoma; LCIS, infiltrative carcinoma *in situ*

Table 2. Association of the control group with the benign and malignant lesions regarding the levels of vitamin D

Variable	Vitamin D		*P* value
	I+D (%)	S (%)	
Controls (N=20)	12 (60%)	8 (40%)	0.363
Benign lesions (N=7)	6 (86%)	1 (14%)	
Controls (N=20)	12 (60%)	8 (40%)	0.255
Malignant lesions (N=57)	49 (86%)	8 (14%)	
Controls (N=20)	12 (60%)	8 (40%)	0.779
Luminals A/B (N=45)	30 (67%)	15 (33%)	
Controls (N=20)	12 (60%)	8 (40%)	0.063
HER2 (N=8)	8 (100%)	0 (0%)	
Controls (N=20)	12 (60%)	8 (40%)	0.140
Triple negative (N=5)	5 (100%)	0 (0%)	

I, insufficient; D, deficient; S, sufficient. *The probability of significance (*P*-value) refers to the chi-square test.

Table 3. Associations between deficient and insufficient 25(OH)D concentrations in control group and cases

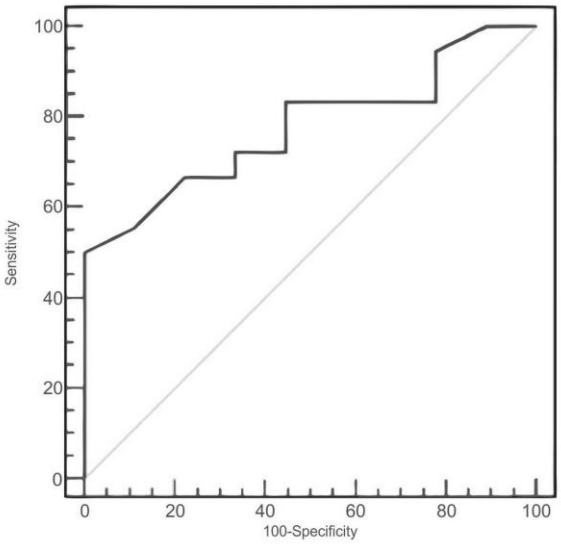
Groups*	Number of cases	Mean (ng/mL)	SD	t-student test	*P*-value**
Benign lesions	6	17.83	7.60	0.336	0.741
Controls	12	18.88	5.84		
Luminal A/B	30	20.29	5.66	0.751	0.457
Controls	12	18.88	5.84		
HER2	8	17.13	7.26	0.621	0.542
Controls	12	18.88	5.84		
Triple Negative	5	17.60	5.03	0.433	0.670
Controls	12	18.88	5.84		
Luminal	30	20.29	5.66	0.908	0.371
Benign lesions	6	17.83	7.60		
HER2	8	17.13	7.26	0.177	0.862
Benign lesions	6	17.83	7.60		
Triple Negative	5	17.60	5.03	0.059	0.955
Benign lesions	6	17.83	7.60		
Luminal A/B	30	20.29	5.66	0.990	0.330
Triple Negative	5	17.60	5.03		
Luminal A/B	30	20.29	5.66	1.308	0.200
HER2	8	17.13	7.26		
Triple Negative	5	17.60	5.03	0.127	0.901
HER2	8	17.13	7.26		

SD, standard deviation

* Only deficiency and insufficiency cases of vitamin D

** The probabilities of significance (*P*-value) refer to the t-Student test for independent samples

Figure 1. ROC curve of association between age and vitamin D quantification for the control group



7. DISCUSSÃO ARTIGO 3 e 4

O sangue representa o principal fluido para pesquisa de biomarcadores de detecção, evolução, e resposta ao tratamento para os cânceres em geral. Entretanto a dificuldade de refinamento e exclusão das proteínas abundantes, fazem com que os candidatos a biomarcadores tumorais apresentem baixa especificidade e sensibilidade no processo de validação clínica. O rastreamento do câncer a partir de proteínas, parte do paradigma que com o crescimento tumoral, a síntese de proteínas neste período sejam capazes de diferenciar do estado, dito normal. Assim, potenciais biomarcadores poderiam ser encontrados entre proteínas, ou peptídeos específicos que estão em baixa e/ou alta expressão no soro/plasma (209,210).

No cenário da patologia mamária, alguns marcadores tumorais presentes no sangue são utilizados como complementares ao diagnóstico clínico e estão rotineiramente sendo avaliados para rastreamento, por exemplo, o antígeno de carboidrato 125 (CA-125) (211). Além do CA-125, o antígeno carcinoembrionário (CEA) (212), a proteína oncogênica RS/DJ-1 (213), o HER2 (214), e os fragmentos circulantes de citoqueratina (antígeno polipeptídico tecidual (TPA), antígeno específico do polipeptídeo tecidual (TPS) e o CYFRA 21-1) têm sido estudados para fins de validação como marcadores de rastreamento para o câncer de mama.

Com o intuito de investigação de biomarcadores circulantes no plasma e na saliva de mulheres com lesões impalpáveis da mama, nós revelamos proteínas diferenciamente expressas a partir da técnica de espectrometria de massas (artigo 3). Entre as proteínas presentes no plasma, nos chamou atenção a baixa expressão da *Vitamin D binding* em todos os grupos avaliados (CDI e fibroadenomas comparados aos controles). Esta proteína possui papel funcional de carrear a 25-hydroxyvitamin D (25OHD), que é a forma mais abundante da vitamina D, e por isso é utilizada para mensuração da sua suficiência. Neste contexto, consequentemente a sua insuficiência/deficiência está envolvida na diminuição da síntese da vitamina D (introdução artigo 3).

Como discutido em ambos os artigos, a deficiência de vitamina D tem sido apontada como fator de risco para diversos tipos de câncer, porém ainda não há consenso sobre a sua suplementação na prevenção e diminuição do risco (215,216). No estudo de Manson et al. (215), mais de 25 mil indivíduos foram randomizados e suplementados com vitamina D, comparados ao grupo placebo. Os resultados do estudo não revelaram qualquer benefício na incidência de câncer ou doença cardivascular. Por outro lado, nos casos com câncer de mama

com suficiência sérica de vitamina D, essa característica tem contribuído para um melhor prognóstico e melhora na sobrevida (216,217).

Devido a sua característica lipossolúvel, a vitamina D tem capacidade de infiltração via membrana celular e ação sob a expressão de importantes genes no processo tumoral (217). Por essa razão, além dos resultados encontrados em nosso estudo proteômico, avaliamos a sua concentração em mulheres com lesões impalpáveis da mama (lesões benignas e malignas), comparadas a um grupo controle (artigo 4). Interessantemente, os resultados demonstraram uma deficiência/insuficiência tanto nos casos, quanto nos controles. Embora não tenhamos encontrado relação significativa entre os baixos níveis de vitamina D e a carcinogênese mamária, demostramos que os níveis da vitamina tendem a cair com maior chance de risco (1.37 vezes mais) a partir dos 45 anos de idade.

A relação entre a deficiência de vitamina D e idade, pode ser justificada por três pontos principais: 1) a deficiência de vitamina D está diretamente relacionada à diminuição da capacidade de produzir vitamina D3, devido à degeneração nos níveis cutâneos de 7-dehidrocolesterol (218,219); b) aumento do tecido adiposo (sobrepeso⁄obesidade) (219); com o aumento do numero de adipócitos, o sequestro da vitamina D para essas células aumenta naturalmente, seja aquela ingerida ou produzida na pele, antes mesmo de ser transportada para o fígado e ser convertida em 25(OH)D3; e c) a diminuição da exposição solar (220). A diminuição à exposição solar, tende a ser uma característica comum aos idosos por usarem mais roupas e passarem mais tempo dentro de casa devido à redução da capacidade de andar (220).

Em relação as proteínas detectadas na saliva são limitados os dados na literatura para comparação. As proteínas identificadas apresentam diversidade de expressão, devido a influência de hábitos sociais como tabagismo e etilismo, além do estadiamento e da presença de metástases no organismo (221-224). Por exemplo, no estudo de Streckfus *et al*. (221) a avaliação do *pool* de 10 casos de mulheres com fibroadenomas, não tabagistas e etilistas, os autores encontraram a α-enolase em alta expressão, enquanto no nosso estudo a mesma proteína, para o mesmo grupo de patologia mamária, apresentou baixa expressão. Outras contradições de expressão também foram vistas para as proteínas *deleted in malignant brain tumors 1* e *leukocyte elastase proteins* na saliva de mulheres com IDC (222,223). Enquanto nos trabalhos de Streckfus *et al*. (222,223) ambas as proteínas apresentaram baixa expressão no pool de saliva de mulheres com IDC, em nosso estudo tais proteínas foram detectadas em baixa expressão (discussão artigo 3).

Assim como concluído nos estudos acima, as proteínas detectadas na saliva e no plasma fornecem evidências das alterações moleculares associadas às lesões mamárias precoces. Enfatizamos que mais estudos devem ser conduzidos com intuito de descrever o fator de risco dos baixos níveis da vitamina D no plasma de mulheres com lesões impalpáveis. Mais além, outras proteínas aqui descritas devem ser mensuradas e validadas em maiores coortes de mulheres com diferentes tipos de lesões mamárias.

CONCLUSÃO

- Nossos dados são originais e representam a heterogeneidade das lesões impalpáveis da mama.

- Os resultados epigenéticos representam uma resposta sistêmica frente a presença do tecido mamário tumoral. Embora não encontrado relações significativas, a frequência de metilações nos promotores dos genes *CDKN2A (p14^{ARF}* e *p16^{INK4A}*) e *ATM* no sangue de casos com lesões benignas e malignas é superior a encontrada nos controles.

- As proteínas identificadas na saliva e no plasma são comuns às patologias mamárias, entretanto demonstram ser fluidos com perfil proteômico próprio.

- Os baixos níveis de vitamina D foram encontrados não somente nos casos com lesões mamárias benignas e malignas, mas nos controles. Este dado evidência maiores análises no que tange a necessidade de exposição ao sol e a necessidade de suplementação da vitamina D.

- As mutações detectadas nos genes *PIK3CA* (éxons 9 e 20), *TP53* (éxons 4-9) e *CDKN2A* (éxons 1-3) no tumor e cfDNA representam a heterogeneidade genética das lesões impalpáveis, dado justificado em partes pela porcentagem de mutações detectadas nas lesões benignas. A coincidência de detecção mutacional no tumor e no cfDNA não foi possível, justificada possivelmente pela sensibilidade do sequenciamento usado (Sanger), paralelamente a ausência de enriquecimento do cfDNA. O estudo deve ser ampliado e comparado com técnicas mais sensíveis, como exemplo *digital PCR* e *NGS*.

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APÊNDICE A- Artigo - mutações no tecido tumoral e cfDNA

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Original Contribution

Mutation profiling in the *PIK3CA*, *TP53*, and *CDKN2A* genes in circulating free DNA and impalpable breast lesions

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ABSTRACT

Breast impalpable lesions have become a clinical dilemma because they are small, presenting a heterogeneous cellular phenotype. The aim of this study was to evaluate the mutational profile of the *PIK3CA*, *TP53*, and *CDKN2A* genes, comparing the mammary tissue with the respective circulating free DNA (cfDNA). The *PIK3CA*, *TP53*, and *CDKN2A* genes were sequenced (PCR-Sanger) in 58 women with impalpable lesions (49 malignant and 9 benign) with the respective cfDNA. The chi-square or Fisher's exact test was used to evaluate statistical significance between the clinical variables and mutational profile. A total of 51 out of 58 samples generated successful mutation profiles in both breast lesion and cfDNA. Of the 37 mutations detected, 10 (27%) and 16 (43%) mutations were detected in benign and malignant breast lesions, respectively, while 2 (5%) and 9 (24%) were found in cfDNA of women with benign and malignant lesions, respectively. The lymph node involvement with mutations in the *PIK3CA* in malignant lesions ($P = 0.001$), and the relationship between mutations in *PIK3CA*, comparing ductal tumors with benign lesions ($P = 0.05$), were statistically significant. This study detected different mutations in *PIK3CA*, *TP53*, and *CDKN2A* genes, which represent, in part, the heterogeneity of impalpable lesions. The results confirm that more studies should be conducted on the functional role of cfDNA in the impalpable lesions.

1. Introduction

The amplification and dissemination of the use of imaging tests for breast cancer screening has increased the number of cases diagnosed with latent and non-palpable (occult) breast lesions [1]. On the other hand, when these lesions are diagnosed, they raise doubts as to the management to be adopted, since they may not be malignant [2-5]. For example, many of these lesions are repeatedly biopsied to define their evolution. In the same way, although these lesions are small, this factor does not limit the potential of metastases (axillar or long distance), leading to the presence of circulating genetic material [6-8]. In this scenario, the liquid biopsy has emerged as a hope for description of the lesion.

The first description of circulating free DNA (cfDNA) was made > 60 years ago [9]. Since then, with the technology advancement, the origin of circulating nucleic acids has been unraveled. Initially, levels of this material were described in the bloodstream as fluctuating in adverse conditions such as pregnancy [10], intense physical activity [11], immunological diseases [12], and tumors in general [13,14].

The cfDNA presence in the bloodstream of healthy individuals originates predominantly from nucleated apoptotic cells. In malignant tumor cases, the cfDNA mixture is more heterogeneous, and may be composed of DNA from the apoptotic and necrotic cells released from the tumor microenvironment [15,16]. The amount of the circulating free tumor DNA (ctDNA) present in the cfDNA mixture may vary. The tumor microenvironment is composed of multiple phenotypes and cell clones; thus, the alterations found in the tumor cannot be easily

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detected in the cfDNA. During tumor evolution, tumor cells may spread from the primary site and when present in the bloodstream will depend on cell degradation and physiological events, such as lymphatic circulation [16] and errors in the immune response [17].

The specific somatic mutation identification for certain tumor types has generated clues to alterations that can be investigated in the cfDNA, responsible for the metastatic site development [18] and resistance to treatment [19]. For breast cancer, the cfDNA role is controversial, but with the introduction of next-generation sequencing (NGS), a mutated gene list has been revealed, including *ESR1* [20-22], *PIK3CA* [18,21], *TP53* [18], *PTEN*, *MYC*, and *CDKN2A* [22]. Many of these genes have been investigated in the cfDNA of breast cancer patients and have shown relevance in the response monitoring treatment [23] and in the risk to metastasis progression [24].

The *PIK3CA* gene is an oncogene responsible for the coding of the p110 kDA subunit, a phosphatidylinositol 3-kinase (PI3K) component, which is responsible for activated protein tyrosine kinases. *PIK3CA* has been described as mutated in breast cancer, with rates ranging from 20 to 40% and 28–43% in tumors and cfDNA, respectively [21,24]. Mutations in *PIK3CA* have been described especially in tumors with estrogen receptor-positive (ER +) [21,24].

The *TP53* gene is a tumor suppressor, acting directly on genome integrity, controlling the cell cycle [25]. It is considered the most mutated gene in sporadic mammary tumors, ranging from 23%–70%, depending on the mammary tumor subtype [26,27]. The mutational rates in cfDNA are similar to those found in the tumor [18,28].

Another gene, *CDKN2A* is responsible for the encoding of p14 (ARF) and p16 (INK4) proteins that will act directly on two pathways responsible for cell cycle control (*TP53* and *RB1*) [29]. It is described as mutated in > 2% of the breast tumors, and is associated with metastatic tumors [30,31] and with the triple negative subtype [31].

In this study, we evaluated the mutational profile of the *PIK3CA*, *TP53*, and *CDKN2A* genes, comparing the mammary tissue (benign and malignant) with the respective cfDNA, to test if they would match. All the cases included in this study were impalpable breast lesions, classified as Breast Imaging Reporting and Data System (Birads) 3 and 4.

2. Subjects and methods

2.1. Study population

Patients who underwent breast cancer screening in the years 2015 to 2016 at the Americas Barra Medical City Clinics in the city of Rio de Janeiro, Brazil, with radiologic diagnosis Birads 3 and 4 were invited to participate in the study. All lesions were selected without any selection bias, being a blind study. The diagnosis of the impalpable lesion was radiological by mammography, and when undetermined the Birads classification, complemented by ultrasonography and/or magnetic resonance.

The study was conducted according to the guidelines of the Declaration of Helsinki and all patients provided written informed consent. This study was approved by the ethics committee of Rio de Janeiro State University Hospital, number 43560115.5.0000.5259. Data on age, tumor classification, grade, size, nodal involvement, and immunohistochemical profile were obtained from histopathological and medical reports. Histologic classification was graded according to current World Health Organization criteria [32], and nuclear grade was defined as grades I to III according to Elston and Ellis [33]. All diagnostic reviews were made by three pathologist physicians (SDOR, CMDN, ASB).

2.2. Methods

2.2.1. Breast lesion and plasma DNA extractions

Ten mL of blood was collected in EDTA before surgery, and centrifuged at room temperature for 10 min at 2000g. Supernatants were centrifuged at 16,000g 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, Hilden, Germany), according to the manufacturer's protocol. The DNA from the tumor tissue was extracted from the formalin-fixed paraffin-embedded (FFPE) samples, using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

For verification of the quality and integrity, the DNA from tissue and plasma samples were quantified with Qubit dsDNA HS Assay Kit (Invitrogen) according to the manufacturer's protocol.

2.2.2. DNA sequencing

The polymerase chain reaction (PCR) was performed in a 25 µL reaction mixture containing 1–10 ng of DNA (breast lesion DNA or cfDNA), STR 1× buffer (Invitrogen, Carlsbad, USA); 200 mM dNTPs (Invitrogen, Carlsbad, USA); 3 mM of $MgCl_2$ (50 mM) (Invitrogen, Carlsbad, USA); primer pairs for each region to be amplified (10 pmol/uL each); and 0.5 U Platinum™ *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, USA) in a final volume of 25 µL. The primers used for *PIK3CA* (exon 9 and 20) and *TP53* (exon 5–8) amplification have been previously described [34,35]. For exon 4 of the *TP53* gene, the primers described by Fernandez et al. [36] were used, due to the limited cfDNA amplification. For amplification of exon 9 of the *TP53* gene the following sequences, forward 5′CCAAGGGTGCAGTTATGCCT3′ and reverse 5′AAAGTTTCCAGTCTCAATCA3′, were used. The primers used for *CDKN2A* amplification (exon 1, 2a, and 3) have been previously described [37,38]. Exon 2 was fragmented into two parts, "a" [37] and "b" [38], due to its long size.

PCR assays were performed in the Veriti™ DX thermal cycler. The PCR program consisted of a pre-denaturation at 94 °C for the first 10 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. The final extension was performed at 72 °C for 10 min.

The PCR products were purified using the GFX™ DNA and Gel Band Purification kit (cat. no. 28903470, GE Healthcare Life Sciences, Chalfont, UK) and QIAquick PCR Purification Kit (cat. no. 28104, Qiagen, Hilden, Germany). Subsequent to purification, sequences were loaded onto an ABI 3730XL DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Comparisons were made between the reference sequences of genes *PIK3CA*, *TP53*, and *CDKN2A* (accession nos. NM_006218.3, NC_000017–10, and NM_000077.4/NM_058197.4, respectively; GenBank) using the sample sequences obtained by sequencing. All samples were sequenced in duplicate. This comparison was performed using the Sequencher version 5.4.6 program (Gene Codes, Ann Arbor, MI, USA).

2.2.3. Statistical analysis

A contingency table was used to associate the mutational profile of each gene evaluated with age, histological grade, infiltration and lymph node involvement, Ki67 proliferation, and molecular classification subtype. Fisher's or Pearson's chi-square test was used to test the statistical significance of the association between such variables. The data were processed in the statistical program Predictive Analytics Software (PASW 18). A level of significance of 5% was considered in all the statistical tests used. Statistically significant data were considered as those whose P value was < 0.05.

3. Results

3.1. Social and clinical data

Following surgery, the histopathological diagnosis revealed 49/58 (84%) malignant lesions and 9/58 (16%) benign lesions. Malignant lesions were 55% IDC, 84% ER-positive, 67% PR-positive, 70% HER2-negative, and 65% Luminal A subtype (Table 1).

The patients with malignant breast lesions ranged in age from 33 to 90 years (mean 61 y, SD 11.42), while the patients with benign breast lesions ranged in age from 27 to 55 (mean 46 y, SD 7.76). The social demografic profile and clinical data of the cases are shown in Table 1.

Table 1
Social demografic and clinical data of the cases available.

	Patients	%
	No = 58	
Characteristic		
Age, years		
Mean	59	
SD	12.67	
≤ 50	17	29%
> 50	39	67%
Not declared	2	4%
Malignant lesions	*N* = 49	
IDC	32	55%
DCIS	3	5%
IDC-DCIS	5	9%
IDC + ILC	1	2%
ILC	2	3%
LCIS	2	3%
ILC/LCIS	1	2%
Micropapillary carcinoma	3	5%
Benign lesions	*N* = 9	
Fibroadenoma	1	11%
Intraductal Papillomas	2	22%
Ductal ectasia and apocrine metaplasia	4	45%
Hyperplasia of columnar cells with and without atypia	2	22%
Nuclear grade	*N* = 49	
I or II	39	80%
III	10	20%
TNM/stage	*N* = 49	
T1N0M0 (stage I)	38	78%
T1N1M0 (stage IIa)	6	12%
TisN0M0 (stage 0)	4	8%
TisN1M0 (stage IIa)	1	2
ER status (malignant lesions)	*N* = 49	
Positive	41	84%
Negative	2	4%
Unknown	6	12%
PR Status		
Positive	33	67%
Negative	10	21%
Unknown	6	12%
HER status		
Positive	7	14%
Negative	34	70%
Unknown	8	16%
Ki 67		
Low (< 20%)	21	43%
Intermediate/high (≥20%)	11	22%
Unknown	17	35%
BC subtype		
Luminal A	32	65%
Luminal B	9	19%
Triple negative	2	4%
Unknown	6	12%

ER = estrogen receptor; PR = progesterone receptor; HER = Human Epidermal Receptor; IDC = infiltrative ductal carcinoma; DCIS = ductal carcinoma in situ; ILC = infiltrative lobular carcinoma; LCIS = lobular carcinoma in situ.

3.2. Mutational profile of mammary DNA and cfDNA

Our study evaluated the mutational profile of the *PIK3CA*, *TP53*, and *CDK2NA* genes in pairs, comparing tumor tissue and the cfDNA in a total of 51 samples. Remaining cases were not possible to evaluate in pairs because of the limited amount of FFPE material, or cfDNA degradation. No concordance in mutations was found in the paired samples.

A total of 37 mutations were found overall, being 8/58 (14%), 18/58 (31%), and 11/58 (19%) for the *PIK3CA*, *TP53*, and *CDKN2A* genes, respectively. The distribution of mutations per gene and specimen evaluated is shown in Table 2.

Among the 8 mutations found in the *PIK3CA* gene, 4/8 (50%), 3/8 (37.5%), and 1/8 (12.5%) were synonymous, missense, and frameshift, respectively. Four of these mutations have been described in the Catalogue of Somatic Mutations in Cancer (COSMIC) database. The mutation distribution per assessed tissue and the nomenclature are shown in Table 3.

For the *TP53* gene, 18 mutations were detected: 9/18 (50%), synonymous; 7/18 (39%), missense; 1/18 (5.5%), stop codon; and 1/18 (5.5%), frameshift. Some synonymous mutations were repeated, with 3/9 (33%), p.G279G; 3/9 (33%), p.Q100Q; and 2/9 (22%), p.R249R. These positions correspond to the hotspot regions. Of the 18 mutations detected, 10 mutations have been described in COSMIC and 15 cataloged in the database of the International Agency for Research on Cancer (IARC). Mutation details, as well as nomenclature, are shown in Table 4. The polymorphisms detected in the samples evaluated for *TP53*, excluding codon 72 polymorphism, can be seen in Supplementary Table 1.

In the *CDKN2A* gene, 11 mutations were detected: 1/12 (8%), synonymous; 8/12 (67%), missense; 1/12 (8%), stop codon; and 1/12 (8%), frameshift. One mutation has been described in COSMIC. The polymorphism p.V28 L was detected in one case and is detailed along with the mutations in Table 5.

The lymph node involvement and mutations in the *PIK3CA* for malignant lesions ($P = 0.001$), and the relationship between mutations in *PIK3CA*, comparing ductal tumors with benign lesions ($P = 0.05$), were statistically significant. The other statistical analysis showed no significant association between mutational findings and correlated clinical pathological variables. The significant *P* value found was above 0.05 (*P* values for each variable are shown in Table 6).

4. Discussion

The screening program expansion and the imaging technology advancement have allowed the detection of early stage breast cancers. In this context, impalpable lesions have become a challenge for the medical field because they are small, and when biopsied represent an unstable tumor phenotype, where lesions in situ and malignant share the same microenvironment [39]. For this reason, many of these lesions are re-biopsied for the tumor cell phenotype confirmation. The discovery of circulating biomarkers would avoid complementary and invasive examinations, in addition to assessing the potential for metastasis and the tumor ability to acquire resistance to treatment.

The data presented here requires careful interpretation. In general, the *TP53* gene was the most mutated, but when observed in the distribution data, the genes showed relatively close mutational rates (see Table 2). The relationship between the mutations and the specimens evaluated were not significant, except for the *PIK3CA* gene in malignant lesions ($P = 0.05$). The mutational frequencies in this study for cfDNA tended to increase in malignant tumors (see Results), but this statement

Table 2
Mutations detected in each gene.

	Benign lesions (*N* = 9) (%)			Malignant lesions (*N* = 49) (%)		
	PIK3CA	*TP53*	*CDKN2A*	*PIK3CA*	*TP53*	*CDKN2A*
Breast lesion	4/9 (44%)	3/9 (33%)	3/9 (33%)	3/49 (6%)	7/49 (14%)	6/49 (12%)
cfDNA	0/9 (0%)	1/9 (11%)	1/9 (11%)	1/49 (2%)	7/49 (14%)	1/49 (2%)

Table 3
Mutations found in the *PIK3CA* gene in breast lesions and cfDNA.

Sample type	Histopathologic grade	TNM	IHC ER/PR/HER	Ki67(%)	Lymph node involvement	Gene *PIK3CA* (Exon)	Codon	Protein	Cosmic
4 – BL	DE/AM	–	–	–	–	9	c.1700A > G	p.N515D	1716810
6 – BL	DE/AM	–	–	–	–	9	c.1700A > G	p.N515D	1716810
16-BL	HCWA	–	–	–	–	20	c.3114A > G c.3250 T > C	p.K986R p.T1031 T	–
3 – BL	IDC grade II	T1N1M0	NI	NI	Yes	20	c.3313A > G	p.T1052 T	4778879
12 – BL	IDC grade II	T1N1M0	+/+/−	10%	Yes	20	c.3250 T > C	p.T1031 T	–
21 – BL	DCIS grade I	TisN0M0	NI	NI	No	9	c.1702_1703insA	p.R1_K1inA	–
17 – cfDNA	IDC grade II	T1N0M0	+/+/+	20%	No	20	c.3232C > T	p.T1025 T	21451[a]
47 – cfDNA	IDC grade I	T1N0M0	+/+/−	5%	No	9	c.1808C > T	p.L551 L	308546

BL = breast lesion; cfDNA = circulating free DNA; DE = Ductal ectasia; AM = apocrine metaplasia; HCWA = Hyperplasia of columnar cells with and without atypia; IHC = immunohistochemistry; COSMIC = Catalogue Of Somatic Mutations In Cancer; ER = estrogen receptor; PR = progesterone receptor; HER = Human Epidermal Receptor; IDC = infiltrative ductal carcinoma; ILC = infiltrative lobular carcinoma; NI = not informative.
[a] SNP (single nucleotide polymorphism) reference rs1784907.

is possible whether applied to a larger study or a different cohort.

The *PIK3CA* gene evaluation in cfDNA from ER+ breast cancer patients, initial ($N = 17$) and metastatic ($N = 69$), revealed that 25% of MBCs (metastatic breast cancers) were mutated [13]. With regard to impalpable lesions, we detected a much lower rate (6%). When cfDNA was evaluated in pairs with metastatic biopsies from 18 advance-stage tumors (breast, ovary, lung, anal, endometrium, gastric, esophagus, pancreas etc.), 28 mutations (97%) were identical. Of the five mammary tumors available in this study, one case treated with paclitaxel presented the p.E545K mutation in *PIK3CA* [18]. Interestingly, in another study, the same mutation was detected in the cfDNA in one of the mammary tumors also treated by paclitaxel [22].

The data presented here for the *PIK3CA* gene do not match mutations between tumor and cfDNA; two mutations (p.N515D and p.T1031T) were found in more than one tumor tissue, confirming the mutational hotspot regions of the gene. In relation to Brazilian data, Mangone et al. [34] found the *PIK3CA* gene mutated in 27% ($N = 23/86$) of the initial mammary tumors. The rates presented here were lower for malignant tumors (6%) and higher for benign lesions (33%).

The relationships between mutations in the *PIK3CA* gene with lymph node involvement ($P = 0.001$) and ductal subtype ($P = 0.05$) were significant in our study. It is known that mutation presence in exon 20 of the *PIK3CA* gene has a relation with worse survival ($P = 0.026$) and disease-free survival (DSF) ($P = 0.079$) [34], but more studies should be conducted to confirm this association.

The *TP53* gene showed a mutation rate of 14% for cases with malignant lesions (breast lesion and cfDNA). This same frequency was reported by Kim et al. [40] in breast tumors of South Korean women with pathological characteristics similar to those evaluated here. Three mutations in our study (p.Q100Q, p.R249R, and p.G279G) were detected more than once (see Results). Among these mutations, hotspot 249 has already been described as mutant in concomitance in a lung adenocarcinoma and the respective cfDNA of the case [41]. Further, p.R249R mutation was detected in 28% of mammary tumors of different histological grades [42], and in hypothesis, it is more frequent in cases exposed to tobacco, pollution, and ingestion of alcohol [43]. The cases detected here with this mutation were not tobacco smokers or those who ingested alcohol, but they were exposed to a pollution environment, a scenario common to large urban centers. The relationship between this mutation and the exposure to these agents can only be affirmed in a populational study.

Although we have not found mutations coincident among the specimens evaluated for the *TP53* gene, it has emerged as the most

Table 4
Mutations found in the *TP53* gene in breast lesions and cfDNA.

Sample type	Grade histopathologic	TNM	IHC ER/PR/HER	Ki67 (%)	Lymph node involvement	Gene *TP53* (Exon)	Codon	Protein	Cosmic	IARC
6 – BL	DE/AM	–	–	–	–	4	c.159A > G	p.W53STOP	–	No
7 – BL	HCWA	–	–	–	–	4	c.326 T > A	p.F109 L	–	Yes
1 - BL	IDC grade II	T1N0M0	+/+/−	20%	No	6	c.604C > A	p.R202S	44174	Yes
19 – BL	IDC grade I	T1N0M0	+/+/−	7%	No	4	c.300G > A	p.Q100Q	5028207	Yes
22 – BL	IDC grade II	T1N0M0	+/−/−	20%	No	7	c.710 T > A	p.M237K	43952	Yes[d]
26[a] – BL	IDC grade II	T1N0M0	+/+/−	10%	No	4	c.229C > T	p.P77S	5991554	Yes
30 – BL	ILC grade II/LCIS	T1N0M0	+/+/−	5%	No	8	c.837G > T	p.G279G	c	Yes
39 – BL	IDC grade I	T1N0M0	+/+/−	10%	No	8	c.837G > T	p.G279G	c	Yes
42 – BL	IDC grade II	T1N0M0	NI	NI	No	8	c.837G > T	p.G279G	c	Yes
54 – BL	IDC grade II	T1N0M0	+/+/−	NI	No	4	c.235G > T	p.A79S	–	Yes
8 – cfDNA	DE/AM	–	–	–	–	7	c.232_233insA	p.T1_H1ins	–	No
12 – cfDNA	IDC grade II	T1N1M0	+/+/−	10%	Yes	7	c.763A > C	p.I255L	–	Yes
14 – cfDNA	DCIS grade I	T1N0M0	+/+/NI	NI	No	7	c.747G > A	p.R249R	44625	Yes
15 – cfDNA	IDC grade I	T1N1M0	+/+/−	5%	Yes	7	c.747G > A	p.R249R	44625	Yes
17 – cfDNA	IDC grade II	T1N0M0	+/+/+	20%	No	4	c.300G > A	p.Q100Q	5028207	Yes
20 – cfDNA	IDC grade I	T1N0M0	+/−/−	5%	No	7	c.732C > A	p.G244G	44787	Yes
21 – cfDNA	DCIS grade I	TisN0M0	NI	NI	No	4	c.300G > A	p.Q100Q	5028207	Yes
27[b] – cfDNA	IDC grade III	T1N0M0	+/+/NI	NI	No	7	c.710 T > A	p.M237K	43952	No

BL = breast lesion; cfDNA = circulating free DNA; DE = Ductal ectasia; AM = apocrine metaplasia; HCWA = Hyperplasia of columnar cells with and without atypia; IHC = immunohistochemistry; ER = estrogen receptor; PR = progesterone receptor; HER = Human Epidermal Receptor; COSMIC = Catalogue Of Somatic Mutations In Cancer; IARC = International Agency for Research on Cancer; IDC = infiltrative ductal carcinoma; ILC = infiltrative lobular carcinoma; NI = not informative.
[a] cfDNA not tested.
[b] Breast lesion not tested.
[c] Described in COSMIC, number 46284, but with exchange G > A.
[d] SNP (single nucleotide polymorphism) no validated (no. 765848205).

Table 5
Mutations found in the *CDKN2A* gene in breast lesions and cfDNA.

Sample Type	Histopathologic Grade	TNM	IHC ER/PR/HER	Ki67(%)	Lymph node involvement	Gene *CDKN2A* (Exon)	Codon[a]	Protein[b]	Cosmic
4 – BL	DE/AM	–	–	–	–	2	c.724_725insT (NM_000077.4)	p.S1_STOP (CCQ43815.1)	–
6 – BL	DE/AM	–	–	–	–	1	c.336G > C (NM_000077.4)	p.E10D (NP_00068.1)	–
7 – BL	HCWA	–	–	–	–	1	c.388G > T (NM_000077.4)	p.V28 L (NP_478104.2)	b
8 – BL	DE/AM	–	–	–	–	2	c.611G > A (NM_000077.4)	p.A54T (CCQ43815.1)	–
1 - BL	IDC grade II	T1N0M0	+/+/–	20%	No	2	c.615G > A (NM_000077.4)	p.G74D (CCQ43815)	–
2 – BL	IDC grade II	T1N0M0	+/+/–	10%	No	2	c.634C > T (NM_000077.4)	p.A61A (CCQ43815.1)	–
10 – BL	DCIS grade II	T1N0M0	+/+/NI	NI	No	2	c.678G > A/c.680G > A (NM_000077.4)	p.R76H/p.D77N (CCQ43815.1)	–
12 – BL	IDC grade II	T1N1M0	+/+/–	10%	Yes	2	C.648C:T (NM_000077.4)	p.P66L (CCQ43815)	–
21 – BL	DCIS grade I	TisN0M0	NI	NI	No	2	c.602G > A (NM_000077.4)	p.R99Q (NP_00068.1)	13618
38 – cfDNA	Fibroadenoma	–	–	–	–	1	c.166G > A (NM_058197.4)	p.A56S (NP_478104.2)	–
41 – cfDNA	IDC grade I	T1N0M0	+/+/–	7%	No	1	c.311_312insA (NM_000077.4)	p.E2insE3 (NP_000068.1)	–

BL = breast lesion; cfDNA, circulating free DNA; DE = Ductal ectasia; AM = apocrine metaplasia; HCWA = Hyperplasia of columnar cells with and without atypia; IHC = immunohistochemistry; COSMIC = Catalogue Of Somatic Mutations In Cancer; IARC = International Agency for Research on Cancer; ER = estrogen receptor; PR = progesterone receptor; HER = Human Epidermal Receptor; IDC = infiltrative ductal carcinoma; ILC = infiltrative lobular carcinoma; NI, not informative.

[a] Access number of the database (Genbank).

[b] Single Nucleotide Polymorphim (SNP), rs876658895 (Genbank).

Table 6
Statistical evaluation between the variables for each gene[a].

		PIK3CA	*TP53*	*CDKN2A*
Breast lesion	Age	0.296	0.355	0.800
cfDNA		0.073	0.491	0.565
Breast lesion	Histologic Grade	0.728	0.345	0.641
cfDNA		0.583	0.229	0.315
Breast lesion	Lymph node involvement	**0.001**	0.266	0.740
cfDNA		0.577	0.168	0.697
Breast lesion	Ki67	0.387	0.946	0.500
cfDNA		0.706	0.460	0.672
Breast lesion	Ductal vs Micropappilar	0.675	0.461	0.825
cfDNA		0.687	0.421	0.778
Breast lesion	Ductal vs Lobular	0.508	0.933	0.931
cfDNA		0.604	0.301	0.717
Breast lesion	Benign lesions vs Ductal	**0.050**	0.968	0.104
cfDNA		0.487	0.620	0.247
Breast lesion	Molecular subtype	0.208	0.662	0.829
cfDNA		0.819	0.604	0.830

[a] Numbers represent p value, $p < 0.05$ is significative.

promising molecular biomarker for investigations of tumor-matching mutations in cfDNA [28,43,44]. The *TP53* gene evaluation by NGS from nine different advanced tumor types (14 lung cancer, 3 ovarian, 2 endometrial, 2 thyroid, 2 hepatocellular, 2 unknown primary, 1 cholangiocarcinoma, 1 gastroesophageal junction adenocarcinoma, and 1 peritoneal adenocarcinoma), when compared to their respective cfDNA, revealed a mutation concordance with sensitivity and specificity of 80% and 87.5%, respectively [28]. Moreover, 20 metastatic colorectal tumors when compared to primary tumor and cfDNA, resulted in a concordance of 39% between the cfDNA and the primary tumor, and 55% between the cfDNA and the metastases [44].

The *CDK2NA* gene evaluation by NGS in 44 cases with MBC (24 triple negative breast cancer, 16 estrogen receptor positive, and 4 human epidermal growth factor receptor 2 positive patients) revealed 3 mutated cases (8%) (2 triple negatives and 1 ER+/HER-) [27]. Considering only the malignant lesion data, our frequency was higher (12%). Further, all our mutated cases for *CDK2N2A* were estrogen and progesterone-positive (see Results).

Recently, the *CDKN2A* gene has been tested as a predictive biomarker of relapse in triple negative breast cancer with residual disease after neoadjuvant chemotherapy. Among the 38 early-stage triple-negative breast cancer patients with matched tumor, blood, and plasma, 33 patients had a mutation identified in their primary tumor, but just one matched mutation in cfDNA (3%) [31]. For the impalpable lesions evaluated here, the frequency was similar (2%). In another study, the frequency was higher (8%), but the mutations were identified in the cfDNA of one lung tumor and one ovary, both at advanced stages [28].

Although we did not find identical mutations in the pairs (cfDNA and lesion), the mutational frequencies in cfDNA were similar to those found in the mammary lesions, demonstrating the possibility of using cfDNA for monitoring and exploring the heterogeneity of the lesion. Beije et al. [43] reported that mutations at estrogen receptor 1 (ESR1) and splicing variants occur more frequently in cfDNA, than in CTC from metastatic breast cancer (MBC) patients progressing on endocrine treatment. The mutation percentages before and after treatment, respectively, were 11% and 5% (cfDNA), and 42% and 8% (CTCs). These same conclusions were observed by Bettegowda et al. [44]. Among the 640 advanced tumors evaluated from different histological subtypes (breast, ovary, hepatocellular, head and neck cancer, among others), the mutational profile of cfDNA was more informative than CTC. ctDNA was often present in patients without detectable CTC, suggesting that these two biomarkers are distinct entities.

In this study, we detected diversified mutations in the impalpable lesions and in corresponding cfDNA. These data demonstrate that non-invasive tests are likely to be successful in evaluating tumor heterogeneity. Although we found representative mutational rates in benign lesions, there are intrinsic factors for each tumor microenvironment that can be

influenced by environmental and behavioral variables that deserve to be studied individually. Further, in some cases, individual genetic alterations are not capable of leading to tissue malignancy [45].

The mutation evaluation in cfDNA by NGS and digital PCR has shown higher sensitivity values, when compared to other conventional methods, as performed here [18-22,27,28,31,43,44]. However, the impalpable breast lesion molecular characteristics are still research objectives and deserve research amplification.

This study is original and detected different mutations in *PIK3CA*, *TP53*, and *CDKN2A* genes, which represent, in part, the heterogeneity from impalpable lesions. We believe that the search for mutations in cfDNA, in parallel with the lesion, may minimize unnecessary biopsies and complement imaging tests, which are not always informative to describe the risk of invasion of the lesion, or to follow the tumor response to treatment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anndiagpath.2018.12.008.

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Author disclosure statement

No competing financial interests exist.

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ANEXO A - Aprovação do Comitê de Ética – INCA

CEP

INCA
INSTITUTO NACIONAL DE CÂNCER

Memo 094/08-CEP-INCA

Rio de Janeiro, 12 de março de 2008

A(o): Dr(a). Gilda Alves Brown
Pesquisador(a) Principal

Registro CEP nº 109/07 (Este nº. deve ser citado nas correspondências referentes a este projeto)
Título do Projeto: "A importância das lesões impalpáveis (lesões III e IV), e das alterações em *TP53* (p53) na detecção precoce do câncer de mama"

Prezada Pesquisadora,

O Comitê de Ética em Pesquisa do Instituto Nacional de Câncer após re-análise aprovou o Protocolo intitulado: **Importância das lesões impalpáveis (lesões III e IV), e das alterações em *TP53* (p53) na detecção precoce do câncer de mama**, bem como seu **Termo de Consentimento Livre e Esclarecido (versão 2)** em 10 de março de 2008.

Ressaltamos que conforme descrito na folha de rosto (item 49), o pesquisador responsável deverá apresentar relatórios semestrais a respeito do seu protocolo que estão previstos para as seguintes datas: *setembro/2008* e *março/2009*.

Estamos encaminhando a documentação pertinente para a CONEP com vistas a registro e arquivamento.

Atenciosamente

Dra. Adriana Scheliga
Coordenadora do Comitê de Ética em Pesquisa
CEP-INCA

C/c – Dra Jane Dobbin – Chefe do Serviço de Hematologia – HC I

ANEXO B - Aprovação do Comitê de Ética – HUGG

UNIVERSIDADE FEDERAL DO ESTADO DO RIO DE JANEIRO
HOSPITAL UNIVERSITÁRIO GAFFRÉE E GUINLE
COMITÊ DE ÉTICA EM PESQUISA

MEMO CEP-HUGG / Nº 80/ 2012

Rio de Janeiro, 11 de setembro de 2012.

A
Ilma. Carolina Maria de Azevedo
Pesquisador Responsável

Conforme decisão do Comitê de Ética em Pesquisa do Hospital Universitário Gaffrée e Guinle, em reunião realizada em 11de setembro de 2012, a Emenda 01 de 18/07/12, entregue ao CEP em 27/08/12 referente ao projeto ***"Detecção Precoce do Câncer de Mama – Estudo da efusão mamilar através do diagnóstico por imagem (mamografia e ultrassonografia), exame citológico e bases moleculares*** registrado no CEP-HUGG sob o nº **07/2007** foi analisada, de acordo com a Resolução CNS nº 196/96, e considerado **APROVADA**.

Ressaltamos que todo envio de documentação e comunicação entre pesquisador e CEP-HUGG deverá ser formalizada através de memorando numerado, datado e assinado pelo pesquisador responsável, **sempre discriminando o número do projeto registrado no CEP-HUGG, bem como o seu título no SISNEP**.

Informamos que, de acordo com a Resolução CNS nº 196/96, cabe ao pesquisador apresentar os **relatórios parciais (semestrais) e final** do projeto aprovado, ***observando os critérios estabelecidos pela CONEP e pelo CEP-HUGG***.

Atenciosamente,

Prof. Dr. Pedro Eder Portari Filho
Coordenador do CEP-HUGG

ANEXO C - Aprovação do Comitê de Ética – HUPE

UERJ - HOSPITAL UNIVERSITÁRIO PEDRO ERNESTO/ UNIVERSIDADE DO

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Pesquisa de marcadores circulantes em pacientes com lesões impalpáveis na mama

Pesquisador: Maria Helena Faria Ornellas de Souza
Área Temática:
Versão: 7
CAAE: 43560115.5.0000.5259
Instituição Proponente: Hospital Universitário Pedro Ernesto/UERJ
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.746.202

Apresentação do Projeto:
Emenda para aprovação de documentação e alteração de informações relativas ao protocolo.

Objetivo da Pesquisa:
Emenda para aprovação de documentação e alteração de informações relativas ao protocolo.

Avaliação dos Riscos e Benefícios:
Emenda para aprovação de documentação e alteração de informações relativas ao protocolo.

Comentários e Considerações sobre a Pesquisa:
Justificativa da Emenda:
O material coletado será utilizado para aplicação em dois testes de ensaio multigênico em parceria com a start-up Bioarray Genetics. Na primeira versão do projeto havia sido informado que o material genômico advindo das células circulantes seria aplicado nestes testes, porém estamos solicitando a extensão dessas análises às amostras parafinadas e sangue, afins de comparação. Solicitamos a autorização do CEP para isenção de aplicação do TCLE, sem prejuízo, ou dano para cada participante.

Considerações sobre os Termos de apresentação obrigatória:
Os documentos enviados a este Comitê estão dentro das boas práticas em pesquisa e apresentando todos dados necessários para apreciação ética.

Endereço: Avenida 28 de Setembro 77 - Térreo
Bairro: Vila Isabel **CEP:** 20.551-030
UF: RJ **Município:** RIO DE JANEIRO
Telefone: (21)2868-8253 **E-mail:** cep.hupe.interno@gmail.com

Conclusões ou Pendências e Lista de Inadequações:

A emenda apresenta todas as informações necessárias para avaliação ética. Diante do exposto e à luz da Resolução CNS nº466/2012, a Emenda pode ser enquadrada na categoria – APROVADO.

Considerações Finais a critério do CEP:

Tendo em vista a legislação vigente, o CEP recomenda ao Pesquisador: Comunicar toda e qualquer alteração do projeto e no termo de consentimento livre e esclarecido, para análise das mudanças; Informar imediatamente qualquer evento adverso ocorrido durante o desenvolvimento da pesquisa; O Comitê de Ética solicita a V. Sª., que encaminhe relatórios parciais de andamento a cada 06 (seis) Meses da pesquisa e ao término, encaminhe a esta comissão um sumário dos resultados do projeto; Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competentes.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1159898_E5.pdf	15/06/2018 15:15:26		Aceito
Projeto Detalhado / Brochura Investigador	Projeto_2018_analise_RNA.doc	15/06/2018 15:13:48	Maria Helena Faria Ornellas de Souza	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	IsencaoTCLE_n2.pdf	15/06/2018 15:12:03	Maria Helena Faria Ornellas de Souza	Aceito
Declaração de Instituição e Infraestrutura	Carta_anuencia.pdf	09/05/2017 14:08:08	Maria Helena Faria Ornellas de Souza	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Regulamento_biorrepositorio.docx	09/05/2017 14:07:47	Maria Helena Faria Ornellas de Souza	Aceito
Outros	Declaracao_de_insercao.pdf	16/02/2017 19:24:55	Maria Helena Faria Ornellas de Souza	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Grupo_Controle.docx	16/02/2017 19:22:56	Maria Helena Faria Ornellas de Souza	Aceito
TCLE / Termos de Assentimento /	SolicitacaodeisencaodeTCLE.pdf	17/11/2016 09:25:45	Maria Helena Faria Ornellas de Souza	Aceito

UERJ - HOSPITAL UNIVERSITÁRIO PEDRO ERNESTO/ UNIVERSIDADE DO

Continuação do Parecer: 2.746.202

Justificativa de Ausência	SolicitacaodeisencaodeTCLE.pdf	17/11/2016 09:25:45	Maria Helena Faria Ornellas de Souza	Aceito
Outros	Declaração do Maurício.pdf	10/03/2015 10:18:56		Aceito
Outros	Declaração de ciência da Disciplina.pdf	10/03/2015 10:18:42		Aceito
Folha de Rosto	Folha de rosto - ca mama (1).jpg	04/02/2015 22:04:52		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RIO DE JANEIRO, 29 de Junho de 2018

Assinado por:
WILLE OIGMAN
(Coordenador)

Endereço: Avenida 28 de Setembro 77 - Térreo
Bairro: Vila Isabel **CEP:** 20.551-030
UF: RJ **Município:** RIO DE JANEIRO
Telefone: (21)2868-8253 **E-mail:** cep.hupe.interno@gmail.com

ANEXO D - Termo de consentimento livre e esclarecido – HUGG e INCA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

PESQUISA DAS LESÕES IMPALPÁVEIS (LESÕES III E IV), E DAS ALTERAÇÕES EM TP53 (p53) NA DETECÇÃO PRECOCE DO CÂNCER DE MAMA.

Nome da Paciente: _____
Número da Matrícula: _____

Você está sendo convidada a participar desta pesquisa da qual serão coletadas amostras da lesão e de sangue e secreção salivar para análise do genes *TP53* (p53), *CDKN2A* (p14 e p16), *ATM*, expressão de marcadores HER2, receptores hormonais (estrogênios e progesteronas) que serão importantes para fatores diagnósticos e prognósticos.

Este projeto tem como coordenadora a Dra. Gilda Alves Brown, Bióloga, Pesquisadora do Laboratório de Genética Aplicada do Serviço de Hematologia do Instituto Nacional de Câncer – INCA.

Para que você possa decidir se quer participar ou não desta pesquisa, precisa conhecer seus benefícios, riscos e implicações.

OBJETIVO DA PESQUISA

A detecção precoce do câncer de mama aumenta consideravelmente as chances de cura.

A interpretação das lesões impalpáveis (III e IV) em imagens de mama, a partir de técnicas de processamento de imagens, é ainda objeto de pesquisa. A associação de estudo criterioso dessas imagens com um importante marcador molecular de câncer de mama não familiar, os genes supressores tumorais *TP53* (*p53*), *CDKN2A* (*p14* e *p16*) e *ATM*, poderá tornar o exame de diagnóstico inicial ou de acompanhamento pós-tratamento mais confiável.

As imagens serão geradas por mamógrafos de alta resolução e/ou digital associando-se à ultra sonografia de alta frequência e morfometria matemática. A análise dos genes *TP53*, *CDKN2A* (*p14* e *p16*), ATM será feita através de PCR-SSCP, seguido de sequenciamento de DNA para detecção das mutações, e as deleções serão analisadas através da técnica de Perda se Heterozigosidade (LOH).

PROCEDIMENTO DA PESQUISA

Se a senhora concordar em participar desta pesquisa, uma amostra de sangue (5 mililitros ou o correspondente a uma colher de sobremesa), uma amostra da lesão e uma amostra de saliva (2 mililitros), serão utilizadas nesta pesquisa. Essas amostras serão enviadas para o Laboratório de Genética Aplicada do INCA para a análise das mutações do *TP53* (p53), *CDKN2A*, *ATM* por PCR-SSCP, sequenciamento de DNA e a LOH, além da análise de expressão do HER2 pelo método de ELISA.

MÉTODOS ALTERNATIVOS

Não há métodos alternativos para a sua participação nesta pesquisa. Se a senhora não concordar com os procedimentos descritos, não poderá participar da mesma.

RISCOS

A coleta de sangue pode resultar em dor no local da punção ou mancha roxa transitória no local, chamada de equimose. Caso isso venha ocorrer, a senhora será orientada a como tratá-la.

BENEFÍCIOS

Esta pesquisa pode não trazer melhora para a sua saúde, entretanto as informações obtidas nesta pesquisa podem contribuir para futura melhoria na precisão do diagnóstico precoce do câncer de mama, aumentando as chances de cura.

ACOMPANHAMENTO – ASSISTÊNCIAS - RESPONSÁVEIS

O seu acompanhamento durante o procedimento será feito pela Dra. Carolina Maria de Azevedo. A responsável pelo processamento e armazenamento das mostras será a Dra. Gilda Alves Brown.

CARÁTER CONFIDENCIAL DOS REGISTROS

Além da equipe de saúde que cuidará da senhora, seus registros médicos poderão ser consultados pelo Comitê de Ética do INCA, do HUGG e pela equipe de pesquisadores envolvidos. Seu nome não será revelado ainda que informações de seu registro médico sejam utilizados para propósitos educativos ou de publicação científica, que ocorrerão independentemente dos resultados obtidos.

TRATAMENTO MÉDICO EM CASO DE DANOS

Seu tratamento e acompanhamento médico independem de sua participação desta pesquisa.

CUSTOS

Não haverá nenhum custo ou forma de pagamento para a senhora pela participação nesta pesquisa.

BASES DA PARTICIPAÇÃO

É importante que a senhora saiba que a sua participação nesta pesquisa é completamente voluntária e que a senhora pode recusar-se a participar ou interromper sua participação a qualquer momento sem penalidade ou perdas de benefícios as quais a senhora tem direito. Em caso de decidir interromper a sua participação nesta pesquisa, a equipe assistente deve ser comunicada e a coleta de amostras para os exames relativos à esta pesquisa será imediatamente interrompida, os dados obtidos de suas amostras não serão utilizados.

O médico responsável pelo procedimento pode interromper a sua participação nesta pesquisa a qualquer momento, mesmo sem a sua autorização, por razões justificadas que lhe serão explicadas.

O seu tratamento, caso não queira participar desta pesquisa, não será alterado.

GARANTIA DE ESCLARECIMENTOS

Nós estimulamos a senhora ou aos seus familiares a fazer perguntas sobre a pesquisa, a qualquer momento. Neste caso, por favor, ligue para a Dra. Gilda Brown no telefone 2506-6622 ou para o CEP do INCA no telefone 3233-1410.

DECLARAÇÃO DE CONSENTIMENTO E ASSINATURA

Li as informações acima e entendi o propósito desta pesquisa, assim como os benefícios e riscos potenciais da participação da mesma. Tive a oportunidade de fazer perguntas e todas foram respondidas.

Eu, por intermédio deste, dou livremente meu consentimento para participar desta pesquisa.

Entendo que poderei ser submetida, a um exame de sangue adicional ao necessário para meu acompanhamento e que não receberei compensação monetária por minha participação nesta pesquisa.

Eu recebi uma cópia assinada deste formulário de consentimento.

_____ _____ / _____ /_____.
(Assinatura da Paciente) dia mês ano

_____
(Nome da paciente – Letra de forma)

_____ _____ / _____ /_____.
(Assinatura de testemunha, se necessário) dia mês ano

Eu, abaixo assinado, expliquei completamente os detalhes relevantes desta pesquisa à paciente indicada acima e/ou a pessoa autorizada para consentir pela paciente.

_____ _____ / _____ /_____.
(Assinatura da Pessoa que obteve o consentimento) dia mês ano

ANEXO E - Questionário clínico - HUGG e INCA

<table>
<tr><th colspan="6">DADOS PESSOAIS</th></tr>
<tr><td>DATA</td><td colspan="4">NOME</td><td>PRONTUÁRIO</td></tr>
<tr><td>IDADE</td><td>DATA DE NASCIMENTO</td><td>SEXO</td><td>COR</td><td>ESTADO CIVIL</td><td>PROFISSÃO</td></tr>
<tr><td colspan="4">ENDEREÇO</td><td colspan="2">TELEFONE
E-MAIL</td></tr>
<tr><th colspan="6">DADOS CLÍNICOS</th></tr>
<tr><td colspan="6">QUEIXA PRINCIPAL</td></tr>
<tr><td colspan="6">EVOLUÇÃO</td></tr>
<tr><td colspan="2">MENARCA</td><td colspan="2">MENOPAUSA</td><td colspan="2" rowspan="2">NORMAL

HISTERECTOMIA</td></tr>
<tr><td colspan="4">GESTA</td></tr>
<tr><td colspan="4">PARA</td><td colspan="2" rowspan="2">DATA DE NASCIMENTO DO 1º FILHO ____/____/ ____

ALEITAMENTO SIM NÃO

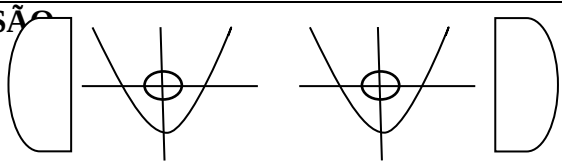
DURAÇÃO
_____</td></tr>
<tr><td colspan="4">ABORTO</td></tr>
<tr><th colspan="6">HISTÓRICO FAMILIAR</th></tr>
<tr><td colspan="6">MÃE _____ IDADE AVÓ _____ IDADE FILHA _____ IDADE
IRMÃ _____ IDADE TIA _____ IDADE</td></tr>
<tr><th colspan="6">TERAPIA HORMONAL</th></tr>
<tr><td colspan="6">TEMPO _____

CAUSA: HISTERECTOMIA OSTEOPOROSE MENOPAUSA OUTROS - _____</td></tr>
<tr><th colspan="6">EXAMES POR IMAGEM REALIZADOS</th></tr>
<tr><td colspan="6">MAMOGRAFIA US OUTROS</td></tr>
<tr><td colspan="6">TIPO DE LESÃO IMPALPÁVEL

DISTORÇÕES ARQUITETURAIS DENSIDADE FOCAL NÓDULO INFRA CENTIMÉTRICO (REDONDO OU OVAL)

MICROCALCIFICAÇÕES
TIPOS (BI-RADS):

III IV V</td></tr>
</table>

LOCALIZAÇÃO DA LESÃO **QSE** **QSI** **QIE** **QII** 
TIPOS DE BIÓPSIA **CIRÚRGICA** **GUIADA POR US** **LOCALIZAÇÃO ESTEREOTÁXICA**
MARCADORES **RE** **RP** **HER**
RESULTADO HISTOPATOLÓGICO

ANEXO F - Termo de consentimento livre e esclarecido - Clínica Dr Maurício Magalhães Costa

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Pesquisa de marcadores circulantes em pacientes com lesões impalpáveis na mama

Nome da Paciente: _____

Número de Registro: _____

Eu estou sendo convidada a participar desta pesquisa na qual serão coletadas amostras da lesão (podendo ser fresca, no ato da cirurgia, ou posteriormente, em bloco de parafina) e de sangue para investigação genética.

Este projeto tem como coordenadora a Dra. Maria Helena Faria Ornellas de Souza, professora e pesquisadora do Laboratório de Marcadores Circulantes do Departamento de Patologia Geral e Laboratórios, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro – UERJ.

Para que eu possa decidir se quer participar ou não desta pesquisa, eu preciso conhecer os meus benefícios, riscos e implicações.

OBJETIVO DA PESQUISA

A detecção precoce do câncer de mama aumenta consideravelmente as chances de cura.

As imagens da mama podem ser duvidosas. A associação de importantes marcadores moleculares de câncer de mama e a detecção de marcadores circulantes poderá tornar o exame de diagnóstico inicial ou de acompanhamento pós-tratamento mais confiável.

Dessa forma, será feito o sequenciamento dos genes *TP53*, *PIK3CA*, *CDKN2A* ($p14^{ARF}$/$p16^{INK4a}$) e *ATM*; investigação de instabilidade e perda de heterozigosidade (LOH) nos microssatélites para os genes *TP53* e *CDKN2A*. Além disso será feita a avaliação da metilação para os genes *CDKN2A* e *ATM*. Essas avaliações serão feitas na amostra de sangue, DNA circulante e no tecido tumoral. Para fins de investigação complementares será feita a

pesquisa de células tumorais circulantes presentes no sangue para aplicação em teste de ensaio multigênico.

PROCEDIMENTO DA PESQUISA

Um fragmento do nódulo fresco ou parafinado da mama e 20 mL de sangue serão coletados para fins de pesquisa. Quando este material estiver em parafina (bloco) este será solicitado a clínica na qual se encontra. Essas amostras serão enviadas para o Laboratório de Marcadores Circulantes do Departamento de Patologia, Faculdade de Ciências Médicas da UERJ para a análise molecular dos genes específicos além da análise de marcação de células tumorais circulantes.

RISCOS

A coleta de sangue e do nódulo podem resultar em dor no local da punção ou mancha roxa transitória no local, chamada de equimose. Caso isso venha ocorrer, a senhora será orientada a como tratá-la.

BENEFÍCIOS

As informações obtidas nesta pesquisa podem contribuir para futura melhoria na precisão e acompanhamento do diagnóstico precoce do câncer de mama, aumentando as chances de cura.

ACOMPANHAMENTO – ASSISTÊNCIAS - RESPONSÁVEIS

O meu acompanhamento durante o procedimento será feito pelo Dr. Maurício Augusto Silva Magalhães Costa, CRM-RJ 52-37484-0. As responsáveis pelo processamento e armazenamento das amostras serão as Doutoras Maria Helena Faria Ornellas de Souza (CRM-RJ 5229307-5) e Gilda Alves Brown (CRBio RJ 453202).

CARÁTER CONFIDENCIAL DOS REGISTROS

Os meus registros médicos poderão ser consultados pela equipe médica que cuidará de mim, pelo Comitê de Ética da UERJ, e pela equipe de pesquisadores envolvidos. O meu nome

não será revelado ainda que informações de meu registro médico sejam utilizados para propósitos educativos ou de publicação científica, que poderão ocorrer independentemente dos resultados obtidos.

TRATAMENTO MÉDICO EM CASO DE DANOS

O meu tratamento e acompanhamento médico independem de minha participação nessa pesquisa.

CUSTOS

Não haverá nenhum custo ou forma de pagamento pela minha participação nesta pesquisa.

BASES DA PARTICIPAÇÃO

Fui informada que a minha participação nesta pesquisa é voluntária e que posso recusar-me a participar ou interromper a minha participação a qualquer momento, sem penalidade ou perdas de benefícios às quais eu tenho direito. Em caso de eu decidir interromper a minha participação nesta pesquisa, a equipe assistente será comunicada e a coleta de amostras para os exames relativos a esta pesquisa será imediatamente interrompida, e os dados obtidos das amostras não serão utilizados.

Fui informada que o médico responsável pelo procedimento pode interromper a minha participação nesta pesquisa a qualquer momento, mesmo sem a minha autorização, por razões justificadas que me serão explicadas, e que o meu tratamento, caso eu não queira participar desta pesquisa, não será alterado.

GARANTIA DE ESCLARECIMENTOS

Todas as perguntas pertinentes à essa pesquisa foram esclarecidas. Mas, para novas perguntas eu poderei ligar para a Dra. Maria Helena Faria Ornellas de Souza pelo telefone 2868-8047 ou para Dr. Maurício Augusto Silva Magalhães Costa pelo telefone 3264-4863 e/ou ainda para o Comitê de Ética em Pesquisa da UERJ pelo telefone 2868-8253.

DECLARAÇÃO DE CONSENTIMENTO E ASSINATURA

Li as informações acima e entendi o propósito desta pesquisa, assim como os benefícios e riscos potenciais da participação da mesma. Tive a oportunidade de fazer perguntas e todas foram respondidas.

Eu, por intermédio deste, dou livremente meu consentimento para participar desta pesquisa.

Entendo que poderei ser submetida a exames de sangue e da lesão adicionais ao necessário para meu acompanhamento e que não receberei compensação monetária por minha participação nesta pesquisa.

Eu recebi uma cópia assinada deste formulário de consentimento.

_____ _____ / _____ /_____.

(Assinatura da Paciente) dia mês ano

_____

(Nome da paciente – Letra de forma)

_____ _____ / _____ /_____.

(Assinatura de testemunha, se necessário) dia mês ano

Eu, abaixo assinado, expliquei completamente os detalhes relevantes desta pesquisa à paciente indicada acima e/ou a pessoa autorizada para consentir pela paciente.

_____ _____ / _____ /_____.

(Assinatura da Pessoa que obteve o consentimento) dia mês ano