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TESE DE DOUTORADO

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EXPRESSÃO DE FATORES RELACIONADOS A CÉLULAS TRONCO CANCEROSAS E DE VASCULOGÊNESE EM CÂNCERES DE MAMA TRIPLO NEGATIVOS BASAIS E NÃO BASAIS

BELO HORIZONTE
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Tese apresentada ao Curso de Pós-Graduação em Patologia da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Patologia

Área de concentração: Patologia Médica

Orientadora: Profa. Dra. Helenice Gobbi

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Interior
Her mind lives in a quiet room,
A narrow room, and tall,
With pretty lamps to quench the gloom
And mottoes on the wall.
There all the things are waxen neat
And set in decorous lines;
And there are posies, round and sweet,
And little, straightened vines.
Her mind lives tidily, apart
From cold and noise and pain,
And bolts the door against her heart,
Out wailing in the rain.

Dorothy Parker

Às minhas pessoas favoritas,
Adseu Álvares de Andrade,
Inez Elizabeth De Brot Andrade
e Louise De Brot Andrade

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Nas palavras de Dorothy Parker:

Mentora, Helenice Gobbi:

The cure for boredom is curiosity. There is no cure for curiosity.

(Porque sempre admirei a sua paixão pela ciência).

Família, Adseu Álvares de Andrade, Inez Elizabeth De Brot Andrade e Louise De Brot Andrade:

For June was nearly spent away

Before my heart was whole.

(…) Now this must be the sweetest place From here to heaven's end;

The field is white and flowering lace,

The birches leap and bend,

The hills, beneath the roving sun,

From green to purple pass,

And little, trifling breezes run

Their fingers through the grass.

Mãe, Inez Elizabeth De Brot Andrade:

A girl's best friend is her mutter.

Love, Frederico Pinheiro Borges:

You - you'd only a lilting song,

Only a melody, happy and high,

You were sudden and swift and strong -

Never a thought for another had I.

SUMÁRIO

LISTA DE ABREVIATURAS

ALDH1- Aldeído desidrogenase 1

BRCA1- Gene do câncer de mama tipo 1(*Breast cancer gene 1*)

CA- Califórnia

CSCs- *Cancer stem cells*

CDI-SOE- Carcinomas ductais invasores sem outra especificação

cDNA- Ácido desoxirribonucléico cíclico

CK- Citoqueratinas

CK5- Citoqueratina 5

CK5/6- Citoqueratinas 5/6

CK17- Citoqueratina 17

c-kit- Receptor tirosina-quinase tipo III

DMV- Densidade microvascular

DMVL- Densidade microvascular linfática

DMVS- Densidade microvascular sanguínea

DNA- Ácido desoxirribonucléico

EDTA- *Ethylenediaminetetraacetic acid*

EGFR- Receptor do fator de crescimento epidérmico

EUA- Estados Unidos da América

EZH2- *Enhancer of Zeste homolog 2*

HR- Hazard ratio

HC- Hospital das Clínicas

HT- Hormonioterapia

HER1- Receptor 1 do fator de crescimento epidérmico

HER2 (c-erB-b2)- Receptor 2 do fator de crescimento epidérmico

HE- Hematoxilina e eosina

I- *Intensity*

IT- Área intratumoural

MDBA- Marina De Brot Andrade

PARP- Poli-ADP-ribose

P- *Percentage*

PP- Área periférica tumoral

PT- Área peritumoral

Q- *Quick score*

QT- Quimioterapia

RE- Receptor de estrógeno

RNA- Ácido ribonucléico

RNAm- Ácido ribonucléico mensageiro

RP- Receptor de progesterona (RP)

RT- Radioterapia

SG- Sobrevida global

SLD- Sobrevida livre de doença

SOE- Sem outra especificação

TEM- Transição epitélio-mesênquima

TGF-β- Fator de crescimento transformante beta

TKIs- Inibidores da tirosina quinase

TMA- Microarranjos de tecido (*tissue microarrays*)

TNM- T=tumor; N=número de linfonodos acometidos pela neoplasia; M= ocorrência de metástases à distância

UFMG- Universidade Federal de Minas Gerais

UK- United Kingdom

VEGF- Fator de crescimento endotelial vascular

VEGFR- Receptor do fator de crescimento endotelial vascular

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RESUMO

Introdução: O câncer de mama triplo negativo é definido pela negatividade para receptores de estrogênio (RE), receptores de progesterona (RP) e receptor do fator de crescimento epidérmico tipo 2 (HER2). O interesse neste tipo de câncer é crescente devido à ausêcia de tratamento sistêmico específico disponível e pela sobreposição com os carcinomas mamários de tipo basal. Embora a maioria destes tumores esteja associada a comportamento biológico agressivo com elevada capacidade metástatica, os fatores envolvidos na sua progressão tumoral e disseminação sistêmica ainda não foram esclarecidos. Estudos emergentes sugeriram que haveria no câncer de mama uma subpopulação de células neoplásicas malignas que estaria associada com o desenvolvimento tumoral, recorrências e metástases - hipótese das células tronco cancerosas. Por outro lado, sabe-se que a ocorrência de metástases também está associada à angiogênese e linfangiogênese.

Objetivos: No presente estudo avaliamos a expressão de marcadores relacionados a células tronco cancerosas (ALDH1 e EZH2) no câncer de mama triplo negativo, investigando sua associação com parâmetros clínicos, variáveis anátomo-patológicas, marcadores de fenótipo basal, sobrevida e prognóstico. Determinanos ainda a densidade microvascular sanguínea (DMVS) e linfática (DMVL), medidas indiretas da angiogênese e linfangiogênese, em carcinomas mamários triplo negativos e de tipo basal, correlacionando-as com parâmetros clínicos-patológicos, ocorrência de metástases axilares e hematogênicas, evolução clínica e sobrevida.

Material e Métodos: Dados clínicos-patológicos foram obtidos para 140 casos de câncer de mama triplo negativo dentre 2235 patientes submitidos a tratamento cirúrgico e estudo imunoistoquímico no periodo de 1985 a 2006. Um microarranjo de tecido foi construído e imunoistoquímica para os seguintes marcadores foi realizada: RE, RP, HER2, CK5, CK14, EGFR, p63, p53, caveolina, ALDH1 e EZH2. A expressão de ALDH1 foi avaliada tanto no componente epitelial quanto no componente estromal (estroma tumoral) dos

tumores. Para o estudo da vasculogênese, os seguintes anticorpos foram aplicados em 133/140 tumores triplo negativos: CD34, CD105 e D2-40. A determinação da DMVS e DMVL foi feita através de análise morfométrica digital computadorizada. Duas características foram observadas na avaliação da vasculogênese: a distribuição/localização de vasos no tumor, em sua periferia e no parênquima mamário adjacente; e a DMVS total / DMVL total. Análise univariada e multivariada foi realizada para avaliar a correlação entre ALDH1, EZH2, DMVS, DMVL com parâmetros clínicos e anátomo-patológicos. Para verificar a associação com a sobrevida das pacientes foi empregado o método de Kaplan-Meier. Um valor de $p < 0.05$ foi considerado significante.

Resultados: Carcinomas ductais invasores sem outra especificação (SOE) representaram a maioria dos cânceres de mama triplo negativos (116/140 casos); 105/140 tumores triplo negativos exibiram o fenótipo basal (expressão de CK5 e/ou CK14). Expressão de ALDH1 foi observada no no componente epitelial de 26/140 tumores, enquanto 117/140 tumores exibiram positividade no componente estromal. A expressão epitelial de ALDH1 não se correlacionou com nenhum dos paramêtros avaliados; por outro lado, expressão estromal foi associada a melhor sobrevida global (SG; p=0.044). A análise multivariada mostrou risco de morte pelo câncer de mama de 2.80 (HR=1/0.357=2.80; 95%CI 0.178–0.714; p=0.004) quando a expressão de ALDH1 foi negativa no componente estromal dos tumores triplo negativos. Expressão elevada de EZH2 foi detectada em 120 de 140 cânceres de mama triplo negativos e não se correlacionou com nenhum dos paramêtros analisados. Em tumores triplo negativos da mama, a maioria dos vasos sanguíneos e linfáticos foram observados nas regiões periférica tumoral (periferia do tumor) e peritumoral (parênquima mamário adjacente). Cânceres triplo negativos com DMVS total e DMVS peritumoral elevadas se correlacionaram significativamente com a presença de recorrências (p=0.05; p=0.01) e com o desenvolvimento de metástases (p=0.01; 0.005), especificamente metástases hematogênicas (p=0.006; p=0.01); também houve associação significativa com menor sobrevida global (p=0.006; p=0.01) e sobrevida livre de doença (p=0.04; p=0.09). DMVS avaliada pela expressão de CD105 se associou com menor

sobrevida global e livre de doença e tal variável manteve seu valor prognóstico após análise multivariada. Em relação à DMVL, não observamos associação significativa da DMVL total e em diferentes regiões tumorais (peritumoral, periférica tumoral e intratumoral) de cânceres de mama triplo negativos com nenhum dos paramêtros analisados, incluindo variáveis anátomo-patológicos, sobrevida global e sobrevida livre de doença.

Conclusões: Expressão de ALDH1 é frequentemente encontrada no estroma tumoral de cânceres de mama triplo negativos de tipo basal, estando associada com melhor evolução clínica; tal achado ressalta a importância de se considerar o microambiente tumoral ao se avaliar o impacto prognóstico da expressão de marcadores relacionados a células tronco cancerosas no câncer de mama. Além disso, vasos sanguíneos e linfáticos se encontram predominantemente nas regiões periférica tumoral e peritumoral destes tipos de tumores. A presença de associação significativa da DMVS em diferentes regiões tumorais com diversos paramêtros clínico-patológicos e pior sobrevida indica que tumores triplo negativos de comportamento biológico agressivo apresentam densidade microvascular sanguínea elevada. O presente estudo mostra, finalmente, que vasos sanguíneos parecem ter papel fundamental na progressão tumoral e capacidade metástatica de cânceres de mama triplo negativos, representando vias de disseminação da doença.

INTRODUÇÃO

1- OS CÂNCERES DE MAMA TRIPLO NEGATIVOS BASAIS E NÃO BASAIS

A heterogenidade do câncer de mama já é reconhecida há algum tempo. Entretanto, foi apenas após o advento das tecnologias moleculares em larga escala que tal consideração destacou-se e tornou-se um dos focos principais das pesquisas do câncer de mama (PEROU *et al*, 2000; RAKHA *et al*, 2008a; SORLIE *et al*, 2001; VAN DE RIJN *et al*, 2002). Apesar desta diversidade, o manejo rotineiro de pacientes continua se baseando na análise histopatológica e no perfil imunoistoquímico do tumor primário. O painel imunoistoquímico composto dos marcadores preditivos receptor de estrogênio (RE), receptor de progesterona (RP) e HER2 (receptor do fator de crescimento epidérmico tipo 2) foi incorporado à prática clínica (ALLRED *et al*, 1998; FITZGIBBONS *et al*, 2000; GOBBI *et al*, 2008; GOLDHIRSCH *et al*, 2005; PAYNE *et al*, 2008; ROCHA *et al*, 2008; WALKER *et al* 2008). Esta abordagem, embora aparentemente simples, tem funcionado, uma vez que a mortalidade do câncer de mama diminuiu nas últimas duas décadas, mesmo com o aumento de sua incidência. Além disso, a estimativa do prognóstico através de algoritmos com múltiplos parâmetros, por exemplo o programa *Adjuvant!* disponível *online*, na maioria das vezes é acurada e corresponde ao curso clínico real dos pacientes (MOOK *et al*, 2009). No entanto, estas condutas convencionais não são suficientes para a implementação de tratamentos individualizados direcionados a um paciente específico (SOTIRIOU *et al*, 2009).

Além da introdução do conceito de medicina individualizada e do desenvolvimento de uma taxonomia molecular para os carcinomas mamários, os estudos de perfis expressão gênicos baseados em microarranjos de DNA/RNA também forneceram evidências de que o câncer de mama RE-positivo e o câncer de mama RE-negativo são doenças distintas (PEROU *et al*, 2000; RAKHA *et al*, 2008a; RAKHA *et al*, 2008b SORLIE *et al*, 2001; SOTIRIOU *et al*, 2009).

O câncer de mama RE-negativo compreende três subtipos de tumores: aqueles com superexpressão de HER2, os de tipo “mama normal”, e os de tipo basal (PEROU *et al*, 2000; RAKHA *et al*, 2008b; SORLIE *et al*, 2001). Vários estudos demonstraram que 80% a 90% dos carcinomas mamários de tipo basal são também cânceres de mama triplo negativos, ou seja, negativos para receptor de estrogênio, receptor de progesterona e HER2 (LIVASY *et al*, 2006; RAKHA *et al*, 2007a). O interesse no câncer de mama triplo negativo é crescente devido à falta de terapêutica específica para pacientes com este tipo de câncer e pela sobreposição com os carcinomas mamários de tipo basal.

O câncer de mama triplo negativo corresponde a cerca de 10% a 17% dos carcinomas mamários, dependendo dos valores de referência empregados para definir a positividade para RE e RP, e do método utilizado para avaliar a superexpressão de HER2 (RAKHA *et al*, 2007a; RAKHA *et al*, 2008b; REIS-FILHO *et al*, 2008). As principais características destes tumores incluem a maior ocorrência entre mulheres mais jovens (<50 anos), frequentemente com apresentação clínica de “carcinomas intervalares” (*interval breast cancers*), comportamento biológico mais agressivo, e taxas de recorrência elevadas, com

menor sobrevida e ausência de resposta a hormonioterapia (CAREY *et al*, 2006; CAREY *et al*, 2007). Histologicamente, a maioria dos tumores triplo negativos são carcinomas ductais invasores pouco diferenciados e com elevada taxa proliferativa. Entretanto, uma significativa proporção destes tumores corresponde a carcinomas mamários invasores mais incomuns, tais como o carcinoma adenóide cístico e o carcinoma secretório. Estes subtipos de tumores têm prognóstico excelente. Além disso, nem todos os tipos de carcinomas triplo negativos pouco diferenciados evoluem mal (BAUER *et al*, 2007; RAKHA *et al*, 2007a; RAKHA *et al*, 2008a; REIS-FILHO *et al*, 2008). Os carcinomas medulares puros, por exemplo, apresentam melhor prognóstico que o esperado (BERTUCCI *et al*, 2006; VINCENT-SALOMON *et al*, 2007). Todas estas peculiaridades apontam para a natureza heterogênea do câncer de mama triplo negativo e para a possibilidade de que subgrupos de tumores triplo-negativos de pior prognóstico poderiam se beneficiar de tratamentos mais agressivos (RAKHA *et al*, 2007a).

Outro ponto relevante é a associação existente entre o fenótipo triplo negativo e o câncer de mama hereditário com mutações germinativas do gene supressor tumoral *BRCA1* (CAREY *et al*, 2010). A maioria dos carcinomas mamários associados a mutações de *BRCA1* exibem o fenótipo triplo-negativo e expressam citoqueratinas basais (ARNES *et al*, 2005; FOULKES *et al*, 2004; LAAKSO *et al*, 2005; PALACIOS *et al*, 2005; RAKHA *et al*, 2008c; RIBEIRO-SILVA *et al*, 2005; TURNER *et al*, 2007). A referida associação fornece a possibilidade de uma nova abordagem terapêutica com a incorporação de agentes inibidores da polimerase de ADP-ribose ou PARP ao tratamento dos pacientes com este tipo de câncer de mama (CAREY *et al*, 2010; GREENBERG & RUGO, 2010 PAL *et al*, 2011). Além

disso, os defeitos de reparo do DNA observados nos carcinomas hereditários contribuem para maior sensibilidade a esquemas quimioterápicos com derivados de antraciclinas e sais de platina, levantando a possibilidade de que os tumores triplo-negativos possam ser mais sensíveis a certos agentes quimioterápicos (ANDERS & CAREY, 2009; JAMES *et al*, 2007; KORSCHING *et al*, 2008; TURNER *et al*, 2007).

Como mencionado anteriormente, há grande sobreposição entre o câncer de mama triplo negativo e o de tipo basal, uma vez que ambos os subgrupos exibem similaridades clínico-patológicas e evolutivas (FADARE & TAVASSOLI, 2007; RAKHA *et al*, 2007a; RAKHA *et al*, 2008a; REIS-FILHO *et al*, 2008). Além disso, os trabalhos pioneiros de Perou *et al* (2000) e Sorlie *et al* (2001) identificaram os carcinomas basais no grupo dos tumores negativos para RE, RP e HER2.

Os tumores de tipo basal, identificados através de estudos de perfis de expressão gênica e imunoistoquímica em grandes séries de pacientes, representam 7% a 19% dos carcinomas mamários invasores (ABD EL-REHIM *et al*, 2005; MATOS *et al*, 2005; NIELSEN *et al*, 2004; PEROU *et al*, 2000; SORLIE *et al*, 2003; VAN DE RIJN *et al*, 2002). No grupo dos tumores triplo negativos a frequência do fenótipo basal é significativamente maior, sendo que 56% a 84% destes tumores expressam marcadores de fenótipo basal, como citoqueratinas basais e EGFR (TISCHKOWITZ *et al*, 2007; RAKHA *et al*, 2007a).

Atualmente, o câncer de mama de tipo basal (também chamados tumores basais ou basalóides) é definido através de perfis de expressão gênica e/ou imunoistoquímica, tendo sido inicialmente descrito como tumores que apresentam perfis gênico e protéico semelhantes aos das células basais/mioepiteliais da

mama normal (PEROU *et al*, 2000; SORLIE *et al*, 2001). Assim, verificou-se neste grupo expressão de citoqueratinas de alto peso molecular, as chamadas citoqueratinas (CK) basais (CK5, CK14 e CK17), além de laminina, c-kit, receptor do fator de crescimento epidérmico tipo 1 (EGFR ou HER1), e P-caderina (ABD EL-REHIM *et al*, 2005; NIELSEN *et al*, 2004).

Como o uso das técnicas de microarranjos de cDNA ainda não é rotineiro, a maioria dos trabalhos define os carcinomas de tipo basal imunoistoquimicamente. Por conseguinte, para auxiliar a padronizar as investigações, uma definição precisa destes tumores é necessária, aumentando a probabilidade de que características prognósticas/preditivas possam ser aplicáveis para todo o grupo. Ainda não há, entretanto, consenso internacional determinando precisamente quais marcadores devem ser empregados para definir e identificar os carcinomas de tipo basal (FADARE & TAVASSOLI, 2007; RAKHA *et al*, 2008d). A maioria dos autores incluiu em suas definições imunopositividade para CK5 ou CK5/6 (ABD EL-REHIM *et al*, 2005; ARNES *et al*, 2005; BANERJEE *et al*, 2006; FOULKES *et al*, 2004; KIM *et al*, 2006; LAAKSO *et al*, 2005; MATOS *et al*, 2005; NIELSEN *et al*, 2004; RAKHA *et al*, 2006a; RAKHA *et al*, 2006b; RAKHA *et al*, 2007b; RIBEIRO-SILVA *et al*, 2005; RODRIGUEZ-PINILLA *et al*, 2006; SIZIOPIKOU *et al*, 2007; VAN DE RIJN *et al*, 2002). Outros marcadores também já requeridos para a inclusão no grupo de tumores basais foram CK14 (ABD EL-REHIM *et al*, 2004; ABD EL-REHIM *et al*, 2005; BANERJEE *et al*, 2006; FULFORD *et al*, 2006; KIM *et al*, 2006; LAAKSO *et al*, 2005; RAKHA *et al*, 2006a; RAKHA *et al*, 2006b; RAKHA *et al*, 2007b), CK17 (BANERJEE *et al*, 2006; VAN DE RIJN *et al*, 2002), EGFR (KIM *et al*, 2006; NIELSEN *et al*, 2004; REIS-FILHO *et al*, 2006; RODRIGUEZ-

PINILLA *et al*, 2006), c-kit (KIM *et al*, 2006; NIELSEN *et al*, 2004), p63 (LAAKSO *et al*, 2005; MATOS *et al*, 2005; RIBEIRO-SILVA *et al*, 2005), P-caderina (ARNES *et al*, 2005; MATOS *et al*, 2005), caveolina 1 (PINILLA *et al*, 2006; SAVAGE *et al*, 2007) e caveolina 2 (SAVAGE *et al*, 2007). A expressão de caveolina 2 foi significativamente associada ao imunofenótipo basal e a pior prognóstico (SAVAGE *et al*, 2007).

A positividade para EGFR em tumores basais foi demonstrada em diferentes trabalhos, com elevadas proporções, que variam de 40% a 70% dos casos (KIM *et al*, 2006; NIELSEN *et al*, 2004; RODRIGUEZ-PINILLA *et al*, 2006). Os carcinomas de tipo basal mostram ainda mutações do gene *TP53* ou expressão imunoistoquímica de p53 em até 85% dos casos (CALZA *et al*, 2006; CAREY *et al*, 2006; KIM *et al*, 2006; SORLIE *et al*, 2001).

Alguns estudos exigiram especificamente a ausência de imunorreatividade para RE, RP e HER2 como critério de definição dos tumores de tipo basal (KIM *et al*, 2006; MATOS *et al*, 2005; RODRIGUEZ-PINILLA *et al*, 2006), enquanto outros não apresentavam esta exigência. Apesar de haver grande sobreposição entre o câncer triplo-negativo e o de tipo basal, esta não é completa (RAKHA *et al*, 2008a). Análises quanto ao *status* de RE, RP e HER2 em carcinomas de mama classificados como basais através de seus perfis de expressão gênica/protéica mostram que estes tumores expressam pelo menos um destes três marcadores em 15% a 54% dos casos (CALZA *et al*, 2006; NIELSEN *et al*, 2004; SOTIRIOU *et al*, 2003). É importante frisar, portanto, que tumores triplo negativos e tumores de tipo basal não são sinônimos (REIS-FILHO & TUTT, 2008).

Morfologicamente, embora não haja achados específicos dos carcinomas de fenótipo basal, algumas características são encontradas com maior freqüência nestes tumores. Vários trabalhos mostraram que os tumores de tipo basal apresentam predominantemente alto grau histológico e observaram que CDI-SOE positivos para marcadores basais são significativamente mais propensos a serem pouco diferenciados ou de grau III (ABD EL-REHIM *et al*, 2004; FOULKES *et al*, 2004; LIVASY *et al* 2006; RAKHA *et al*, 2006; RIBEIRO-SILVA *et al*, 2005; KIM *et al*, 2006; RODRIGUEZ-PINILLA *et al*, 2006; RODRIGUEZ-PINILLA *et al*, 2007; LAAKSO *et al*, 2005). Há ainda relatos de maior propensão a apresentarem padrão de crescimento expansivo, com bordas não infiltrativas e infiltrado inflamatório linfoplasmocitário peritumoral (FULFORD *et al*, 2006; LIVASY *et al*, 2006; MATOS *et al* 2005). É relevante ainda destacar que cerca de 30% a 75% dos carcinomas metaplásicos da mama apresentam fenótipo basal (LEIBL *et al*, 2005; REIS-FILHO *et al*, 2006), detectando-se a expressão de diversos marcadores, como p63, P-caderina, actina, EGFR, CK14 e CK5 (KIM *et al*, 2006; LEIBL *et al*, 2005; REIS-FILHO *et al*, 2006).

O câncer de mama de tipo basal, como o câncer de mama triplo negativo, tem sido consistentemente associado a comportamento clínico agressivo, com sobrevida global (SG) e sobrevida livre de doença (SLD) baixas (ABD EL-REHIM *et al*, 2004; NIELSEN *et al*, 2004; VAN DE RIJN *et al*, 2002). Alguns estudos demonstraram a expressão de CK basais como fator prognóstico independente da dimensão tumoral, grau histológico e *status* linfonodal (ABD EL-REHIM *et al*, 2004; RAKHA *et al*, 2007b). Entretanto, até recentemente, quando comparados com tumores não basais RE-negativos (JUMPPANEM *et al*, 2007) ou de grau

histológico semelhante (FULFORD *et al*, 2007), o fenótipo basal não seria, por si só, um indicador de pior prognóstico.

2- PROGNÓSTICO E PADRÕES DE METÁSTASE

O câncer de mama triplo negativo é heterogêneo em termos da sua biologia, prognóstico e resposta terapêutica (ADAMO & ANDERS, 2011; ANDERS & CAREY, 2009; PEROU *et al*, 2000; RAKHA & ELLIS, 2009; SORLIE *et al*, 2003). Pacientes com tumores triplo negativos apresentam pior evolução clínica quando comparados a pacientes com tumores não triplo negativos. Entretanto, o primeiro grupo exibe elevadas taxas de resposta patológica completa (RPc) após quimioterapia neoadjuvante. A presença ou ausência de doença residual após quimioterapia pré-operatória foi descrita como um forte preditor de sobrevida no câncer triplo-negativo. Liedtke e colegas (2008) encontraram que a sobrevida de 3 anos em pacientes com tumores triplo negativos com e sem doença residual após quimioterapia neoadjuvante é de 68% e 94%, respectivamente. A pior sobrevida, mesmo com altas taxas de RPc, parece ser causada pelas elevadas taxas de recidiva entre os pacientes cujos tumores não foram erradicados completamente (ADAMO & ANDERS, 2011; LIEDTKE *et al*, 2008).

Para definir o prognóstico de pacientes com câncer triplo negativo, classificado através de parâmetros clínicos (negativos para RE/RP/HER2), uma grande coorte de pacientes foi estudada, incluindo mais de 1.600 mulheres diagnosticas com carcinoma mamário invasor e tratadas em Toronto de 1987 a 1997 (DENT *et al*, 2007). Dentre as 11,2% pacientes diagnosticadas com tumores triplo-negativos,

tanto a probabilidade de recorrência com metástase à distância (hazard ratio [HR], 2.6; $p < 0,0001$) quanto a de morte por câncer de mama nos primeiros 5 anos do diagnóstico (HR, 3.2; $p < 0,0001$) foram mais elevadas quando comparadas a pacientes com tumores não triplo-negativos da mama. Recorrência à distância e mortalidade câncer-específica aconteceram tipicamente nos primeiros 5 a 7 anos após o diagnóstico inicial - pico nos primeiros 3 anos, com declínio rápido após este período (DENT *et al*, 2007).

Um estudo avaliando resposta a quimioterapia neoadjuvante entre mais de 1.000 pacientes tratadas no *M. D. Anderson Cancer Center* no período de 1985 a 2004 corroborou os achados descritos acima. Os resultados demonstraram uma redução de 3 anos na sobrevida livre de doença e na sobrevida global de pacientes com câncer de mama triplo negativo, comparadas àquelas com câncer de mama não triplo negativo. Como em trabalhos prévios, recorrência e taxas de mortalidade foram mais altas para o câncer de mama triplo negativo apenas nos primeiros 3 anos do diagnóstico inicial. Tal padrão é consistente com a natureza precoce e agressiva das recidivas observadas em pacientes com câncer de mama triplo negativo (LIEDTKE *et al*, 2008).

Além dos padrões observados quanto ao tempo de ocorrência das recidivas, locais preferenciais de recorrência também foram identificados em pacientes com câncer de mama triplo negativo e de tipo basal (ANDERS & CAREY, 2009; FULFORD *et al*, 2006; FULFORD *et al*, 2007; LUCK *et al*, 2008; RAKHA *et al*, 2007). Dent *et al* (2007) relataram que poucas mulheres com tumores triplo-negativos têm recorrência local antes que metástases à distância ocorram. Mais especificamente, Liedtke *et al* (2008) descreveram que pacientes com estes

tumores têm taxas mais elevadas de recorrência em órgãos viscerais e partes moles, e menores taxas de metástases para ossos, quando comparadas a pacientes com tumores hormônio-positivos (p=0,027).

Em outros trabalhos ressalta-se que o câncer de mama triplo-negativo e de tipo basal apresentam comportamento biológico diferente dos demais carcinomas mamários pouco diferenciados, com padrão de disseminação distinto. Comparados com outros tumores de alto grau, os carcinomas de tipo basal têm menor tendência a evoluir com metástases para linfonodos axilares, ossos e fígado. Há, por outro lado, maior incidência de metástases hematogênicas para pulmões e cérebro (ANDERS & CAREY, 2009; FULFORD *et al*, 2007; LUCK *et al*, 2008; RAKHA *et al*, 2007). Gaedcke *et al* (2007) demonstraram que a maioria dos carcinomas mamários metastatizantes para o cérebro apresentam fenótipo triplo negativo e/ou basal.

Em suma, o câncer de mama triplo negativo e o de tipo basal apresentam várias características moleculares e morfológicas semelhantes. Embora a maioria dos tumores esteja associada a comportamento biológico agressivo e pior prognóstico, os fatores envolvidos na patogênese deste comportamento agressivo ainda não foram esclarecidos (ADAMO & ANDERS, 2011; RAKHA & ELLIS, 2009; REIS-FILHO & TUTT, 2008).

Uma das hipóteses levantadas para a capacidade metastática dos carcinomas é a da chamada transição epitélio-mesênquima (TEM), que é definida como um processo dinâmico de transição de células epiteliais imóveis e polarizadas para células tipo mesenquimais altamente móveis, por meio da reorganização do citoesqueleto e da perda de junções celulares. Este mecanismo permitiria

carcinomas adquiram um fenótipo mais agressivo, promovendo a progressão e invasão pelas células neoplásicas. A TEM é regulada por fatores de transcrição como o *SNAIL*, que reduz a atividade de genes epiteliais e aumenta a de genes mesenquimais. Na progressão tumoral tal fator funciona através da inibição da expressão de E-caderina, promovendo a aquisição de propriedades invasivas e migratórias. Entretanto, a TEM parece ocorrer em uma pequena porcentagem de células neoplásicas (MORENO-BUENO *et al*, 2008).

O papel da TEM na progressão tumoral e ocorrência de metastáses já foi demonstrado no câncer de mama e a indução desta via pode estar envolvida também no desenvolvimento e proliferação das chamadas células tronco cancerosas, ou *cancer stem cells* (SANTISTEBAN *et al*, 2009). Por outro lado, sabe-se que a ocorrência de metástases também está associada à angiogênese e linfangiogênese, sendo a formação de novos vasos sanguíneos e linfáticos processos críticos para o crescimento tumoral, invasão e disseminação do câncer (CHOI *et al*, 2005).

5- A HIPÓTESE DAS CÉLULAS TRONCO CANCEROSAS NO CÂNCER DE MAMA

A heterogeneidade biológica é um fenômeno já bem caracterizado em tumores sólidos, uma vez que estes são constituídos por um espectro de células malignas fenotipicamente diversas (PAYNE *et al*, 2008; RAKHA *et al*, 2008b; STINGL & CALDAS, 2007). De fato, o conceito de que tumores contêm um subgrupo de células semelhantes a células tronco epiteliais foi proposto recentemente,

juntamente com estudos emergentes sugerindo que algumas malignidades obedeceriam a uma hierarquia celular equivalente à observada em tecidos normais (CLARKE & FULLER, 2006; JORDAN *et al*, 2006; REYA *et al*, 2001). De acordo com a hipótese das células tronco cancerosas, haveria uma subpopulação altamente tumorigênica de células neoplásicas malignas que estaria associada com o desenvolvimento tumoral, resistência a agentes quimioterápicos, desenvolvimento de recorrências e de metástases, tornando-se potenciais alvos terapêuticos para o tratamento do câncer de mama (CARIATI & PURUSHOTHAM, 2008). As células tronco cancerosas seriam organizadas de forma hierárquica e a transformação a partir de suas próprias células tronco progenitoras resultaria em carcinogênse, crescimento e progressão tumoral (CARIATI & PURUSHOTHAM *et al*, 2008; CLARKE *et al*, 2006; NAKSHATRI *et al*, 2009). Postula-se que as células tronco cancerosas, ou *cancer stem cells* (CSCs), possuiriam capacidade de auto-renovação, divisão celular lenta, e a habilidade de gerar células novas diferenciadas, em um paralelo com as propriedades de células tronco normais (JORDAN *et al*, 2006; KAKARALA & WICHA 2007; WICHA *et al*, 2006). Esta subpopulação de células cancerosas corresponderia a uma pequena porcentagem das células malignas em tumores sólidos. Esta pequena porcentagem de células neoplásicas com propriedades de células tronco originaria todas as outras células mais diferenciadas que compõem o tumor (JORDAN *et al*, 2006; VISVADER *et al*, 2008; WICHA *et al*, 2006). Tais células parecem exibir características diferentes daquelas apresentadas pelas demais células em um determinado tumor (FILLMORE & KUPERWASSER, 2007). Como as CSCs são resistentes a radioterapia e a quimioterapia (CLARKE & FULLER, 2006; KAKARALA & WICHA

2007; MORRISON *et al*, 2008), recorrência do câncer e falência terapêutica refletiriam a quiescência e a resistência a drogas intrínsecas destas células (DIEHN *et al*, 2006; LI *et al*, 2008; O'BRIEN *et al*, 2008).

No decorrer dos últimos anos, as CSCs foram identificadas em diferentes tipos de câncer, incluindo carcinomas mamários primários/metastáticos e inúmeras linhagens de células mamárias cancerosas (AL-HAJJ *et al*, 2003; CLARKE *et al*, 2006; WICHA *et al*, 2006). Tanto as células tronco normais mamárias quanto as células tronco cancerosas mamárias foram purificadas em sistemas de cultura *in vitro* através da identificação de seus antígenos de superfície (AL-HAJJ *et al*, 2003; LIU *et al*, 2005). Embora progresso considerável tenha sido obtido na identificação de células tronco mamárias humanas, o fenótipo exato destas células permanece indefinido (CLARKE *et al*, 2006; CLARKE & FULLER, 2006; VILLADSEN *et al*, 2007; WICHA *et al*, 2006). O antígeno de superfície celular CD44 tem sido empregado para identificar células tronco cancerosas no câncer de mama. Foi demonstrado em modelos experimentais que a população celular com o fenótipo CD44+/CD24- apresentam os critérios necessários para serem classificadas como CSCs. Citometria de fluxo seguida de separação celular, desenvolvimento de mamosferas e injeção de um pequeno número de células cancerosas mamárias CD44+/CD24- em camundongos resultou em crescimento tumoral (AL-HAJJ *et al*, 2003; SHERIDAN *et al*, 2006). Alguns estudos observaram ausência da população celular com fenótipo CD44+/CD24- em linhagens de células mamárias positivas para RE. Tal população de células CD44+/CD24- parece estar restrita a linhagens de células mamárias mesenquimais/triplo-negativas (MANI *et al*, 2008; SHERIDAN *et al*, 2006). Foi

também demonstrado que o fenótipo CD44+/CD24- está associado a tumores de tipo basal (HONETH *et al*, 2008). Além disso, relatos confirmam que tumores com expressão do fenótipo CD44+/CD24- estão associados a pior prognóstico (HONETH *et al*, 2008; SHERIDAN *et al*, 2006). Outros antígenos de superfície celular foram reconhecidos em células tronco cancerosas mamárias, entre eles a alfa 6 Integrina, CD133, e beta 1 Integrina/CD29 (HONETH *et al*, 2008; WRIGHT *et al*, 2008).

É provável que o fenótipo das CSCs seja definido pela sua célula de origem (células tronco ou células progenitoras) e pelos eventos oncogênicos que contribuem para a sua transformação neoplásica (JAMIESON *et al*, 2004). Um dos possíveis biomarcadores de CSCs é a aldeído desidrogenase 1 (*aldehyde dehydrogenase 1* - ALDH1), enzima responsável pela oxidação intracelular de aldeídos e crucial durante a embriogênese (DUESTER *et al*, 2000). Pesquisas mostraram que a ALDH1 tem papel importante na fase inicial de diferenciação das células tronco através da conversão de retinol em ácido retinoico (CHUTE *et al*, 2006). Aumento da atividade de ALDH1 foi demonstrada em células tronco hematopoiéticas humanas/células progentitoras e populações de células tronco do mieloma múltiplo (MATSUI *et al*, 2004). Em um estudo conduzido por Ginestier *et al* (2007), ensaios de ALDEFLUOR foram utilizados para mostrar que células com atividade enzimática de ALDH1 isoladas da mama humana normal exibem características fenotípicas e funcionais de células tronco mamárias. Curiosamente, um pequeno número de células ALDEFLUOR-positivas foi suficiente para originar tumores em camundongos (GINESTIER *et al*, 2007). Este trabalho também demonstrou que tanto células tronco mamárias normais quanto malignas podem

ser identificadas através de imunoistoquímica (GINESTIER *et al*, 2007). Em estudos prévios envolvendo carcinomas mamários em mulheres, a expressão de ALDH1 foi associada a pior prognóstico, negatividade para receptores hormonais e expressão de citoqueratinas basais (CROKER *et al*, 2009; GINESTIER *et al*, 2007; NEUMEISTER *et al*, 2010; RESETKOVA *et al*, 2010; ZHOU *et al*, 2010). Além disso, alguns trabalhos evidenciaram que a expressão de ALDH1 não está restrita a células epiteliais, tendo sido observada ainda em células do estroma mamário (GINESTIER *et al*, 2007; RESETKOVA *et al*, 2010). Restkova e colegas (2010) não somente detectaram expressão de ALDH1 no estroma tumoral, como também relataram a sua correlação com melhor evolução clínica em duas coortes de pacientes com câncer de mama triplo-negativo.

Recentemente foi proposto que uma população secundária mais agressiva de células tronco cancerosas, denominadas células cancerosas iniciadoras ou *tumor-initiating cancer cells*, se originaria a partir da população primária de células tronco cancerosas através da aquisição de mutações genéticas adicionais. Tais células conduziriam a progressão tumoral (CHANG *et al*, 2011). Chang *et al* (2011) relataram que a expressão de EZH2 (Enhancer of Zeste homolog 2), essencial na renovação de células tronco, está ligada à regulação e crescimento das *tumor-initiating cancer cells* no câncer de mama. Por sua vez, EZH2 é uma enzima histona-lisina N-metiltransferase da família do grupo *Polycomb* (PcG), que em humanos é codificada pelo gene EZH2. Este é um gene silenciador que controla a metilação de DNA. A proteína EZH2 é também importante na preservação da identidade celular e manutenção da pluropotência e plasticidade das células tronco embrionárias (COLLET *et al*, 2006; KLEER *et al*, 2003). A expressão de

EZH2 foi associada a carcinomas mamários agressivos e a mau prognóstico (COLLET *et al*, 2006; KLEER *et al*, 2003). O estudo de Chang e colegas (2011) identificou que a expressão de EZH2 inibe o reparo de DNA, favorencendo a carcinogênese. No mesmo trabalho, uma nova droga anti-neoplásica foi testada – o Sorafenib. Esta droga elimina células tronco mamárias cancerosas EZH2-positivas (CHANG *et al*, 2011). Tais achados apontam para o valor potencial de EZH2 como um possível alvo terapêutico no câncer de mama.

Há poucos trabalhos na literatura investigando a expressão de marcadores de células tronco em amostras teciduais de carcinomas mamários, especialmente em tumores triplo negativos e de tipo basal (HONETH *et al*, 2008; HENNESSY *et al*, 2009). Um estudo pré-clínico recente evidenciou que um inibidor de tirosina-quinase, o dasatinib, tem efeito antitumoral mais potente em células cancerosas de fenótipo triplo negativo/basal através de sua ação contra a população de células tronco cancerosas (KUREBAYASHI *et al*, 2010).

No presente estudo, pretendemos avaliar a expressão dos marcadores relacionados a células tronco cancerosas ALDH1 e EZH2 em cânceres de mama triplo negativos, investigando sua associação com parâmetros clínicos, variáveis anátomo-patológicas e marcadores de fenótipo basal. Além disso, nós descrevemos a presença e o significado de células ALDH1-positivas no estroma de carcinomas mamários triplo negativos.

6- ESTUDO DA ANGIOGÊNESE E LINFANGIOGÊNESE NO CÂNCER DE MAMA

Como abordado anteriormente, o câncer de mama triplo-negativo e o de tipo basal apresentam curso clínico, comportamento biológico e padrão de disseminação distintos. Comparados com outros tumores de alto grau histológico, os carcinomas triplo-negativos e de tipo basal têm menor tendência a evoluir com metástases para linfonodos axilares, ossos e fígado. Há, por outro lado, maior incidência de metástases por via hematogênica para pulmões e cérebro (CAREY *et al*, 2006; FULFORD *et al*, 2007; RAKHA *et al*, 2007b).

Sabe-se que a ocorrência de metástases está associada à angiogênese, já tendo sido demonstrada relação da disseminação peri-operatória de células neoplásicas com a densidade microvascular intratumoral (MCCULLOCH *et al*, 1995). Desta forma, a formação de novos vasos sanguíneos é um dos mecanismos que permite o crescimento tumoral, disseminação hematogênica e ocorrência de metástases. Por outro lado, os mecanismos envolvidos na disseminação neoplásica via sistema linfático são pouco conhecidos (RAN *et al*, 2010).

O termo *angiogenic switch* representa um dos pontos-chave de malignidade, quando o tumor inicia o recrutamento de seu suprimento sanguíneo próprio, a partir do desequilíbrio entre fatores pró-angiogênicos e antiangiogênicos. A angiogênese tumoral é controlada por citocinas e fatores genéticos (LONGATTO FILHO *et al*, 2010). Entre estes, o fator de crescimento endotelial vascular (VEGF) e seus receptores têm papel crucial. A família da proteína VEGF consiste de

glicoproteínas estruturalmente relacionadas (VEGFA a E), sendo VEGFA (também conhecida como VEGF) uma glicoproteína que tem múltiplos efeitos durante a angiogênese: atua como mitógeno das células endoteliais; estimula secreção e ativação de enzimas envolvidas na degradação da matriz extracelular; e tem papel na sobrevida, migração e proliferação das células endoteliais e neoplásicas. As funções do VEGFA são mediadas através da ligação com os receptores VEGFR1 e VEGR2. O VEGFB se liga e ativa o VEGFR1, enquanto VEGFC e VEGFD se ligam ao VEGFR2 e VEGFR3 e atuam na linfangiogênese (BANERJEE *et al*, 2007; RAN *et al*, 2010).

No câncer de mama, a expressão de VEGF é estimulada por fatores de crescimento, citocinas, hormônios, perda funcional de p53, ativação do oncogene RAS e amplificação de HER2. A ativação do EGFR através da via de sinalização do VEGF também tem efeitos nas células tumorais e endoteliais. Em estudos experimentais, o uso de inibidores de EGFR (gefitinib) resultou em redução dose-dependente da expressão de VEGF pelas células tumorais e concomitante diminuição da angiogênese (CIARDIELLO *et al*, 2001). Este dado é particularmente importante para pacientes com tumores de tipo basal, em que já se verificou expressão de EGFR em 40% a 70% dos casos (NIELSEN *et al*, 2004; REIS-FILHO *et al*, 2006). Portanto, estas proteínas representam alvos terapêuticos potenciais e já há ensaios testando drogas (vatalinib) que bloqueiam simultaneamente tais moléculas e que poderiam ter maiores efeitos antitumorais e antiangiogênicos (SINI *et al*, 2005).

Os métodos de avaliação da angiogênese e linfangiogênese consistem na contagem microscópica direta de vasos (densidade microvascular) ou

indiretamente por meio da medida de fatores angiogênicos/linfangiogênicoas e seus receptores (HASAN *et al*, 2002). A estimativa microscópica da densidade microvascular (DMV) nos cortes histológicos dos tecidos, através do estudo imunoistoquímico com marcadores endoteliais, é amplamente disponível e empregada em departamentos de Patologia (CHOI *et al*, 2005; KERBEL, 2008). A medida da DMV também pode ser realizada através de sistemas digitais de análise de imagens (MOHAMMED *et al*, 2009). Vários marcadores do endotélio de vasos sanguíneos têm sido empregados, incluindo CD31, CD34, antígeno relacionado ao fator VIII e endoglina (CD105). O anticorpo anti-CD105 é considerado um marcador de proliferação vascular ou angiogênese (CHARPIN *et al*, 2004; DALES *et al*, 2003; OXMANN *et al*, 2008), uma vez que se liga preferencialmente a células endoteliais ativadas *in vitro* (WANG *et al*, 1993). A endoglina, por sua vez, é uma das glicoproteínas que compõem a família do fator de crescimento transformante beta, TGF-β (CHEIFETZ *et al*, 1992). Este tem a função de inibir a proliferação endotelial. Quando superexpressa em células endoteliais ativadas de vasos tumorais, a endoglina age inibindo a ação do TGF-β (SHE *et al*, 2004). A expressão de CD105 em carcinomas mamários invasores foi associada a pior prognóstico e menor sobrevida das pacientes (CHARPIN *et al*, 2004; DAKHAL *et al*, 2008; *et al*, 2008; DALLAS DALES *et al*, 2003; DALES *et al*, 2004; OXMANN *et al*, 2008). Este dado pode ter aplicações clínicas considerando-se a disponibilidade de novos anticorpos monoclonais que inibem a endoglina (SEON *et al*, 1997; TSUJIE *et al*, 2008; WU *et al*, 2005). Um destes anticorpos, referido com TRC105, se liga ao CD105 humano e inibe a angiogênese e o crescimento tumoral. Ensaios clínicos encontram-se em andamento para testar a

eficácia desta anticorpo em pacientes com cancer (MENDELSON *et al*, 2010). Há ainda evidência de que a expressão de marcadores relacionados a angiogênese estaria relacionada a resistência a quimioterapia (BERESFORD *et al*, 2006; ESCORCIA *et al*, 2010; GLUTZ *et al*, 2011).

Inicialmente, poucos estudos avaliaram vasos linfáticos, densidade microvascular linfática (DMVL) e linfangiogênese em tumores sólidos, devido à menor especificidade da maioria dos anticorpos disponíveis até então (CHOI *et al*, 2005; RAN *et al*, 2010). Entretanto, marcadores seletivos de endotélio linfático foram desenvolvidos, como LYVE-1, prox1 e podoplanina (CUNNICK *et al*, 2002). O anticorpo monoclonal D2-40 permitiu a detecção seletiva do endotélio de vasos linfáticos em mama e tonsilas palatinas (KAHN & MARKS, 2002; KAHN *et al*, 2002), sendo altamente sensível e específico na identificação destes vasos. Estudos de nosso grupo mostraram que a invasão neoplásica em vasos linfáticos, identificados pela imunoistoquímica com o anticorpo D2-40, relaciona-se positivamente com a ocorrência de metástases axilares e com outros indicadores de pior prognóstico (MARINHO *et al*, 2008).

Controvérsias ainda existem em relação à topografia dos vasos linfáticos em carcinomas mamários, sua utilidade como fatores prognósticos, sua relação com a angiogênese e a ocorrência de linfangiogênese intratumoral ativa (MOHAMMED *et al*, 2009).

Muitos trabalhos relataram que a DMV intratumoral é um indicador útil de sobrevida livre de doença (SLD) e sobrevida global (SG) no câncer de mama (WEIDNER *et al*, 1992; LONGATTO FILHO *et al*, 2010). Gasparini *et al* (1995) demonstraram que a DMV seria um indicador preditivo independente de menor

SLD e SG em pacientes com tumores de mama e comprometimento de linfonodos axilares tratadas com hormonioterapia (HT) e quimioterapia (QT) adjuvantes. Ensaios clínicos mostraram que VEGF é um preditor independente de SLD e SG após QT, HT e radioterapia (RT) adjuvantes no câncer de mama avançado (LINDERHOLM *et al*, 2000). Dhakal *et al* (2008), através da contagem de vasos marcados com CD34 e CD105, mostraram que em pacientes linfonodo-negativas a elevada contagem vascular (CD34 e CD105 altos) identificou aquelas com evolução clínica desfavorável.

Considerando-se que a angiogênese é fator importante para o crescimento tumoral, invasão e metástase, a inibição deste processo seria uma estratégia interessante para o tratamento do câncer de mama. Pesquisas recentes têm se concentrado em buscar novos agentes antiangiogênicos utilizados isoladamente ou em combinação com quimioterapia, como o bevacizumab (Avastin®) e inibidores da tirosina quinase ou TKIs (BANERJEE *et al*, 2007; VERMEULEN *et al*, 2010). O uso de bevacizumab, um anticorpo monoclonal humanizado dirigido contra o fator pró-angiogênico mais potente, o VEGF, ainda representa uma abordagem promissora (ALVAREZ 2010; NIELSEN *et al*, 2010).

Recentemente foi demonstrado que carcinomas mamários invasores com densidade microvascular linfática elevada se correlacionam com o desenvolvimento de metástases para linfonodos axillares e estadio avançado do câncer. Densidade microvascular linfática elevada também indicou pior sobrevida entre o grupo de pacientes estudado. Entretanto, a significância estatística desta variável não se manteve em análise multivariada. Por outro lado, o mesmo grupo descreveu que tumores com densidade microvascular sanguínea elevada exibem

alto grau histológico, acometimento de linfonodos axilares e negatividade para receptores hormonais (MOHAMMED *et al*, 2009).

Há poucos estudos que avaliaram fatores envolvidos na angiogênese em carcinomas mamários triplo negativos e de tipo basal (RIBEIRO-SILVA *et al*, 2006). Também são raros os trabalhos que investigaram DMVS e DMVL nestes grupos de tumores. Um trabalho de Li e colaboradores (2011) mostrou que densidade microvascular sanguínea elevada está associada com pior sobrevida em pacientes com câncer de mama tripo-negativo. Entretanto, DMVS não reteve sua significância estatística em análise multivariada (LI *et al*, 2011). Mais recentemente, Mohammed e colegas (2011) observaram que, entre cânceres de mama linfonodo-negativos, tumores triplo negativos e basais apresentam DMVS mais elevada que tumores não triplo negativos e não basais. Tal achado sugere que cânceres de mama tripo negativos e de tipo basal poderiam, particularmente, se beneficiar de terapia anti-angiogênica (JUBB *et al*, 2006; MACKEY *et al*, 2012; SIDDIQUI-JAIN *et al*, 2010).

Dadas as interações biológicas existentes entre neoformação vascular sanguínea e linfática, crescimento tumoral, invasão e metástases, a quantificação da angiogênese e linfangiogênese em tumores triplo negativos e de tipo basal poderá fornecer informações importantes sobre a progressão da doença e também ser fonte de estudo para novos alvos terapêuticos.

Neste trabalho pretendemos estudar a angiogênese e linfangiogênese em cânceres de mama triplo negativos e de tipo basal, e correlacioná-las com as características clínicas, anátomopatológicas, padrões de metástases e evolução das pacientes.

OBJETIVOS

Os objetivos deste estudo são:

1- Avaliar a expressão de marcadores relacionados a células tronco cancerosas da mama (ALDH1 e EZH2) cânceres de mama triplo negativos, investigando sua associação com parâmetros clínicos, variáveis anátomo-patológicas, marcadores de fenótipo basal e sobrevida das pacientes.

2- Estudar a topografia e distribuição de vasos sanguíneos e linfáticos em cânceres de mama triplo negativos basais e não basais.

3- Examinar a densidade microvascular sanguínea (DMVS), medida indireta de angiogênese, em cânceres de mama triplo negativos, correlacionando-a com parâmetros clínicos, variáveis anátomo-patológicas, marcadores de fenótipo basal, ocorrência de metástases axilares e hematogênicas e prognóstico.

4- Determinar a densidade microvascular linfática (DMVL), medida indireta de linfangiogênese, em cânceres de mama triplo negativos, correlacionando-a com parâmetros clínicos, variáveis anátomo-patológicas, marcadores de fenótipo basal, ocorrência de metástases axilares e a distância, e prognóstico.

MATERIAIS E MÉTODOS

Como parte de trabalho prévio de Mestrado, intitulado “Carcinomas Mamários de Tipo Basal: Estudo Clínico-Patológico e Imunoistoquímico em Mulheres Brasileiras”, pacientes com história documentada de câncer de mama triplo negativo foram identificados entre 2235 casos de cânceres de mama submetidos a tratamento cirúrgico no Hospital das Clínicas da Universidade Federal de Minas Gerais (UFMG) no período de 2001 a 2006. Incluímos ainda casos de tumores mamários examinados no Departamento de Patologia do Hospital do Câncer/A.C. Camargo de São Paulo. Selecionamos todos os casos cujos laudos imunoistoquímicos revelaram negatividade para receptores hormonais (RE e RP) e HER2 (câncer de mama triplo negativo triplo negativo).

Assim, para a inclusão neste estudo, os casos preencheram os seguintes requisitos:

1- Ter diagnóstico histológico original de carcinoma mamário invasor;

2- Ter testes imunoistoquímicos prévios revelando negatividade para RE, RP e HER2;

3- Ter disponibilidade de blocos de parafina do tumor para reavaliação histológica e novo estudo imunoistoquímico.

1- DADOS CLÍNICOS

Os dados clínicos foram obtidos a partir das requisições de exames histopatológicos arquivados no Laboratório de Patologia Mamária e de pesquisa nos prontuários médicos dos pacientes, arquivados no Serviço de Arquivo Médico do Hospital das Clínicas da UFMG (HC/UFMG). Também foram coletados dados dos arquivos médicos do Departamento de Patologia do Hospital do Câncer/A.C.Camargo, para os casos provenientes deste serviço. Foram obtidos os seguintes dados: nome da paciente; registro geral; ano da biópsia; data do diagnóstico inicial e da última consulta e/ou óbito; idade ao diagnóstico inicial; cor da pele (branca, negra, parda ou amarela); período reprodutivo (pré ou pós-menopausa); história familiar para câncer de mama; tratamentos complementares (hormonioterapia, quimioterapia, radioterapia); recidivas (data e local); tempo de seguimento clínico.

2- ESTUDO ANÁTOMO-PATOLÓGICO

2.1- Dados anátomo-patológicos dos laudos originais

Os dados foram obtidos dos laudos anátomo-patológicos e/ou relatórios prévios arquivados nos referidos serviços de Patologia, sendo coletados: tamanho do tumor, *status* linfonodal e estadiamento anátomo-patológico.

A classificação do tumor com relação ao tamanho foi efetuada tendo como base o sistema TNM, considerando-se a dimensão descrita no laudo original.

2.2- Revisão histopatológica

Todas as lâminas originais coradas pelo método de hematoxilina-eosina (HE), referentes aos casos selecionados, foram reavaliadas pelo mesmo patologista (MDBA). A revisão histopatológica incluiu reavaliação do tipo e grau histológicos dos tumores.

2.2.1- Classificação e graduação histológicas

Para a classificação dos tumores quanto ao tipo histológico, foram empregadas as recomendações de PAGE *et al* (1998), adotadas pelo Colégio Americano de Patologia (FITZGIBBONS *et al*, 2000), onde os carcinomas são divididos em ductal sem outra especificação (SOE), tipo “especial puro” e tipo “especial variante”. Casos incluídos neste estudo estao ilustrados na FIGURA 1.

Para a avaliação do grau histológico, utilizamos o Sistema de Nottinghan (ELSTON & ELLIS, 1991; ELSTON & ELLIS, 1998), onde o tumor é classificado como bem diferenciado (baixo grau ou grau I), moderadamente diferenciado (grau intermediário ou grau II) ou pouco diferenciado (alto grau ou grau III). Estas categorias são avaliadas através de notas atribuídos a três fatores (formação tubular, pleomorfismo nuclear e contagem mitótica), os quais geram valores de 1 a 3 que somados, resultam em um *score* final de 3 a 9.

3- ESTUDO IMUNOISTOQUÍMICO

A imunoistoquímica foi feita em cortes histológicos sequenciais obtidos a partir de um microarranjo de tecido ou em cortes histológicos inteiros provenientes dos blocos de parafina de cada caso selecionado. Todo o procedimento imunoistoquímico foi semi-automatizado utilizando-se o *Link 48 Autostainer* (DAKO, Carpinteria, CA, EUA). O sistema de detecção usado foi o sistema *Flex Plus* e, para visualização das reações, usamos Diaminobenzidina e contra-coloração com Hematoxilina.

3.1- Construção do microarranjo de tecido ("tissue microarray" - TMA)

Um microarranjo de tecido (TMA) foi construído como parte do projeto de Mestrado referido acima. A partir das lâminas originais coradas pelo método da hematoxilina-eosina, duas áreas mais representativas e mais preservadas do tumor foram marcadas. Os blocos de parafina referentes aos casos selecionados foram utilizados como "blocos doadores", a partir dos quais foram retirados dois cilindros de tecido das áreas previamente marcadas. Os cilindros obtidos foram colocados em um bloco receptor, organizados em coordenadas definidas em uma planilha, permitindo a identificação precisa de cada amostra. O diâmetro de cada disco de tecido foi de 1mm, de modo que o microarranjo de tecido confeccionado contém cilindros com amostras representativas de todos os tumores selecionados.

A montagem do TMA foi realizada utilizando-se um equipamento manual, da marca *Beecher Instruments*®.

3.2- Imunoistoquímica para avaliação do fenótipo triplo negativo e basal

Lâminas contendo cortes sequenciais do TMA foram coradas para RE, RP e HER2 para confirmação do diagnóstico de câncer de mama triplo negativo. Em lâminas sequenciais, foram aplicados anticorpos dirigidos aos marcadores de diferenciação basal (CK5 e CK14), e também outros marcadores, como EGFR, p63, caveolina e p53. A expressão destes marcadores está apresentada na FIGURA 2. Anticorpos primários, diluições, fabricantes, métodos de recuperação antigênica e valores de corte utilizados no estudo imunoistoquímico para avaliação do fenótipo basal estão apresentados na TABELA 1. Para a avaliação e graduação das reações imunoistoquímicas dos marcadores de fenótipos triplo negativo e basal, empregamos critérios publicados previamente na literatura (GOLDHIRSCH *et al*, 2005; HARVEY *et al*, 1999; RAKHA *et al*, 2007b SAVAGE *et al*, 2007; WOLFF *et al*, 2007; RAKHA *et al*, 2007b).

3.3- Definição do câncer de mama de tipo basal

Para classificar os tumores como basais, adotamos os critérios de Rakha *et al* (2007b), que propuseram que a definição do fenótipo basal seja baseada apenas na expressão de CK basais (CK5 e/ou CK14), a despeito da expressão de outros marcadores.

TABELA 1- Anticorpos primários e diluições utilizados no estudo imunoistoquímico para avaliação do fenótipo basal

Anticorpo	Clone	Diluição	Fabricante	Recuperação Antigênica	Valores de Corte
RE	6F11	1: 1000	Novocastra, Newcastle, UK	*Steamer*/Citrato pH 6,0	< 1% (negativo)
RP	PGr312	1: 1000	Novocastra, Newcastle, UK	*Steamer*/Citrato pH6,0	< 1% (negativo)
HER2	CB11	1: 80	Novocastra, Newcastle, UK	Sem pré-tratamento	0 or 1+ (negativo)
CK5	XM26	1: 100	NeoMarkers, Freemont, CA, EUA	Panela de pressão/Citrato pH6,0	≥ 10% (positivo)
CK14	LL002	1: 400	Biogenex, San Ramon, CA, EUA	Panela de pressão/Citrato pH6,0	≥ 10% (positivo)
EGFR	31G7	1:200	Zymed, San Francisco, CA, EUA	Digestão Enzimática Proteinase K	≥ 10% (positivo)
p63	4A4	1: 2000	DAKO, Carpenteria, CA, EUA	Banho maria EDTA/TRIS pH9,0	≥ 10% (positivo)

Anticorpo	Clone	Diluição	Fabricante	Recuperação Antigênica	Valores de Corte
Caveolina	E-249	1:500	Epitomics Burlingame, CA, EUA	Panela de pressão/Citrato pH6,0	> 10% (positivo)
p53	D0-7	1:2000	DAKO, Carpenteria, CA, EUA	Panela de pressão/Citrato pH6,0	> 5 % (positivo)

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3.4- Avaliação da expressão de marcadores relacionados a células tronco cancerosas da mama

O estudo imunoistoquímico com os marcadores relacionados a células tronco cancerosas da mama ALDH1 e EZH2 foi realizado em lâminas sequenciais obtidas a partir do TMA (TAB.2).

A expressão de ALDH1 foi avaliada tanto no componente epitelial dos tumores, como no componente estromal (estroma tumoral). Para o componente epitelial, a marcação foi categorizada estimando-se a porcentagem (P) de células tumorais epiteliais mostrando a coloração citoplasmática característica (de 0% a 100%) e a intensidade da coloração (I) (1=fraca; 2=moderada; 3=forte). Como previamente publicado por outros autores (GINESTIER *et al*, 2002; GINESTIER *et al*, 2007; RESETKOVA *et al*, 2010), os resultados foram obtidos pela multiplicação da porcentagem de células positivas pela intensidade da marcação para gerar um *score* numérico, o *quick score* ($Q = P \times I$). Nós consideramos os casos com pontuação final igual a zero como negativos, e aqueles com pontuação final maior ou igual a um como positivos.

A expressão de ALDH1 no estroma tumoral foi classificada como ausente, fraca (<25% de coloração citoplasmática em células estromais), moderada (25%-75% de coloração citoplasmática em células estromais), ou forte (>75% de coloração citoplasmática em células estromais).

De acordo com critérios já publicados (KLEER *et al*, 2003), a expressão de EZH2 foi graduada através da avaliação da marcação nuclear em células epiteliais tumorais, como a seguir: negativo (*score*=1, ausência de coloração); fraca (*score*=2, <25% das células com coloração nuclear); moderada (*score*=3, 25-75%

das células com coloração nuclear); ou forte (*score*=4, >75% das células com coloração nuclear). Pontuação final de 3 ou 4 foi considerada expressão elevada de EZH2; pontuação final de 1 ou 2 foi considerada expressão baixa de EZH2.

TABELA 2 - Anticorpos primários, diluições, fabricantes, recuperação antigênica e valores de corte utilizados no estudo da expressão de marcadores relacionados a células tronco cancerosas da mama

Anticorpo	Clone	Diluição	Fabricante	Recuperação Antigênica	Valores de Corte
ALDH1	EP1932Y	1: 150	Epitomics, Burlingame, CA, EUA	PT-Link pH 9,0	*Score*=0 (negativo) Score > 1 (positivo)
EZH2	Policlonal	1: 2000	Zymed, San Francisco, CA, EUA	PT-Link pH 9,0	*Score*=0 (negativo) *Score*=1 or 2 (baixo) *Score*=3 or 4 (alto)

3.5- Avaliação da angiogênese e linfangiogênese

Para cada caso, todas as lâminas arquivadas coradas pelo método de H&E foram inicialmente revistas com o objetivo de selecionar aquela que tivesse melhor representação do carcinoma mamário invasor (região intratumoral), borda tumoral ou periferia do tumor (região periférica tumoral) e parênquima mamário não tumoral/normal (região de parênquima mamário adjacente ao tumor ou região peritumoral). A seguir, cortes histológicos de 4 μm de espessura foram obtidos a partir do bloco de parafina selecionado e os seguintes anticorpos foram aplicados: CD34, CD105, e D2-40 (TAB.3).

3.5.1- Determinação da densidade microvascular (DMV)

As lâminas referentes a todos os casos foram escaneadas através do *ScanScope® XT*, *Aperio Digital Pathology System*. Por meio de escaneamento, imagens digitalizadas de todas as lâminas foram obtidas, permitindo a análise morfométrica digital computadorizada de todos os casos selecionados. Para a análise destas imagens empregamos o sistema de patologia digital da *Aperio* (*software release* 10.2, 2009), que inclui o *Aperio ImageScope*^{TM}. O *ImageScope*^{TM} (*version* 10.02.02.2317) é o sistema de visualização de imagens digitais obtidas a partir do escaneamento das lâminas. Este sistema permite que as imagens digitais obtidas sejam analisadas através de algoritmos customizados. Para a avaliação da densidade microvascular aplicamos o algoritmo *Microvascular Density v1*, a partir do qual foram analisados os seguintes parâmetros: número de vasos, densidade microvascular, área endotelial total, área vascular total e área luminal total. A presença de invasão vascular angiolinfática também foi registrada.

3.5.2- Determinação da DMV sanguínea (DMVS)

Foram aplicados, em lâminas sequenciais contendo cortes histológicos com 4μm de espessura, os anticorpos dirigidos a células endoteliais CD34 e endoglina/CD105. Duas características foram observadas no estudo da angiogênese: a distribuição/localização de vasos sanguíneos no tumor e em sua periferia; e a DMVS total.

Primeiramente, lâminas coradas pelo CD34 e CD105 referentes a cada caso foram escaneadas. Em seguida, cada lâmina digital obtida foi avaliada por um patologista na magnificação de x20 para a divisão do espécime em três áreas: região intratumoral, região periférica tumoral e região peritumoral. Subsequentemente, em magnificação de x100, áreas com maior número de vasos distintos ("hot spots") foram identificadas e marcadas através de ferramentas de seleção milimetradas, empregando os critérios propostos por WEIDNER *et al*. (1991). Células endoteliais isoladas ou grupos delas, com ou sem lúmen, foram considerados como vasos individuais.

Esta seleção foi feita separadamente nas três áreas de cada espécime, com área total de análise de 6 mm^2 em cada região estudada. Após a seleção, o algoritmo *Microvascular Density v1* foi aplicado separadamente em cada região – intratumoral, tumoral periférica e peritumoral – permitindo que a DMVS (vasos/mm^2) de cada área fosse calculada. A soma das DMVS de cada área forneceu a DMVS total do espécime.

3.5.3- Determinação da DMV linfática (DMVL)

A avaliação da DMVL foi feita em lâminas sequenciais coradas pelo D2-40, anticorpo específico para o endotélio de vasos linfáticos. Células endoteliais isoladas ou grupos delas, com ou sem lúmen, foram considerados como vasos individuais, segundo as recomendações do First International Consensus on the Methodology of Lymphangiogenesis Quantification in Solid Human Tumors (VAN der AUWERA *et al,* 2006). Duas características foram observadas no estudo da linfangiogênese: a distribuição/localização de vasos linfáticos no tumor e em sua periferia; e a DMVL total. A maioria dos estudos prévios avaliou a DMVL contando vasos linfáticos em 3 a 5 *hot spots*, semelhante ao método empregado na determinação da DMVS.

Como estávamos interessados em avaliar a distribuição de linfáticos em todo o tumor, nós optamos por utilizar um método de análise modificado em que todos os vasos linfáticos presentes em um corte histológico do tumor são contados (MOHAMMED *et al*, 2009). Primeiramente, cada corte foi examinado em pequeno aumento e dividido em três áreas através de ferramentas de seleção milimetradas: área intratumoural (IT – dois terços centrais da massa tumoral; área análise de 34 mm^2), área periférica tumoral (PP – terço externo da massa tumoral; área de análise de 34 mm^2) e área peritumoral (PT – área de parênquima mamário adjacente que circunda o tumor; área de análise de 34 mm^2). Tal método está ilustrado na FIGURA 3. Após a seleção, o algoritmo *Microvascular Density v1* foi aplicado separadamente em cada região. Os vasos linfáticos foram contados em cada área e a DMVL (vasos/mm^2) foi obtida para cada uma delas. A soma das DMVL de cada área forneceu a DMVL total para cada espécime.

TABELA 3 - Anticorpos primários e diluições utilizadas no estudo da angiogênese (DMVS) e da linfangiogênese (DMVL)

Anticorpo	Clone	Diluição	Fabricante/País	Método de reativação antigênica
CD34	QBEnd10	1:200	Dakocitomation, Denmark	*Steamer*/Citrato pH6,0
Endoglina (CD105)	4G11	1:50	Novocastra, UK	Microondas/EDTA pH9,0
D2-40	D2-40	1:400	DAKO, Carpinteria, CA, EUA	Banho Maria EDTA/TRIS pH9,0

expressão de marcadores basais. Os testes de Mann Whitney, teste *T*, ANOVA e Kruskal Wallis foram empregados quando apropriado.

Para avaliar a associação da DMVS e DMVL com parâmetros clínicos-patológicos e expressão de marcadores do fenótipo basal, foi feita análise univariada utilizando o teste exato de Fisher ou o teste do qui-quadrado. As medidas de DMVS/DMVL total e em cada área estudada foram categorizadas em DMVS/DMVL elevada *versus* DMVS/DMVL baixa, de acordo com o valor das medianas.

Para verificar a associação das diferentes variáveis com a sobrevida das pacientes foi empregado o método de Kaplan-Meier. As curvas de sobrevida foram construídas e comparadas através dos testes de Log-rank e Wilcoxon.

Análise multivariada pelo modelo de regressão Cox foi realizada, englobando todas as variáveis estatisticamente significativas. Um valor de $p < 0.05$ foi considerado significativo.

RESULTADOS E DISCUSSÃO

Os resultados obtidos e discussão serão apresentados como artigos científicos submetidos a periódicos. Os manuscritos estão estruturados com base nas normas dos periódicos a que foram ou serão submetidos, e acompanhados dos respectivos comprovantes de aceite para publicação e/ou submissão.

Após os artigos, apresentaremos as conclusões gerais que atendem aos objetivos propostos, lista de Referência Bibliográficas referentes à Introdução e Anexos.

1- ARTIGO 1

Prognostic impact of the cancer stem cell related markers ALDH1 and EZH2 in triple negative and basal-like breast cancers

MARINA DE BROT, RAFAEL M. ROCHA, FERNANDO A. SOARES AND HELENICE GOBBI

Manuscrito aceito para publicação na Revista *Pathology* em 06 de Novembro de 2011

Pathology decision on MS PAT-D-11-00259R1

Pathology <journal@rcpa.edu.au>

To: Marina De Brot <debrot@gmail.com>

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Pathology journal@rcpa.edu.au via editorialmanager.com

06 Nov 2011

PAT-D-11-00259R1

Prognostic impact of the cancer stem cell related markers ALDH1 and EZH2 in triple negative and basal-like breast cancers

Dear Dr De Brot,

Thank you for revising the manuscript above for publication in Pathology. I am pleased to inform you that your manuscript has been accepted for publication without further revision and it will appear in the next available issue, most likely June 2012.

Proofs of your article will be e-mailed to you in PDF format by our publisher, Wolters Kluwer Health / Lippincott, Williams and Wilkins. Please check these proofs thoroughly, paying particular attention to figure legends, tables and references.

Please note that if proofs are not promptly returned, the Editor reserves the right to either delay publication until a subsequent issue, or to proceed to press without your corrections.

In the meantime, don't hesitate to contact the journal office if you have any queries (journal@rcpa.edu.au).

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Kind regards,

Professor Brett Delahunt

Editor

Patholog

2- ARTIGO 2

Microvessel density as determined by computerised image analysis of CD34 and CD105 expression correlates with poor outcome in triple-negative breast cancer

MARINA DE BROT, RAFAEL M. ROCHA, FERNANDO A. SOARES AND HELENICE GOBBI

Manuscrito submetido para publicação à revista Journal of Pathology em 21 de Maio de 2012

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Microvessel density as determined by computerised image analysis of CD34 and CD105 expression correlates with poor outcome in triple-negative breast cancer

Authors:

De Brot, Marina Rocha, Rafael Soares, Fernando Gobbi, Helenice

Date Submitted:

22-May-2012

3- ARTIGO 3

Objective evaluation of lymphatic and blood vessels in triple-negative breast cancer: a morphometric analysis with prognostic implications

MARINA DE BROT, RAFAEL M. ROCHA, FERNANDO A. SOARES AND HELENICE GOBBI

Primeiro rascunho de manuscrito a ser submetido para publicação futuramente

CONCLUSÕES

O presente estudo caracterizou a expressão dos marcadores relacionados a células tronco cancerosas da mama, ALDH1 e EZH2, em cânceres de mama triplo negativos basais e não basais, avaliando o potencial uso de ambos como indicadores prognósticos em pacientes com estes tipos de tumores mamários. Nossos resultados mostraram que a expressão de ALDH1 no componente epitelial dos tumores e a expressão de EZH2 não se correlacionou com nenhuma das variáveis clínico-patológicas estudadas. Por outro lado, a expressão de ALDH1 no componente estromal dos tumores se associou significativamente com melhor sobrevida global em pacientes com câncer de mama triplo negativo. Tal achado fortalece a ideia de que a interação tumor-estroma e o estroma tumoral possuem papel relevante na progressão das neoplasias malignas. Desta forma, demonstramos que o microambiente tumoral deve ser levado em consideração nos estudos de impacto prognóstico de marcadores relacionados a células tronco cancerosas no câncer de mama. Finalmente, alterações envolvendo o estroma tumoral oferecem novos alvos a serem explorados no tratamento de pacientes com cânceres de mama triplo negativos.

Quanto a topografia e distribuição de vasos sanguíneos e linfáticos em cânceres de mama triplo negativos e de tipo basal, nossos achados mostram que tanto os primeiros como os últimos localizam-se principalmente na região periférica dos tumores. Vasos sanguíneos estão distribuídos uniformemente tanto

no estroma interlobular quanto no estroma intralobular do parênquima mamário normal. Os vasos linfáticos, ao contrário, não estão presentes no estroma intralobular, embora sejam frequentemente observados no compartimento interlobular da mama.

Densidade microvascular sanguínea (DMVS) elevada, tanto DMVS total quanto DMVS em diferentes áreas tumorais, se correlacionou com diversas variáveis clínico-patológicas e pior evolução em carcinomas mamários triplo negativos. Além disso, carcinomas mamários de tipo basal apresentaram DMVS mais elevada em comparaçao a tumores triplo negativos não basais. Tais associações indicam que carcinomas mamários triplo negativos de comportamento biológico mais agressivo exibem DMVS elevada. Ainda, DMVS determinada pela expressão de CD105 é fator prognóstico independente em cânceres de mama triplo negativos, estando associada tanto com menor sobrevida global quanto com menor sobrevida livre de doença. Tal correlação reflete a importância da angiogênese, mais especificamente da neoangiogênese, no crescimento tumoral e desenvolvimento de metástases. Estes resultados ressaltam que a neovascularização tumoral está localizada primariamente na região periférica de tumores e que estes vasos tumorais neoformados podem ter papel essencial na progressão do câncer, representando vias de disseminação da doença.

Finalmente, densidade microvascular linfática (DMVL), medida indireta de linfangiogênese, não é indicador de prognóstico em pacientes com cânceres de mama triplo negativos. Não detectamos associações estatisticamente significativas entre DMVL, parâmetros clínico-patológicos, desenvolvimento de

metástases e sobrevida. Também não observamos correlação entre DMVL e DMVS, mostrando que tais processos são regulados por vias moleculares

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ANEXOS

ANEXO 1 - PRODUÇÃO CIENTÍFICA RELACIONADA À TESE

1- APRESENTAÇÕES EM CONGRESSOS E ENCONTROS

De Brot M, Rocha RM, Soares FA, Gobbi H. Prognostic impact of cancer stem cell markers ALDH1 and EZH2 in triple negative breast cancer. Histopathology 2010; 57 (suppl): 35.

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2- ARTIGOS COMPLETOS PUBLICADOS EM PERIÓDICOS INDEXADOS

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ANEXO 2 - PRODUÇÃO CIENTÍFICA NÃO RELACIONADA À TESE

1- APRESENTAÇÕES EM CONGRESSOS E ENCONTROS

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ANEXO 3 – CÓPIA DA ATA DE DEFESA DE TESE E DECLARAÇÃO DE APROVAÇÃO DA DEFESA DE TESE

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ATA DA DEFESA DE TESE DE DOUTORADO de **MARINA DE BROT ANDRADE,** nº de registro 2008713665. Às quatorze horas do dia **05 de junho de dois mil e doze**, reuniu-se na Faculdade de Medicina da UFMG, a Comissão Examinadora de defesa de tese, indicada pelo Colegiado do Programa de Pós-Graduação em Patologia da UFMG, para julgar, em exame final, o trabalho intitulado: **"EXPRESSÃO DE FATORES RELACIONADOS A CÉLULAS TRONCO CANCEROSAS E DE VASCULOGÊNESE EM CÂNCERES DE MAMA TRIPLO NEGATIVO E DE TIPO BASAL",** requisito final para a obtenção do grau de doutor em Patologia, pelo Programa de Pós-Graduação em Patologia da UFMG - Área de Concentração em Patologia Médica. Abrindo a sessão, a Presidente da Comissão, Profa. Helenice Gobbi, após dar a conhecer aos presentes o teor das normas regulamentares do trabalho final passou a palavra à candidata para apresentação do seu trabalho. Seguiu-se a arguição pelos examinadores com a respectiva defesa da candidata. Logo após, a Comissão se reuniu sem a presença da candidata e do público para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações:

Profa. Helenice Gobbi/Orientadora	Instituição: UFMG	Indicação: Aprovada
Prof. Dawidson Assis Gomes	Instituição: UFMG	Indicação: Aprovado
Profa. Alessandra Clarizia	Instituição: UNI-BH	Indicação: Aprovada
Prof. Rafael Malagoli Rocha	Instituição H. A.C. Camargo:	Indicação: APROVADA
Profa. Angela Logullo Waitzberg	Instituição: UNIFESP	Indicação: Aprovada

Pelas indicações, a candidata foi considerada Aprovada.

O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 05 de junho de 2012.

Profa. Helenice Gobbi Helenice Gobbi

Prof. Dawidson Assis Gomes Dawidson A Gomes

Profa. Alessandra Clarízia Clariz

Prof. Rafael Malagoli Rocha

Profa. Angela Logullo Waitzberg

Prof. Wagner Luiz Tafuri (Coordenador) Wagner Luiz Tafuri

Prof. Wagner Luiz Tafuri
Coordenador do Programa de
Pós-Graduação em Patologia
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Obs.: Este documento não terá validade sem a assinatura e carimbo do Coordenador.

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DECLARAÇÃO

A Comissão Examinadora, abaixo assinada, composta pelos professores doutores: Helenice Gobbi, Dawidson Assis Gomes, Alessandra Clarízia, Rafael Malagoli Rocha e Angela Logullo Waitzberg, aprovou a defesa da tese intitulada: **"EXPRESSÃO DE FATORES RELACIONADOS A CÉLULAS TRONCO CANCEROSAS E DE VASCULOGÊNESE EM CÂNCERES DE MAMA TRIPLO NEGATIVO E DE TIPO BASAL"**, apresentada pela doutoranda **MARINA DE BROT ANDRADE** para obtenção do título de doutora em Patologia, pelo Programa de Pós-Graduação em Patologia - Área de Concentração em Patologia Médica, da Universidade Federal de Minas Gerais, realizada em 05 de junho de 2012.

Profa. Helenice Gobbi
Orientadora

Prof. Dawidson Assis Gomes

Profa. Alessandra Clarízia

Prof. Rafael Malagoli Rocha

Profa. Angela Logullo Waitzberg

I hate writing,
I love having
written
Dorothy Parker

Pathology (June 2012) **44(4)**, pp. 303–312

ANATOMICAL PATHOLOGY

Prognostic impact of the cancer stem cell related markers ALDH1 and EZH2 in triple negative and basal-like breast cancers

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Summary

Aims: We assessed the expression of ALDH1 and EZH2, cancer stem cell (CSC) related markers, in triple negative and basal-like breast cancers, investigating their association with clinicopathological features and outcome.

Methods: Clinicopathological data were obtained from 140 cases of triple negative breast cancer. A tissue microarray was constructed and immunohistochemistry for ER, PR, HER2, ALDH1, EZH2, CK5, CK14, EGFR, p63, caveolin, and p53 was performed. Tumour cell and stromal expression of ALDH1 were evaluated. Multivariate analysis was conducted, including all significant variables.

Results: The majority of triple negative breast cancers were invasive ductal carcinomas of no special type (NST) (116/140). Tumour cells exhibited cytoplasmic expression of ALDH1 in 26 of 140 cases, while stromal expression was detected in 117 of 140 cases. Tumour cell expression did not correlate with any of the parameters. Conversely, stromal expression was associated with better overall survival (p=0.044). Assessment by Cox Regression Model showed a HR of 2.80 (HR = 1/0.357 = 2.80; 95%CI 0.178–0.714; p = 0.004) for breast cancer death when ALDH1 was not found in the stromal compartment of tumours, independent of age, histological type/grade, nodal status, stage, relapse, and expression of basal markers. High EZH2 expression was noted in 120 of 140 triple negative breast cancers and was not associated with other variables. Basal-like cancers comprised 75% (105/140) of triple negative breast cancers. Interestingly, we found association between EZH2 and CK14 expression (p = 0.041).

Conclusions: ALDH1 expression is frequent in tumour-associated stromal cells of triple negative breast cancer and is associated with better outcome. Tumour microenvironment should be considered when studying prognostic impact of CSCs in breast cancer.

Key words: ALDH1, basal-like breast cancer, breast neoplasms, EZH2, follow-up, immunohistochemistry, neoplastic stem cells, survival, triple negative breast cancer.

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BACKGROUND

Heterogeneity is an acknowledged phenomenon in solid tumours, as they are composed of a range of phenotypically diverse malignant cells.[1–3] Indeed, the concept that cancers contain a subset of cells similar to epithelial stem cells was proposed several years ago, along with emerging studies suggesting that some malignancies may obey a cancer cell hierarchy similar to that observed in normal tissues.[4–6] According to the controversial 'cancer stem cell hypothesis', cancer cells are hierarchically organised, and transformation from their own progenitor stem cells results in carcinogenesis, tumour growth and spread.[7–9] It is postulated that cancer stem cells (CSCs) possess self-renewal capacity, slow cell division, and the ability to generate a differentiated progeny, paralleling the properties of normal stem cells.[6,10,11] This subpopulation of cancer cells correspond to a very small percentage of cells in solid tumours which would give rise to the diversity of differentiated cells that comprise the bulk of the tumour.[6,11,12] Such cells were also thought to exhibit distinct properties compared to the rest of the cells in a given tumour.[13] Moreover, CSCs would hold selective resistance to radio- and chemotherapy.[4,10,14] Relapse of cancer and treatment failure could consequently echo the intrinsic quiescence and drug resistance of CSCs.[15–17]

Over the last few years, CSCs have been identified in different human cancers including primary and metastatic breast carcinomas, in addition to a number of established breast cancer cell lines.[9,11,18] Mammary stem cells and breast CSCs have been purified in *in vitro* culture systems by cell surface antigen identification.[18,19] Although considerable progress has been made towards identification of human mammary stem cells, the exact phenotype of these cells still remains poorly defined.[5,9,11,20] The CD44 cell-surface marker has been used to identify putative cancer stem cells in breast cancer. It has been shown in xenograft models that a CD44+/CD24– cell population meets the criteria for CSCs. Flow cytometry, followed by cell sorting, developing mammospheres and injecting a small number of these CD44+/CD24– breast tumour cells into nude mice, resulted in tumour growth.[18,21] Some studies have observed that ER+ breast cancer cell lines lack a CD44+/CD24– population of cells and that these cells appear to be restricted to mesenchymal/triple negative cell lines.[21,22] The CD44+/CD24– phenotype has also been shown to be enriched in basal-like breast tumours.[23] In addition, reports have confirmed poor prognosis of CD44+/CD24– expressing tumours.[21,23] Other cell surface markers have been linked to a CSC phenotype and were found in various subsets of breast cancer cells, including alpha 6 integrin, CD133, and beta 1 integrin/CD29.[21,24]

It is probable that CSCs have a phenotype defined by the cell of origin (stem cells or early progenitor cells) and by the oncogenic events that contribute to transformation.[25] A candidate marker of CSCs is aldehyde dehydrogenase 1 (ALDH1), an enzyme responsible for oxidising intracellular aldehydes and crucial during embryogenesis.[26] ALDH1 has been reported to

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have a role in early differentiation of stem cells, converting retinol to retinoic acid.[27] Increased ALDH1 activity has been demonstrated in human haematopoietic stem/progenitor cells and in stem cell populations in multiple myeloma.[28] In a study by Ginestier *et al.*,[29] the Aldefluor assay was utilised to show that cells with ALDH1 enzymatic activity isolated from normal human breast have phenotypic and functional properties of mammary stem cells. Interestingly, a small number of Aldefluor positive cells were capable of generating tumours in animal models.[29] They have also demonstrated that both normal and malignant human mammary stem cells may be identified *in situ* by immunohistochemistry.[29] In prior studies of human breast cancers, ALDH1 expression has been related to poor clinical outcome, absence of oestrogen and progesterone receptors, and expression of basal cytokeratins.[29–31] Moreover, some studies have shown that expression of ALDH1 is not restricted to epithelial cells but it has also been observed in stromal cells.[29,31,32] A study by Resetkova *et al.*[31] showed that not only has ALDH1 expression been identified in the tumoural stroma, it was associated with better clinical outcome in two cohorts of triple negative breast cancers.

Recently, it has been proposed that an aggressive secondary cancer stem cell population, the so-called 'tumour-initiating cancer cells', arises from a primary cancer stem cell population through acquisition of additional genetic mutations and drives cancer progression.[33] Chang *et al.*[33] reported that over-expression of EZH2, essential in stem cell self-renewal, is linked to the regulation and growth of breast tumour initiating cells. EZH2 is a Polycomb group (PcG) protein homologous to Drosophila Enhancer of Zeste which has been associated with breast cancer aggressiveness and poor outcome.[34,35] Novel research by Chang *et al.* identified that EZH2 expression mediates down-regulation of DNA damage repair. They have also tested an anti-cancer drug, sorafenib, which eliminates EZH2-promoted breast cancer stem cells.[33] These findings shed light on the value of EZH2 as a target for breast cancer therapy.

There is limited literature on expression of stem cell related markers in triple negative breast cancer, a subgroup that lacks expression of oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2).[36] Triple negative breast cancer shares morphological and genetic abnormalities with basal-like breast cancer, a subgroup of breast cancer primarily defined by gene-expression profiling.[36,37] Basal-like breast cancer has been said to express cytokeratins typical of basal cells and other non-luminal (basal) genes.[38,39] Although heterogeneous, triple negative and basal-like breast cancers typically occur in younger women and are associated with a range of adverse biological features including high grade, high mitotic count and p53 positivity.[40,41] While responsive to conventional chemotherapy, triple negative and basal-like breast cancers tend to relapse and metastasise early and have a worse prognosis than other tumour subtypes.[39,42] Thus, both subgroups represent high risk cancers that lack the benefit of specific treatment and for which we have no known targeted agents.[39,43] A recent preclinical study has shown that a multiple tyrosine kinase inhibitor, dasatinib, has a more potent antitumour effect on triple negative/basal-like breast cancer cells with a significant loss of putative cancer stem cell population.[44] Here, we sought to assess the potential utility of ALDH1 and EZH2 as prognostic markers in triple negative and basal-like cancers. We examine the expression pattern of ALDH1 and EZH2, investigating their association with clinical parameters, pathological variables, and basal-like markers. Furthermore, we describe the presence and significance of ALDH1 positive cells in the stroma of triple negative invasive breast carcinomas.

METHODS

Cases for this study were drawn from 2235 patients submitted to surgical treatment from 1985 to 2006 at the A. C. Camargo Cancer Hospital (São Paulo, Brazil), and at the Federal University of Minas Gerais (UFMG) Clinic Hospital (Belo Horizonte, Brazil). Cases were selected based on the availability of clinical data and paraffin blocks; original histological diagnosis of invasive breast carcinoma; and a previous pathology report showing negativity for ER, PR and HER2 (triple negative cancer). Formalin fixed, paraffin embedded (FFPE) tumours of 140 patients were obtained from the archives of the Pathology Department. Lack of expression for ER, PR, and HER2 was confirmed by a new immunohistochemical study.

This study was approved by the Research Ethics Committee of our institution under protocol number ETIC 466/06.

Clinicopathological data

Patients' clinical history and tumour characteristics were retrieved from the medical records and correlated with ALDH1 and EZH2 expression. The assessment included date of diagnosis; age at initial diagnosis; race; menopausal status; family history of breast cancer; nodal status; and pathological stage at diagnosis [2009 American Joint Committee on Cancer (AJCC) TNM Staging System]. Survival data were comprised of survival time, disease-free interval, date and type of relapse, development of distant metastasis, date and cause of death. Follow-up data were available for 132 patients, and the follow-up period ranged from 1 to 148 months (median 39 months; mean 51 months).

All original haematoxylin and eosin (H&E) stained sections of representative tumour blocks were reviewed in detail and histological type and grade were re-evaluated by a single pathologist (MDB).

Our triple negative cohort consisted of 140 women (mean age 55 years; range 32–86 years) with invasive breast carcinoma (116 invasive ductal carcinomas of no special type, 6 carcinomas with medullary features, 5 metaplastic carcinomas, 4 micropapillary carcinomas, 4 papillary carcinomas, 3 apocrine carcinomas, 1 medullary carcinoma, and 1 adenoid cystic carcinoma).

Construction of the tissue microarray

The tissue microarray (TMA) was constructed by extracting 1.0 mm diameter cores of histologically confirmed invasive breast carcinoma from the original paraffin blocks using a tissue core extractor (Beecher Instruments, USA) and re-embedding these cores into a gridded paraffin block. One such recipient paraffin block was constructed, containing two tissue cores from each selected tumour. Control tissue cores from normal liver were placed at two ends of this recipient paraffin block for orientation. Slides containing sections of a positive breast tumour were included in all batches as external control. Two cores were taken from tumour dense areas from the original biopsy block from each of the 140 patients. Each tissue core was assigned a unique TMA location, and was entered into an Excel database. After construction, 4 μm tissue sections were cut using a microtome and adhered to Fisher SuperFrost Plus glass slides.

Immunohistochemistry and scoring

Sequential slides from the TMA were deparaffinised in xylene, rehydrated in graded alcohol and stained with commercially available antibodies: ER (Novocastra, UK), PR (Novocastra), HER2 (Novocastra), ALDH1 (Epitomics, USA), EZH2 (Zymed, USA), cytokeratin 5 (NeoMarkers, USA), cytokeratin 14 (Biogenex, USA), EGFR (Zymed), p63 (Dako, USA), caveolin (Epitomics), and p53 (Dako). Antigen retrieval varied according to the primary antibody following the supplier's specifications. Negative controls were produced by omitting the primary antibody. All primary antibodies used, clones, dilutions, and suppliers are shown in Table 1. All immunohistochemistry (IHC) procedures were performed on a Link 48 Autostainer (Dako) using the Flex Plus visualisation system as per manufacturer's specifications. Stains were visualised using diaminobenzidine (Dako) and haematoxylin (Dako) counterstain.

Firstly, slides were stained for ER, PR, and HER2 to confirm the triple negative diagnosis. Then, basal cytokeratins (cytokeratins 5 and 14) and other

Table 1 Primary antibodies, dilutions, sources, antigen retrieval, and cut-off values used in the immunohistochemical study

Antibody	Clone	Dilution	Source	Antigen retrieval method and duration	Cut-off values
ER	6F11	1:1000	Novocastra, UK	Steamer/citrate pH 6.0; steamer 25 min; cooling 30 min	<1% (negative)
PR	PGr312	1:1000	Novocastra, UK	Steamer/citrate pH 6.0; steamer 25 min; cooling 30 min	<1% (negative)
HER2	CB11	1:80	Novocastra, UK	No pre-treatment	0 or 1+ (negative)
ALDH1	EP1932Y	1:150	Epitomics, USA	PT-Link pH 9.0; 15 min	Epithelial: quick score; Stromal: percentage and intensity*
EZH2	Polyclonal	1:2000	Zymed, USA	PT-Link pH 9.0; 15 min	Score = 0 (negative); score = 1 or 2 (low); score = 3 or 4 (high)†
CK5	XM26	1:100	NeoMarkers, USA	Pressure pan/citrate pH 6.0; pressure pan 25 min; cooling 30 min	≥10% (positive)
CK14	LL002	1:400	Biogenex, USA	Pressure pan/citrate pH 6.0; pressure pan 25 min; cooling 30 min	≥10% (positive)
EGFR	31G7	1:200	Zymed, USA	Enzymatic digestion; proteinase K; 7 min	≥10% (positive)
p63	4A4	1:2000	Dako, USA	Water bath EDTA/TRIS pH 9.0; 25 min	≥10% (positive)
Caveolin	E-249	1:500	Epitomics, USA	Pressure pan/citrate pH 6.0; pressure pan 25 min; cooling 30 min	>10% (positive)
p53	D0-7	1:2000	Dako, USA	Pressure pan/citrate pH 6.0; pressure pan 25 min; cooling 30 min	>5% (positive)

* ALDH1 expression was evaluated in the epithelial component of tumours as well as the tumoural stroma. For the epithelial component, immunoreactivity was categorised by quick score ($Q = Percentage \times Intensity$). We considered cases with a score 0 to be negative, and those with a score >1 to be positive. Stromal ALDH1 expression was categorised as none or weak (<25% of cytoplasmic staining in stromal cells), moderate (25–75% of cytoplasmic staining in stromal cells), and strong (>75% of cytoplasmic staining in stromal cells).
† EZH2 was assessed by scoring nuclear staining in epithelial tumour cells as negative (score = 1, no staining); weak (score = 2, <25% of nuclei staining); moderate (score = 3, 25–75% of nuclei staining, any intensity); and strong (score = 4, >75% of nuclei staining, any intensity). Scores 3 and 4 were considered high EZH2; scores 1 and 2 were defined as low EZH2.

markers such as EGFR, p63, caveolin, and p53 were applied. Immunoreactivity was assessed according to criteria published in previous reports;[45–48] cut-off values used in the current study are shown in Table 1.

ALDH1 expression was evaluated in the epithelial component of tumours as well as the tumoural stroma. For the epithelial component, immunoreactivity was categorised by estimating the percentage (P) of tumour cells showing characteristic cytoplasmic staining (from undetectable level or 10%, to homogeneous staining or 100%) and by recording the intensity (I) of staining (1, weak staining; 2, moderate staining; 3, strong staining). As previously published by other groups, results were scored by multiplying the percentage of positive cells by the intensity to generate a numerical score, the quick score ($Q = P \times I$).[29,31,49] Due to the low number of positive cases, the statistical analysis was performed using a negative (score 0) and positive (score >1) cut-off. Following published criteria, stromal ALDH1 expression was classified as none, weak (<25% of cytoplasmic staining in stromal cells), moderate (25–75% of cytoplasmic staining), and strong (>75% of cytoplasmic staining).[31,49]

Following published criteria, expression of EZH2 was assessed by scoring nuclear staining in epithelial tumour cells as negative (score = 1, no staining); weak (score = 2, <25% of nuclei staining); moderate (score = 3, 25–75% of nuclei staining, any intensity); and strong (score = 4, >75% of nuclei staining, any intensity).[35] Scores 3 and 4 were considered high EZH2; scores 1 and 2 were defined as low EZH2.[35]

Given that two cores were analysed from each tumour, each core was scored individually and the mean of two readings was calculated.

Definition of basal phenotype

We considered basal-like cancers to be those tumours showing expression of basal cytokeratins (cytokeratin 5 and/or cytokeratin 14) in our series of triple negative invasive breast carcinomas.[47]

Statistical analysis

We examined the association between expression of basal markers and other prognostic indicators via chi-squared test and Fisher's exact test. Data were examined by Statistical Package for Social Sciences v.17.0 (SPSS Institute, USA) software. Univariate analysis was performed to analyse the correlation between ALDH1, EZH2, and clinical, pathological, and immunohistochemical variables. Mann–Whitney test, *t*-test, ANOVA and Kruskal–Wallis test were carried out as appropriate. Multivariate analysis using the Cox proportional hazards model was performed to assess the independent prognostic significance of ALDH1 and EZH2. Proportional hazards model of statistically significant covariates was developed by removing non-significant parameters in a step-wise manner; regression analysis included expression of ALDH1 (epithelial cell expression and stromal expression), EZH2, basal markers, age, menopausal status, histological type and tumour grade, nodal status, pathological stage, and relapse. A p value of <0.05 was considered significant. Survival analyses used the Kaplan-Meier method and survival curves were compared using the log rank test and the Wilcoxon rank test.

RESULTS

In the current study, 2235 cases of invasive breast carcinoma submitted to immunohistochemistry during the period of investigation were informative for the markers oestrogen receptor, progesterone receptor, and HER2. Of these informative cases, 252 of 2235 (11.3%) had a previous pathology report showing negativity for ER, PR and HER2 (triple negative cancer). Among these 252 cases, 106 were not included in our cohort. For 77 of 106 cases, suitable archival FFPE tumour blocks were not available for TMA construction; 29 of 106 tumours were excluded because of the presence of artefacts of autolysis; and 20 of 106 cases had been classified as triple-negative based on a different threshold used to define ER and PR negativity. Because we adopted the ER scoring criteria validated by Harvey *et al.*,[45] cases with as few as 1% of tumour cells showing weak immunostaining nuclear signal (i.e., the definition of ER positivity) were not included in our cohort. After excluding the uninformative TMA cores, 140 of 2235 (6.3%) tumours were available for immunohistochemical analysis, had their triple negative phenotype confirmed and formed the basis of this study.

Triple negative breast cancer: clinicopathological and immunohistochemical characteristics

A positive family history was present in 27 of 140 patients (19.2%). Pre-menopausal women represented 25.0% (35/140 patients) of cases, and 5.7% (8/140) of these were younger than 35 years old. Regarding race, 70 of 140 patients (50.0%) were reportedly white, five of 140 patients (3.6%) were black, 25 of 140 patients (17.8%) were brown-skinned (multiethnic), and three of 140 patients (2.1%) were of Asian origin.

The majority of triple negative tumours were invasive ductal carcinomas of no special type (116/140; 82.8%) of high

Table 4 Multivariate analysis using the Cox proportional hazards model

	Adjustment of co-variates			Cox final model		
Parameter	HR	*p* value	95%CI	HR	*p* value	95%CI
Age						
≤35 years	1.00			1.00		
>35 years	0.295	0.006	0.123–0.704	0.501	0.144	0.198–1.267
Histological type						
Special types	1.00				Not selected ($p > 0.20$)	
IDC NST	1.846	0.398	0.446–7.641			
Histological grade						
Low	1.00				Not selected ($p > 0.20$)	
High	1.834	0.584	0.695–2.050			
Nodal status						
Positive	1.00			1.00		
Negative	0.576	0.090	0.305–1.089	0.664	0.330	0.661–3.424
Relapse						
Present	1.00			1.00		
Absent	0.055	0.000	0.021–0.139	0.092	0.023	0.012–0.718
Stage						
I and II	1.00			1.00		
III and IV	1.656	0.015	1.102–2.488	1.517	0.072	0.963–2.388
Stromal ALDH1						
Negative	1.00			1.00		
Positive	0.510	0.050	0.260–0.999	0.357	0.004	0.178–0.714
EZH2						
Negative	1.00				Not selected ($p > 0.20$)	
Positive	1.089	0.834	0.489–2.425			
CK5						
Positive	1.00				Not selected ($p > 0.20$)	
Negative	0.747	0.412	0.371–2.701			
CK14						
Positive	1.00				Not selected ($p > 0.20$)	
Negative	0.725	0.283	0.404–1.304			
EGFR						
Positive	1.00				Not selected ($p > 0.20$)	
Negative	0.766	0.352	0.437–1.342			

Cox regression analysis of co-variates was developed by removing non-significant parameters ($p > 0.20$) in a step-wise manner; the final Cox hazard model shows that the prognostic value of stromal ALDH1 is independent of age, histological type/grade, nodal status, pathological stage, and expression of EZH2 and basal-like markers. A final p value of <0.05 was considered significant.

In our series, survival analysis showed no correlation between ALDH1 expression in tumour cells and disease-free survival or overall survival. Tumour cell expression was not associated with any of the parameters examined. The role of ALDH1 as a prognostic or predictive marker is still undetermined. Ginestier *et al.*[29] examined 577 patients from two TMA cohorts and verified that expression of ALDH1 was correlated with poor clinical outcome. They also showed that ALDH1 expression was strongly associated with absence of ER and PR expression. Alternatively, Neumeister *et al.*[51] demonstrated that ALDH1 expression alone, measured by automated quantitative analysis (AQUA), did not significantly predict outcome in a cohort of 626 patients. However, these studies did not focus solely on triple negative cancer and did not evaluate stromal expression of ALDH1. In the study by Resetkova *et al.*[31] tumour cell ALDH1 expression correlated only with basal-like and HER2 tumour types but not with other clinical parameters, in a series of 245 invasive breast carcinomas. In contrast, high degree of stromal expression was significantly associated with better disease-free survival in their triple negative cohort (45 TMA cases and whole tissue sections from 40 patients). Similarly, we separately analysed the stromal ALDH1 expression and we found that positive (high/moderate) ALDH1 stromal expression was significantly associated with the best outcome in our triple negative cohort of 140 patients. In a multivariate Cox regression analysis, ALDH1 stromal expression was an independent predictor of prognosis.

While previous published data have revealed an association between expression of ALDH1 and basal cytokeratins in breast cancer[29,31] we did not demonstrate this correlation in our triple negative cohort. We examined a number of basal-like markers (CK5, CK14, EGFR, caveolin, and p63) and none of them correlated significantly with ALDH1 expression. Of interest, we did see expression of ALDH1 in our series of 105 basal-like cancers, both in tumour cells (21/105 cases; 20.0%) and tumoural stroma (87/105 cases; 82.9%).

ALDH1 is an enzyme responsible for oxidation of intracellular aldehydes and it is also engaged in the synthesis of retinoids.[36] Retinoic acid has been described to inhibit the growth of several breast cancer cell lines in culture and to reduce breast tumour growth in animal models.[52]

Expression of ALDH1 in stromal fibroblasts of the breast had already been described by Ginestier *et al.*[29] Resetkova and colleagues evaluated the significance of this observation.[31] Likewise, we report an association of stromal ALDH1 with better outcome, indicating that the stromal compartment of tumours could be as relevant as the epithelial compartment in establishing disease prognosis. In addition, this finding suggests that interactions between tumour cells and cells with stem cell properties in the tumour-associated stroma might have a tumour growth-suppressing effect.

More recently, Chang *et al.* have shown that the protein EZH2 is heavily expressed in the tumour microenvironment.[33] Oxygen-starved portions of the tumour stimulate over-expression of EZH2, improving self-renewal, survival and growth of breast CSCs and thus contributing to breast cancer progression. They have also reported a clinical trial drug (sorafenib) that eliminates EZH2-promoted breast cancer stem cells. Although we failed to demonstrate a correlation between expression of EZH2 and clinical parameters, high EZH2 expression was frequently seen in our triple negative cohort (119/140 tumours; 85.0%), as well as in our series of basal-like cancers (91/105 tumours; 86.6%). Interestingly, expression of EZH2 significantly correlated with the expression of CK14 (39/91 tumours; 42.8%; $p = 0.041$). Our results raise the possibility that patients with triple negative cancer and a basal-like phenotype could specially benefit from this novel anti-cancer drug.

CONCLUSIONS

Our study characterised the expression of cancer stem cell related markers ALDH1 and EZH2 in triple negative and basal-like tumours, examining the utility of both of them as prognostic markers in patients with triple negative cancer.

Our results showed that tumour cell expression of ALDH1 did not correlate with nodal status, outcome, or expression of basal markers. Nonetheless, stromal expression of ALDH1 was significantly associated with better overall survival in our triple negative cohort. Even though additional confirmatory studies are required, our work supports the view that the tumour-associated stroma is an important co-conspirator rather than a passive bystander in breast cancer progression. Moreover, tumour microenvironment should be taken into account when determining the prognostic impact of stem/progenitor cells in human breast cancer. Finally, alterations within the stroma can offer novel avenues for cancer treatment.

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Microvessel density as determined by computerised image analysis of CD34 and CD105 expression correlates with poor outcome in triple-negative breast cancer

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Microvessel density as determined by computerised image analysis of CD34 and CD105 expression correlates with poor outcome in triple-negative breast cancer

Short Title: Prognostic impact of microvessel density in triple-negative breast cancer

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ABSTRACT

Triple-negative and basal-like breast cancers tend to relapse and metastasise early with a distinct pattern of dissemination. Although the occurrence of metastasis is related to angiogenesis, literature on angiogenic characteristics of these tumours is limited.

We examined blood vessels topography and microvessel density (MVD) in triple-negative breast cancer (TNC), investigating their association with clinicopathological criteria, basal phenotype and prognosis. Sections from 133 TNCs were stained with CD34 and CD105. Total MVD and MVD in three areas (intratumoural, peripheral tumoural, peritumoural) were assessed using a computerised image analyser. Blood vessels were located largely in the peripheral area of tumours and TNCs with a basal phenotype showed higher MVDs (p=0.017). Tumours with high total CD34 MVD and CD34 MVD at the peritumoural area (PT-MVD) were significantly associated with visceral metastasis (p=0.006; p=0.019), shorter overall survival (OS; p=0.003; p=0.016) and disease-free interval (DFI, p=0.040; p=0.007). Also, high CD34 PT-MVD was associated with stage (p=0.028) and lymph node (LN) metastasis (p=0.048). While high CD105 MVD at the peripheral area (PP-MVD) was associated with distant metastasis (p=0.031), CD105 PT-MVD was correlated with vascular invasion (p=0.015). High CD105 PP-MVD predicted poorer OS and DFI (p=0.001). In multivariate analysis for OS, total CD34 MVD (HR=2.20; 1.11-4.35; p=0.023) and CD105 PP-MVD (HR=2.78; 1.41-5.49; p= 0.003) retained significance after adjustment for stage, LN *status* and distant metastasis. For DFI, CD34 PT-MVD (HR=1.99; 1.03-3.87; p=0.040) and CD105 PP-MVD (HR=2.90; 1.54-5.48; p=0.001) showed significance in multivariate analysis. Our findings demonstrate significant associations between MVD in different tumoural areas and various parameters in triple-negative breast tumours, indicating that TNCs with aggressive features have higher MVDs. Also, CD105 MVD is a strong, independent prognostic factor in TNC. Neovessels are mainly located in the peripheral area

of TNCs and may have a critical role in tumour progression by representing means for disease dissemination.

KEYWORDS

triple-negative breast cancer; basal-like breast cancer; blood vessels; microvessel density; computerised image analysis; metastasis; survival; prognosis

INTRODUCTION

Triple-negative breast cancer (TNC) – defined by the lack of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type 2 (HER2) expression – is known for its distinguishable epidemiological, clinical and biological features [1-4]. TNC shares many characteristics with the so-called basal-like breast cancer (BLC), a subgroup of tumours primarily defined by gene-expression profiling that often express basal cytokeratins, epidermal growth factor receptor (EGFR) and p53 [5-12]. Although heterogeneous, TNC and BLC are typically characterised by unfavourable outcome, as they are associated with a range of adverse biological features including high histological grade, presence of metaplastic elements and atypical medullary features [4,5,7,11-15]. Additionally, both present higher rates of recurrence and a distinct pattern of spread with an increased propensity for haematogenous metastasis [15-17].

Several studies have recognized that the occurrence of metastasis is related to angiogenesis, a complex process of forming new blood vessels from existing vascular networks, which allow tumours to initiate recruitment of their own blood supply [18-20]. This is a fundamental event for tumour growth, invasion and metastasis in many tumour types, including breast cancer [20-22]. The acknowledgment that disease progression is dependent on angiogenesis has prompted the investigation of its prognostic significance in breast cancer. This process has been often assessed directly by counting blood vessels in tumour sections stained with vascular markers such as CD34 or CD31; i.e., measurement of microvessel density (MVD) [22-27]. Also, it has been demonstrated that computer-aided image analysis is a good and objective technique for MVD evaluation [27-29].

Of interest, anti-CD105 antibody was found to bind preferentially to activated endothelial cells in vitro [30]. CD105 (endoglin), a component of the transforming growth factor-β receptor (TGF-β) complex, is overexpressed in proliferating endothelial cells of tumour

vessels and prevents TGF-β1-mediated inhibition of endothelial cell proliferation [31,32]. Expression of CD105 has been associated with poor prognosis in breast carcinomas [33,34]. Also, anti-CD105 radiolabeled monoclonal antibodies (mAbs) have been developed for cancer diagnosis and treatment [35-39].

Intratumoural MVD has been considered an indicator of survival in node-positive and node-negative breast cancer [40,41]. High MVD has also been associated with features of unfavourable outcome, such as larger tumour size and poor differentiation [42,43]. Furthermore, it has been recently demonstrated that tumours with high MVD are significantly associated with higher grade, lymph node metastasis and negativity for hormonal receptors [44]. Data regarding MVD in triple-negative and basal-like breast cancers are limited. A work by Li *et al* showed that high MVD was correlated with worse survival in triple-negative breast cancer patients. Nevertheless, MVD did not retain its significance as an independent prognostic factor by multivariate analysis [45]. More recently, Mohammed and colleagues reported that lymph node negative basal and triple-negative carcinomas have a significantly higher MVD than lymph node negative non-basal and non-triple-negative carcinomas [46], suggesting that such groups may preferentially benefit from anti-angiogenic therapy [47-50]. Studies to enhance our understanding of TNC and BLC are necessary to improve current management, and ultimately outcomes, of patients with these types of breast cancer.

Here we sought to study, in detail, the topography and characteristics of blood vessels in TNC and to examine their association with clinicopathological criteria, basal-like phenotype and prognosis in a series of triple-negative breast carcinomas with follow-up.

METHODS

Patients with a documented history of triple-negative breast cancer were identified from 2235 cases of invasive breast carcinomas submitted to surgical treatment from 1985 to 2006 at the A.C. Camargo Cancer Hospital (São Paulo, Brazil), and at the Federal University of Minas

Gerais Clinic Hospital (Belo Horizonte, Brazil). This study was approved by the Research Ethics Committee of our institution under protocol number ETIC 466/06, on March 21st, 2007.

Patients and specimens

Cases were selected based on the availability of archival material; original diagnosis of invasive breast carcinoma; and a previous pathology report showing negativity for ER, PR and HER2 (triple-negative breast cancer). Amongst 2235 cases informative for the markers ER, PR and HER2, 252/2235 (11.3%) had a previous pathology report showing the triple-negative immunophenotype. Of these 252 cases, 133 had archival formalin-fixed, paraffin-embedded (FFPE) tumour blocks suitable for analysis and formed the basis of this study. Lack of expression for ER, PR, and HER2 was confirmed by a new immunohistochemical analysis.

Clinicopathological data

Patients' clinical history and tumour characteristics were obtained from medical records. Original hematoxylin-eosin stained sections representative of tumour blocks were reviewed and histological type and grade were re-evaluated by a single pathologist (M. D. B.). Tumours were graded according to a modified Bloom–Richardson scoring system [51,52]. Our series consisted of 133 women with TNC (106 invasive ductal carcinomas of no special type [IDC NST], 5 carcinomas with medullary features, 6 metaplastic carcinomas, 6 micropapillary carcinomas, 5 papillary carcinomas, 3 apocrine carcinomas, 1 medullary carcinoma and 1 adenoid cystic carcinoma). The median age at time of diagnosis was 55 years (range, 32–86 years) and 80 (60.2%) patients had positive axillary lymph nodes (LN). The main types of adjuvant therapy were chemotherapy (8%), radiotherapy (7%), or both (52%). Other clinicopathological characteristics are summarised in Table 1.

Construction of the tissue microarray

A tissue microarray (TMA) was constructed by extracting 1.0 mm diameter cores of histologically confirmed invasive breast carcinomas from the original paraffin blocks using a tissue core extractor (Beecher Instruments, Silver Springs, MD) and re-embedding these cores into a gridded paraffin receptor block. Two cores were taken from tumour dense areas for each one of the 133 cases.

Immunohistochemistry and Scoring

Sequential slides from the TMA were deparaffinized in xylene, rehydrated in graded alcohol and stained with commercially available antibodies: ER, PR, HER2, cytokeratin 5 (CK5), cytokeratin 14 (CK14), EGFR, p63 and p53. Antigen retrieval varied according to the primary antibody following supplier's specifications. Negative controls were produced by omitting the primary antibody. Immunohistochemistry (IHC) was performed on a Link 48 Autostainer (DAKO, Carpinteria, CA, USA) using the Flex Plus visualization system per manufacturer's specifications. Stains were visualised using diaminobenzidine and hematoxylin counterstain provided by DAKO, Carpinteria, CA, USA. Immunoreactivity was assessed according to previously published criteria [53-55]. Primary antibodies, clones, dilutions, suppliers and cutoff values are shown in Table 2.

Definition of basal phenotype

Basal-like breast cancers were defined by the expression of basal cytokeratins (cytokeratin 5 and/or cytokeratin 14) in our series of TNCs [55].

Assessment of angiogenic characteristics

For visualisation of blood vessels, 4µm-thick whole tissue sections from each specimen were stained with CD34 and CD105 and specifications are shown in Table 3. IHC procedure was performed on a Link 48 Autostainer as described previously.

Two characteristics were noted for blood vessels: (1) distribution/ location within and around tumours; (2) MVD, a surrogate marker of angiogenesis.

Assessment of MVD was conducted in sections stained with CD34 and CD105 according to Weidner *et al* using a computerised image analyser [56,57]. First, slides were scanned with *ScanScope® XT- Aperio Digital Pathology System*, which creates high-resolution digital slides by whole slide scanning [58]. Using *Aperio ImageScopeTM*, each digital slide was examined by a pathologist (M.D.B) at low magnification (x20) and divided into three areas (Fig. 1): the central core of the tumour (intratumoural area; IT); the outer 1/3 of the tumour tissue (peripheral tumoural area; PP); and normal breast parenchyma surrounding the tumour (peritumoural area; PT). By screening the whole section at 40x magnification, 3 to 5 of the most vascularised areas (the so called "hot spots") were identified and selected with millimetered drawing tools in each area (IT, PP, PT - surface area of 6.0 mm^2 each). Selections were made without knowledge of the patient's clinical outcome. Subsequently, each area was analysed separately by applying a microvessel density analysis algorithm (*Microvessel Density v1, Aperio Digital Pathology System*). This method provided the MVD in the peritumoural area (PT-MVD), in the peripheral tumoural area (PP-MVD) and in the intratumoural area (IT-MVD). Total MVD for each specimen was the sum of MVDs in the three areas examined. Other parameters measured in each area are illustrated in Table 4. Microvessel analysis algorithm performance was controlled by a set of input parameters: maximum vessel area and vessel wall thickness threshold (large vessels with tunica media are excluded); region joining parameter (stained endothelial cell clusters separate from adjacent vessels are joined into a single microvessel, even in the absence of vessel lumen); endothelial stain; and background stain. A dual-level thresholding process screened out non-specific staining, when present.

Vascular invasion (VI) was identified when tumour clusters were detected within CD34- or CD105-positive vessels.

Statistical analysis

Statistical analysis was performed using the software package SPSS for windows, version 17 (SPSS Institute, Chicago, IL, USA). We examined the associations between MVD and other parameters by categorizing the specimens into two groups (high MVD x low MVD) according to the median value of MVD. Correlation between MVD and clinicopathological criteria was evaluated in univariate analysis using chi-squared test, Mann Whitney test or *t*-test, as appropriate. Survival analysis was conducted using the Kaplan–Meier method and differences between curves were evaluated by the long-rank and Wilcoxon rank tests. Multivariate analysis was performed and a P value of < 0.05 was considered significant.

RESULTS

Overall, 101 (75.9%) TNCs were found to have a basal-like phenotype. Follow-up data were available for 123 patients and time ranged from 1 to 161 months (median, 40 months; mean, 53 months). Overall, 53 (39.1%) patients had developed a recurrence by the time of last follow-up and 45 (33.8%) had died from breast cancer. Local recurrence was detected in 11 (8.3%) patients and 49 (36.8%) women developed distant metastasis (non-regional lymphatic metastasis, 2/49[4.1%]; hematogenic metastasis, 41/49 [83.7%]; both, 6/49 [12.2%]). Lungs, brain and bones represented the main sites of metastases (51.0%, 18.4%, and 28.5%, respectively). Other sites were liver (8.2%), pleura (6.1%), meninges (2.0%) and mediastinum (2.0%). Overall survival (OS) ranged from 1 to 161 months (median=40 months; mean=53 months); median disease-free interval (DFI) was 37 months (mean=48 months; range 0-161 months); 5 (3.8%) patients presented with disseminated disease at diagnosis.

Distribution of CD34-positive and CD105-positive blood vessels in triple-negative breast cancers

The distribution of blood vessels in areas of normal breast parenchyma surrounding the tumour as well as in tumoural areas was examined. Blood vessels were found to be evenly distributed in both the interlobular and intralobular compartments of the normal breast (Fig. 2A). In tumours, blood vessels were located largely in the peripheral tumoural area. Vascular invasion was detected in 64 (48.1%) specimens (Fig. 2B).

For vessels highlighted with CD34 staining, MVD in the peritumoural area (CD 34 PT-MVD) ranged from 8.21 to 318.65/mm2 (median 92.54/mm^2; mean 100.37/mm^2); CD34 MVD in the peripheral area (CD34 PP-MVD) ranged from 34.58 to 278.06/mm^2 (median 99.19/mm^2; mean 102.19/mm^2); while CD34 MVD in the intratumoural area (CD34 IT-MVD) ranged from 10.63 to 232.66/mm^2 (median 53.05/mm^2; mean 58.74/mm^2). Total CD34 MVD for the specimen ranged from 61.21 to 606.69/mm^2 (median 253.67/mm^2; mean 261.30/mm^2).

For CD105, PT-MVD ranged from 8.55 to 317.44/mm^2 (median 70.88/mm^2; mean 79.56/mm^2); CD105 PP-MVD ranged from 43.42 to 306.79/mm^2 (median 139.53/mm^2; mean 139.84 /mm^2); CD105 IT-MVD ranged from 11.55 to 363.09/mm^2 (median 124.88/mm^2; mean 128.88/mm^2); lastly, total CD105 MVD ranged from 132.64 to 783.73/mm^2 (median 345.77/mm^2; mean 345.46/mm^2).

Distribution of blood vessels in tumoural areas is illustrated in Figures 3, 4 and 5.

Relationship between CD34 MVD, clinicopathological characteristics and patient prognosis

Tumours with high total CD34 MVD, high CD34 MVD at the peritumoural area and high CD34 MVD at the peripheral area were significantly associated with the development of distant metastasis (p=0.017; p=0.005; p=0.020), specifically with visceral metastasis

(p=0.006; p=0.013; p=0.019). Also, high CD34 PT-MVD was associated with recurrence (p=0.010), advanced tumoural stage (p=0.028) and positive axillary LN *status* (p=0.048). Interestingly, TNCs with a basal phenotype presented a higher CD34 PT-MVD when compared to all TNCs (p=0.017). No association was found between CD34 IT-MVD and any of the parameters analysed. The relationships between CD34 MVD in different areas, clinicopathological criteria and expression of basal-like markers are summarised in Table 5.

As illustrated by the Kaplan–Meier survival curves (Fig. 5), total CD34 MVD and CD34 PT-MVD were significantly associated with shorter OS (p=0.003; p=0.016) and shorter DFI (p=0.040; p=0.007). In addition, a significant correlation was found between CD34 PP-MVD and poorer OS (p=0.017). In multivariate analysis of CD34 MVD for OS, only total CD34 MVD retained significance after adjustment for significant variables (p=0.023). Multivariate analysis for DFI using the same parameters showed significance for CD34 PT-MVD (p=0.040). Results for the Cox regression analysis are demonstrated in Table 6.

Relationship between CD105 MVD, clinicopathological characteristics and patient prognosis

High total CD105 MVD and high CD105 MVD at the peripheral area were significantly associated with histological type (p=0.016; p=0.029). Significant correlation was also observed between high CD105 PP-MVD, recurrence (local and distant; p=0.038) and development of distant metastasis (p=0.031). High CD105 PT-MVD was associated with expression of EGFR (p=0.028) and vascular invasion (p=0.015). The relationships between CD105 MVD, other variables and the basal phenotype are demonstrated in Table 7.

Survival analysis showed that CD105 PP-MVD was strongly associated with poorer OS (p=0.001) and DFI (p=0.001), Figure 6. In multivariate analysis for OS and DFI, CD105 PP-MVD retained its prognostic significance (p= 0.003; p=0.001; Table 6).

DISCUSSION

Triple-negative and basal-like breast cancers tend to relapse and metastasize early and demonstrate a peculiar pattern of dissemination with an increased tendency for visceral metastases to the brain and lungs rather than to the liver and bones [16,17,59-61]. Likewise, patients in our series of TNCs showed a high rate of recurrence (39.1%) and distant metastases (36.8%) to lungs and brain (51.0% and 18.4%, respectively). Previous reports established that the occurrence of metastasis is related to angiogenesis [20,24]. However, there is limited literature on angiogenesis and microvessel density in TNCs and BLCs. In the present study, we examined the angiogenic characteristics of triple-negative breast cancers and investigated their association with clinicopathological parameters and outcome. Considering that microvessel density is a surrogate marker of angiogenesis, it was also of interest to assess MVD in three distinct regions of each specimen: peritumoural, peripheral tumoural and intratumoural areas.

Numerous studies have investigated the prognostic impact of microvessel density in solid tumours, including breast cancer [22-27]. It has been previously demonstrated that breast tumours with high MVD are significantly associated with positive axillary node *status* and poor prognosis [44,62,63]. In concordance with prior data, our results showed a significant association between high CD34 MVD, advanced tumour stage, LN *status* and visceral metastasis.

Also, intratumoural MVD has been described as an indicator of poorer survival in node-positive breast cancer patients treated with adjuvant therapy; similarly, high MVD in node-negative tumours was correlated with an unfavourable outcome [40,41,62-64]. Moreover, a population-based study consisting of 836 breast cancer patients demonstrated that CD34 MVD was associated with a reduced recurrence-free survival and an increased risk of death [25]. Likewise, we found that total CD34 MVD was significantly associated with shorter OS.

Its prognostic power was strong enough to retain significance in multivariate analysis with an increased risk of death of 2.2 (p=0.023). Additionally, CD34 PT-MVD significantly predicted poor DFI in univariate and multivariate analyses (p=0.04).

A study by Mohammed *et al.* revealed a significant association between high MVD and negativity for hormonal receptors. The same group has recently reported that basal-like and triple-negative tumours exhibit a higher MVD when compared to non-basal and non-triple negative tumours [46]. Equally, we found that TNCs with a basal phenotype had a higher CD34 PT-MVD and CD105 PT-MVD.

Other studies have shown that increased vascular density as assessed by CD105 staining as well as elevated serum CD105 were associated with worse outcome in breast cancer [34,66,67]. A work by Dales *et al.* described a significant correlation between CD105-positive microvessels, high risk for metastasis and poor OS in a series of 929 breast cancers [33]. Our results demonstrate that TNCs of high CD105 PP-MVD were significantly associated with the development of distant metastasis. Moreover, CD105 PP-MVD was an independent, strong prognostic factor in multivariate analysis for both OS and DFI. Considering that CD105 seems to be more specific for malignant angiogenesis, our findings indicate that newly formed vessels are mostly located at the peripheral area of tumours. Also, tumoural neovascularization as assessed by CD105 MVD had a survival impact in TNC patients. Attempts to target endoglin and tumour microvasculature in tumour-bearing mice have yielded promising results [38,39]. Notably, TRC105 is a mAb that binds to human CD105 and inhibits angiogenesis and tumour growth. TRC105 is now in clinical trials in cancer patients [37,38,68]. There is also evidence that expression of angiogenic markers may reflect resistance to chemotherapy in breast cancer, particularly within the context of dose intensified chemotherapy [69-71].

Whilst many reports have concluded that MVD is a useful indicator of survival in invasive breast carcinomas, others reached the opposite conclusion. Consequently, the usefulness of microvessel density as a prognostic factor remains controversial. A meta-analysis of 87 breast cancer angiogenesis publications by Uzzan *et al*. revealed that 25 reported a significant association between MVD and survival [72]. Lastly, it should be noted that the use of MVD measurement in practice is still restricted due to the considerable variability in methods of evaluation, inter- and intraobserver variation, and differences between regions within tumours (e.g. periphery or center) [72-74]. By examining MVD separately in 3 regions of each specimen using a computerised image analyser, we sought to minimise those difficulties. Although clinical applications of MVD assessment is limited, such understanding has biological implications and can shed light on optimal management of breast cancer and on finding the appropriate tailored therapy for each patient.

In conclusion, the current study shows significant associations between microvessel density in different tumoural areas and various clinicopathological parameters in triple-negative breast tumours. These associations indicate that TNCs with aggressive features have higher MVDs. Furthermore, CD105 MVD is a strong, independent prognostic factor in triple-negative breast cancer; such correlation reflects the importance of angiogenesis in tumour growth and development. Finally, our findings highlight that tumoural neovasculature is mainly located at the peripheral area of triple-negative breast cancers and may have a critical role in tumour progression by representing means for disease dissemination.

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COMPETING INTERESTS

The authors declare that they have no conflicts of interest.

STATEMENT OF AUTHOR CONTRIBUTIONS

MDB and HG conceived the study, participated in its design and coordination, and drafted the manuscript. RMR performed immunoassays and was involved in revising the manuscript critically. FAS contributed to the acquisition of data and participated in the reviewing process of the manuscript. All authors have read and approved the final manuscript.

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TABLES

Table 1 – Clinicopathological characteristics of patients with triple-negative breast cancers in our series

Clinicopathological Criteria	Frequency n (%)
Age	
≤50 years old	48 (36.1)
>50 years old	85 (63.9)
Menopausal status	
Premenopause	41(30.8)
Post menopause	92 (69.2)
Family History of Breast Cancer	
Present	35 (26.3)
Absent	98 (73.7)
Stage*	
I	13 (9.8)
II	47 (35.3)
III	65 (48.9)
IV	5 (3.8)
Tumour type	
IDC NST	106 (79.7)
Other types	27 (20.3)
Tumour grade	
1	2 (1.5)
2	18 (13.5)
3	113 (85.0)
Recurrence	
No	70 (52.6)
Local	4 (3.0)
Distant	42 (31.6)
Both	7 (5.3)
Lost to follow up	10 (7.5)
Follow up	
No evidence of disease	70 (52.6)
Alive with disease	10 (7.5)
Death from breast cancer	43 (32.3)
Lost to follow up	10 (7.5)

* For 3 patients, complete clinical data could not be retrieved from medical records. Follow-up data were available for 123 patients. For each variable, missing information was regarded as censored in univariate analyses. Cox multivariate analysis took into consideration the presence of censored data.

Table 2 – Immunohistochemical study for 133 triple-negative breast cancers represented on the tissue microarray: primary antibodies, dilutions, sources, antigen retrieval and cutoff values

Antibody	Clone	Dilution	Source	Antigen retrieval method and duration	Cutoff values
ER	6F11	1: 1000	Novocastra, Newcastle, UK	Steamer/Citrate pH 6.0 Steamer 25 min Cooling 30 min	< 1% (negative)
PR	PGr312	1: 1000	Novocastra, Newcastle, UK	Steamer/Citrate pH 6.0 Steamer 25 min Cooling 30 min	< 1% (negative)
HER2	CB11	1: 80	Novocastra, Newcastle, UK	No pre-treatment	0 or 1+ (negative)
CK5	XM26	1: 100	NeoMarkers, Freemont, CA, USA	Pressure pan/Citrate pH 6.0 Pressure pan 25 min Cooling 30 min	≥ 10% (positive)
CK14	LL002	1: 400	Biogenex, San Ramon, CA, USA	Pressure pan/Citrate pH 6.0 Pressure pan 25 min Cooling 30 min	≥ 10% (positive)
EGFR	31G7	1:200	Zymed, San Francisco, CA, USA	Enzymatic Digestion Proteinase K 7 min	≥ 10% (positive)
p63	4A4	1: 2000	DAKO, Carpinteria, CA, USA	Water bath EDTA/TRIS* pH 9.0 25 min	≥ 10% (positive)
p53	D0-7	1:2000	DAKO, Carpinteria, CA, USA	Pressure pan/Citrate pH 6.0 Pressure pan 25 min Cooling 30 min	> 5 % (positive)

* EDTA, Ethylenediaminetetraacetic Acid; Tris buffer, Tris(hydroxymethyl)aminomethane

Table 3 – Immunohistochemistry performed on paraffin-embedded 4µm-thick whole tissue sections representative of 133 triple-negative breast cancers: primary antibodies, dilutions, sources, antigen retrieval and pattern of staining

Antibody	Clone	Dilution	Source	Antigen retrieval method and duration	Pattern of Staining
CD34	QBEnd10	1: 200	Dakocitomation, Denmark	Steamer/Citrate pH 6.0 Steamer 25 min Cooling 30 min	Membranous and/or cytoplasmic positivity
CD105	4G11	1:50	Novocastra, Newcastle, UK	Microwave/EDTA* pH 8.0 Microwave 6 min Cooling 30 min	

* EDTA, Ethylenediaminetetraacetic Acid.

Table 4 – Blood vessel parameters assessed by the microvessel analysis algorithm applied on high-resolution digital slides representative of 133 triple-negative breast cancers; digital slides were obtained by whole slide scanning of tissue sections stained with CD34 and CD105

Region	CD34			CD105		
	IT	PP	PT	IT	PP	PT
Total Surface Area	6mm^2	6mm^2	6mm^2	6mm^2	6mm^2	6mm^2
Median Number of Vessels	318.00	585.00	555.00	748.50	836.50	419.50
Median Microvessel Density	53.05	99.19	92.54	124.88	139.53	70.88
Median Vascular Area*	135.00	139.00	108.00	116.00	115.00	117.50
Median Vessel Area**	70.00	77.00	56.00	61.00	62.00	65.50
Median Lumen Area	18.00	23.00	19.00	20.00	21.50	23.00

IT, intratumoural area; PP, peripheral tumoural area; PT, peritumoural area. *The vascular area represents the sum of all endothelial cells. **The vessel area equals vascular area/2 + lumen area.

Table 5 – Association between MVD at the three tumour areas, total MVD and clinicopathological criteria

	CD34 IT-MVD				CD34 PP-MVD				CD34 PT-MVD				CD34 Total MVD			
	Low	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**
Age																
< 50 yrs	25(52)	23(48)	48	0.622	26(54)	22(46)	48	0.392	20(42)	28(58)	48	0.148	21(44)	27(56)	48	0.340
≥ 50 yrs	40(47)	45(53)	85		39(46)	46(54)	85		45(53)	40(47)	85		44(52)	41(48)	85	
Total			133				133				133				133	
Size																
≤ 2cm	6(38)	10(62)	16	0.316	5(31)	11(69)	16	0.124	7(44)	9(56)	16	0.593	7(44)	9(56)	16	0.639
> 2 cm	58(51)	56(49)	114		59(52)	55(48)	114		58(51)	56(49)	114		57(50)	57(50)	114	
Total*			130				130				130				130	
Stage																
I / II	30(50)	30(50)	60	0.935	29(48)	31(52)	60	0.915	24(40)	36(60)	60	**0.028**	26(43)	34(57)	60	0.183
III / IV	34(49)	36(51)	70		34(49)	36(51)	70		41(59)	29(41)	70		38(54)	32(46)	70	
Total			130				130				130				130	
LN *status*																
Positive	41(51)	39(49)	80	0.560	40(50)	40(50)	80	0.824	47(59)	33(41)	80	**0.048**	43(54)	37(46)	80	0.192
Negative	23(46)	27(54)	50		24(48)	26(52)	50		18(36)	32(64)	50		21(42)	29(58)	50	
Total*			130				130				130				130	
Tumour type																
IDC NST	53(50)	53(50)	106	0.864	54(51)	52(49)	106	0.547	50(47)	56(53)	106	0.143	50(47)	56(53)	106	0.262

	CD34 IT-MVD				CD34 PP-MVD				CD34 PT-MVD				CD34 Total MVD			
	Low	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**
Other Types	13(48)	14(52)	27		12(44)	15(56)	27		17(68)	10(32)	27		16(59)	11(41)	27	
Total			133				133				133				133	
Grade																
I	1(50)	1(50)	2	0.139	1(50)	1(50)	2	0.139	1(50)	1(50)	2	0.658	1(50)	1(50)	2	0.722
II	5 (28)	13(72)	18		5 (28)	13(72)	18		7 (39)	11(61)	18		7 (39)	11(61)	18	
III	60(53)	53(47)	113		60(53)	53(47)	113		59(52)	54(48)	113		58(51)	55(49)	113	
Total			133				133				133				133	
VI																
Positive	35(55)	29(45)	64	0.261	29(45)	35(55)	64	0.338	33(52)	31(48)	64	0.792	32(50)	32(50)	64	0.933
Negative	31(39)	48(61)	79		37(54)	32(46)	69		33(49)	36(53)	69		34(49)	35(51)	69	
Total			133				133				133				133	
CK5																
Positive	52(51)	49(49)	101	0.446	54(53)	47(47)	101	0.115	45(45)	56(55)	101	**0.017**	48(48)	53(52)	101	0.390
Negative	14(44)	18(56)	32		12(37)	20(63)	32		22(69)	10(31)	32		18(56)	14(44)	32	
Total			133				133				133				133	
CK14																
Positive	19(46)	22(54)	41	0.613	21(51)	20(49)	41	0.806	20(49)	21(51)	41	0.806	19(46)	22(54)	41	0.613
Negative	47(51)	45(49)	92		45(49)	47(51)	92		47(51)	45(49)	92		47(51)	45(49)	92	
Total			133				133				133				133	
EGFR																
Positive	25(54)	21(46)	46	0.428	24(52)	22(48)	46	0.669	24(52)	22(48)	46	0.763	24(52)	22(48)	46	0.669

	CD34 IT-MVD				CD34 PP-MVD				CD34 PT-MVD				CD34 Total MVD			
	Low	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**
Negative	41(47)	46(53)	87		42(48)	45(52)	87		43(49)	44(51)	87		42(55)	45(45)	87	
Total			133				133				133				133	
p63																
Positive	16(42)	22(58)	38	0.273	19(50)	19(50)	38	0.956	17(45)	21(55)	38	0.411	15(39)	23(61)	38	0.139
Negative	50(53)	45(47)	95		47(49)	48(51)	95		50(53)	45(47)	95		51(54)	44(46)	95	
Total			133				133				133				133	
p53																
Positive	43(48)	47(52)	90	0.538	43(48)	47(52)	90	0.538	44(49)	46(51)	90	0.620	42(47)	48(53)	90	0.324
Negative	23(53)	20(47)	43		23(53)	20(47)	43		23(53)	20(47)	43		24(56)	19(44)	43	
Total			133				133				133				133	
Relapse																
Yes	27(50)	26(50)	53	1.000	32(60)	21(40)	53	0.067	33(63)	20(37)	53	**0.010**	31(58)	22(42)	53	0.053
No	35(50)	35(50)	70		30(43)	40(57)	70		28(40)	42(60)	70		28(40)	42(60)	70	
Total*			123				123				123				123	
DM																
Yes	26(53)	23(47)	49	0.632	31(63)	18(37)	49	**0.020**	32(65)	17(35)	49	**0.005**	30(61)	19(39)	49	**0.017**
No	36(49)	38(51)	74		31(42)	43(58)	74		29(39)	45(61)	74		29(39)	45(61)	74	
Total*			123				123				123				123	
HM																

	CD34 IT-MVD				CD34 PP-MVD				CD34 PT-MVD				CD34 Total MVD			
	Low	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**
Yes	26(55)	21(45)	47	0.391	30(64)	17(36)	47	**0.019**	30(64)	17(36)	47	**0.013**	30(64)	17(36)	47	**0.006**
No	36(47)	40(53)	76		32(42)	44(58)	76		31(41)	45(59)	76		29(38)	47(62)	76	
Total*			123				123				123				123	
NR-LM																
Yes	3 (37)	5(63)	8	0.491	5(63)	3(37)	8	0.717	6(75)	2(25)	8	0.163	3 (37)	5(63)	8	0.719
No	59(51)	56(49)	115		57(49)	58(51)	115		55(48)	60(52)	115		56(49)	59(51)	115	
Total*			123				123				123				123	

IT-MVD, microvessel density in the intratumoural area; PP-MVD, microvessel density in the peripheral tumoural area; PT-MVD, microvessel density in the peripheral tumoural area; LN, lymph node; IDC NST, invasive ductal carcinoma of no special type; VI, vascular invasion; CK5, cytokeratin 5; CK14, cytokeratin 14; EGFR, epidermal growth factor receptor; DM, distant metastasis; HM, haematogenous metastases; NR-LM, non-regional lymphatic metastasis. *P* values in bold indicate statistically significant associations.

***** For 3 patients, complete clinical data could not be retrieved from medical records. Follow-up data were available for 123 patients. For each variable, missing information was regarded as censored in survival analysis. Cox multivariate analysis took into consideration the presence of censored data.

Table 6 – Multivariate analysis for OS and DFI using the Cox proportional hazard model

	Adjustment of Covariates			Multivariate Analysis for OS		
	HR	**Significance**	**95% CI**	**HR**	**Significance**	**95% CI**
Stage at diagnosis	1.91	0.044	1.02-3.58	-	0.600	-
Lymph node *status*	1.46	0.030	1.22-1.93	-	0.137	-
Absence of distant metastasis	0.01	0.000	0.00-0.08	0.01	**0.000**	0.00-0.08
Absence of haematogenous metastasis	0.03	0.000	0.01-0.10	-	0.910	-
Absence of non-regional lymphatic metastasis	0.29	0.002	0.13-0.64	-	0.214	-
CD34 Total MVD	2.12	0.020	1.13-3.97	2.20	**0.023**	1.11-4.35
CD34 PT-MVD	2.35	0.008	1.25-4.40	-	0.455	-
CD34 PP-MVD	2.13	0.020	1.12-4.03	-	0.843	-
CD105 PP-MVD	2.13	0.018	1.14-3.98	2.78	**0.003**	1.41-5.49

	Adjustment of Covariates			Multivariate Analysis for DFI		
	HR	**Significance**	**95% CI**	**HR**	**Significance**	**95% CI**
Stage at diagnosis	1.90	0.034	1,05-3.44	-	0.651	-
Lymph node *status*	2.13	0,024	1.10-4.11	-	0.263	-
Absence of distant metastasis	0.03	<0.0001	0.01-0.09	0.03	**0.000**	1.01-1.09
Absence of haematogenous metastasis	0.04	0.000	0.02-0.10	-	0.766	-
Absence of non-regional lymphatic metastasis	0.37	0.025	0.16-0.88	-	0.595	-
CD34 Total MVD	1.80	0.045	1.01-3.20	-	0.897	-
CD34 PT-MVD	2.16	0.011	1.19-3.91	1.99	**0.040**	1.03-3.87
CD105 PP-MVD	2.13	0.011	1.19-3.81	2.91	**0.001**	1.54-5.48

OS, overall survival; DFI, disease-free interval; HR, hazard ratio; CI, confidence interval; PT-MVD, microvessel density in the peritumoural area; PP-MVD, microvessel density in the peripheral tumoural area. Cox regression analysis of covariates was developed by removing nonsignificant parameters in a step-wise manner; the final Cox hazard model included statistically significant variables (stage at diagnosis, axillary lymph node *status*, presence of distant metastasis, haematogenous metastasis and non-regional lymphatic metastasis, CD34 Total MVD, CD34 PT-MVD, CD34 PP-MVD and CD105 PP-MVD). Numbers in bold indicate statistically significant associations in multivariate analysis.

Table 7 – Association between MVD at the three tumour areas, total MVD and clinicopathological criteria

	CD105 IT-MVD				CD105 PP-MVD				CD105 PT-MVD				CD105 Total MVD			
	Low	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**
Age																
< 50 yrs	21(44)	27(56)	48	0.307	28(58)	20(42)	48	0.392	25(52)	23(48)	48	0.148	21(44)	27(56)	48	0.340
≥ 50 yrs	46(54)	39(46)	85		40(47)	45(53)	85		40(47)	45(53)	85		44(52)	41(48)	85	
Total			133				133				133				133	
Size																
≤ 2cm	8(50)	8(50)	16	1.000	7(44)	9(56)	16	1.000	7(44)	9(56)	16	0.687	7(44)	9(56)	16	0.896
> 2 cm	57(50)	57(50)	114		57(50)	57(50)	114		56(49)	58(51)	114		57(50)	57(50)	114	
Total*			130				130				130				130	
Stage																
I / II	30(50)	30(50)	60	0.868	29(48)	31(52)	60	0.851	31(52)	29(48)	60	0.603	27(45)	33(55)	60	0.416
III / IV	33(47)	37(53)	70		34(49)	36(51)	70		32(46)	38(54)	70		37(53)	33(47)	70	
Total*			130				130				130				130	
LN *status*																
Positive	41(51)	39(49)	80	0.560	40(50)	40(50)	80	0.824	38(48)	42(52)	80	0.617	42(52)	38(48)	80	0.345
Negative	23(46)	27(54)	50		24(48)	26(52)	50		26(52)	24(48)	50		22(44)	28(56)	50	
Total*			130				130				130				130	
Tumour type																
IDC NST	52(49)	54(51)	106	0.662	48(45)	58(55)	106	**0.029**	51(48)	55(52)	106	0.381	47(44)	59(56)	106	**0.016**

	CD105 IT-MVD				CD105 PP-MVD				CD105 PT-MVD				CD105 Total MVD			
	Low	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**
Other Types	15(56)	12(44)	27		19(70)	8(30)	27		15(56)	12(44)	27		19(70)	8(30)	27	
Total			133				133				133				133	
Grade																
I	2(100)	0(0)	2	0.359	2(100)	0(0)	2	0.166	2(100)	0(0)	2	0.117	2(100)	0(0)	2	0.722
II	8 (44)	10(56)	18		7(39)	11(61)	18		11(39)	7(61)	18		9 (50)	9(50)	18	
III	57(51)	56(49)	113		58(52)	55(48)	113		53(47)	60(53)	113		55(48)	58(52)	113	
Total			133				133				133				133	
VI																
Positive	35(55)	29(45)	64	0.296	34(53)	30(47)	64	0.486	39(61)	25(39)	64	**0.012**	36(56)	28(44)	64	0.141
Negative	32(40)	37(60)	69		33(48)	36(52)	69		27(39)	42(61)	69		30(43)	39(57)	69	
Total			133				133				133				133	
CK5																
Positive	51(51)	50(49)	101	0.837	54(53)	47(47)	101	0.151	47(47)	54(53)	101	0.151	50(49)	51(51)	101	0.961
Negative	16(50)	16(50)	32		12(38)	20(62)	32		20(62)	12(38)	32		16(50)	16(50)	32	
Total			133				133				133				133	
CK14																
Positive	24(59)	17(41)	41	0.188	24(59)	17(41)	41	0.188	23(56)	18(44)	41	0.347	22(54)	19(46)	41	0.534
Negative	43(47)	49(53)	92		43(47)	49(53)	92		43(47)	49(53)	92		44(48)	48(52)	92	
Total			133				133				133				133	
EGFR																
Positive	26(57)	20(43)	46	0.273	26(57)	20(43)	46	0.273	17(37)	29(63)	46	**0.028**	26(57)	20(43)	46	0.247

	CD105 IT-MVD				CD105 PP-MVD				CD105 PT-MVD				CD105 Total MVD			
	Low	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**
Negative	41(47)	46(53)	87		41(47)	46(53)	87		49(56)	38(44)	87		42(48)	45(52)	87	
Total			133				133				133				133	
p63																
Positive	23(60)	15(40)	38	0.124	23(60)	15(40)	38	0.124	17(45)	21(55)	38	0.701	18(47)	20(53)	38	0.742
Negative	44(46)	51(54)	95		44(46)	51(54)	95		47(49)	48(51)	95		48(51)	47(49)	95	
Total			133				133				133				133	
p53																
Positive	47(52)	43(48)	90	0.577	43(48)	47(52)	90	0.353	43(48)	47(52)	90	0.353	45(50)	45(50)	90	0.900
Negative	20(47)	23(53)	43		24(56)	19(44)	43		24(56)	19(44)	43		21(49)	22(51)	43	
Total			133				133				133				133	
Relapse																
Yes	25(47)	28(53)	53	0.855	32(60)	21(40)	53	**0.038**	24(45)	29(55)	53	0.479	26(49)	27(51)	53	0.796
No	34(49)	36(51)	70		29(41)	41(59)	70		36(51)	34(49)	70		32(46)	38(54)	70	
Total*			123				123				123				123	
DM																
Yes	23(47)	26(53)	49	0.913	28(57)	21(43)	49	0.149	22(45)	27(55)	49	0.531	22(45)	27(55)	49	0.683
No	36(49)	38(51)	74		33(45)	41(55)	74		38(51)	36(49)	74		36(49)	38(51)	74	
Total*			123				123				123				123	
HM																

	CD105 IT-MVD				CD105 PP-MVD				CD105 PT-MVD				CD105 Total MVD			
	Low	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**
Yes	22(47)	25(53)	47	0.898	27(57)	20(43)	47	0.148	21(45)	26(55)	47	0.520	21(45)	26(55)	47	0.666
No	37(49)	39(51)	76		34(45)	42(55)	76		39(51)	37(49)	76		37(49)	39(51)	76	
Total*			123				123				123				123	
NR-LM																
Yes	5(63)	3(37)	8	0.476	5(63)	3(37)	8	0.717	4(50)	4(50)	8	1.000	5 (63)	3(37)	8	0.474
No	54(47)	61(53)	115		56(49)	59(51)	115		56(49)	59(51)	115		53(46)	62(54)	115	
Total*			123				123				123				123	

IT-MVD, microvessel density in the intratumoural area; PP-MVD, microvessel density in the peripheral tumoural area; PT-MVD, microvessel density in the peripheral tumoural area; LN, lymph node; IDC NST, invasive ductal carcinoma of no special type; VI, vascular invasion; CK5, cytokeratin 5; CK14, cytokeratin 14; EGFR, epidermal growth factor receptor; DM, distant metastasis; HM, haematogenous metastases; NR-LM, non-regional lymphatic metastasis. *P* values in bold indicate statistically significant associations.

***** For 3 patients, complete clinical data could not be retrieved from medical records. Follow-up data were available for 123 patients. For each variable, missing information was regarded as censored in survival analysis. Cox multivariate analysis took into consideration the presence of censored data.

FIGURES

Figure 1 - Specimen section with dashed lines demonstrating the division into three areas: intratumoural (the central core of the tumour), peripheral (the outer 1/3 of the tumour) and peritumoural (normal tissue surrounding the tumour), x10 magnification.

Figure 2 – Sections stained with CD34 (A) and CD105 (B) illustrating the peritumoural and peripheral areas of a triple-negative breast cancer specimen.

(A) Normal breast parenchyma with a terminal duct lobular unit showing vessels (arrows) stained with CD34 and distributed both in the interlobular and the intralobular stroma, 200x magnification.

(B) Multiple vascular invasions (arrows) at the peripheral tumoural area of a triple-negative breast invasive carcinoma; vessels are positive for CD105, 400x magnification.

Figure 3 – Sections of a triple-negative breast cancer specimen showing CD34-positive vessels in the three areas examined at 100x magnification: intratumoural (A), peripheral tumoural (B) and peritumoural (C).

Figure 4 – Histograms showing distribution of intratumoural MVD (A), peripheral MVD (B), peritumoural MVD (C) and total MVD (D) in 133 triple-negative breast cancer specimens stained with CD34.

Figure 5 – Histograms showing distribution of intratumoural MVD (A), peripheral MVD (B), peritumoural MVD (C) and total MVD (D) in 133 triple-negative breast cancer specimens stained with CD105.

Figure 6 - Survival analysis showing significant associations between high MVD assessed with CD34 staining and overall survival (OS) and disease-free interval (DFI) using Kaplan–Meier survival curves.

(A) Positive relationship between high total MVD, shorter overall survival ($p = 0.003$) and disease-free interval ($p=0.040$).

(B) Positive relationship between high MVD at the peritumoural area, shorter overall survival (p = 0.003) and disease-free interval (p=0.040).

Figure 7 - Kaplan–Meier survival curves demonstrating a significant association between survival and high MVD at the peripheral tumoural area (assessed with CD105 staining).

(A) Overall survival (OS), p=0.001.

(B) Disease-free interval (DFI), p=0.001.

Figure 8 – Measurement of microvessel vessel density by applying the microvessel density analysis algorithm in sections of a triple-negative breast cancer specimen stained with CD34, 100x magnification. Vessels highlighted in green represent vascular structures counted by the image computerised analyser.

(A) Intratumoural area

(B) Peripheral tumoural area

(C) Peritumoural area

Objective evaluation of lymphatic and blood vessels in triple-negative breast cancer: a morphometric analysis with prognostic implications

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INTRODUCTION

Lymph vessels and blood vessels within and around malignant tumours play a critical role in disease progression in solid tumous, including breast cancer [1-3]. Although regional lymph node status represents one of the main parameters in determining treatment strategies and prognosis in breast cancer, lymph vessel research has evolved slowly compared with that in blood vessels [4]. Histological features and immunohistochemical markers which could precisely distinguish blood endothelial cells from lymphatic endothelial cells were limited until recently. Over the last decade, however, robust lymphatic markers have become available and made the distinction between lymphatic and blood vessels possible. Some of these markers include podoplanin/D2-40, LYVE-1, Prox-1 and VEGFR-3 [4-9]. The advent of lymphatic endothelial markers has increased the interest in exploring the role of lymphatics in cancer progression. Research in this area has linked lymphangiogenesis and its surrogate marker, lymph vessel density, to axillary lymph node status and disease spread [10-12]. Nevertheless, prognostic significance of lymph vessel density remains controversial [12]. Moreover, whether lymphatic are located within tumours or only at their periphery is still arguable. Initial reports have described an absence of both intratumoural lymphatics and of lymphangiogenesis in human breast carcinomas [13-15]. Conversely, intratumoural and peripheral lymphatics have been detected by others [16-19].

Lymphangiogenesis and angiogenesis may have mutual regulatory characteristics [20]. Although angiogenesis has been found to be associated with features of tumour aggressiveness and poor outcome, much less is known on the role of lymph vessels in tumour progression and patient prognosis [10-12, 21,22].

Determination of microvessel density (MVD) and lymph vessel density (LVD) is the most commonly used technique to quantify angiogenesis and lymphangiogenesis, respectively [10, 23-27]. These are assessed directly by counting blood and lymph vessels in tumour sections

stained with endothelial cell markers, such as CD31, CD34 and D2-40 [23-27]. Also, it has been demonstrated that computer-aided image analysis is a good and objective technique for evaluation of vessel density [27-29].

Numerous studies have investigated the prognostic impact of microvessel density in breast cancer [23-27, 30-32]. It has been previously demonstrated that breast tumours with high MVD are significantly associated with positive axillary node status and poor prognosis [30-32]. Also, intratumoural MVD has been described as an indicator of poorer survival in node-positive and node-negative breast cancer patients [30-34]. As for lymph vessel density, high LVD has been correlated with lymph node metastasis and shorter survival in invasive breast carcinomas [10-12]. Recently, a work by Mohammed *et al* demonstrated that lymph node negative basal and triple-negative breast carcinomas have a significantly higher MVD than lymph node negative non-basal and non-triple-negative carcinomas [35], suggesting that such groups may preferentially benefit from anti-angiogenic therapy [36-39]. Unlike microvessel density, no significant difference was detected in lymph vessel density between the basal or triple-negative groups compared with their respective controls [35].

There is limited literature on angiogenesis and lymphangiogenesis in triple-negative and basal-like breast cancers, high-risk tumours for which no specific therapy is available [40-44].

Studies to enhance our understanding of triple-negative breast cancer (TNC) and basal-like breast cancer (BLC) are necessary to improve current management, and ultimately outcomes, of patients with these types of tumours.

The aims of the present study were to examine, in detail, the topography and characteristics of lymph vessels in a series of triple-negative breast cancers; to investigate the association of lymph vessel density with clinicopathological criteria, basal-like phenotype and prognosis;

and to assess microvessel density in the same specimens to evaluate if tumours share common angiogenic and lymphangiogenic characteristics.

METHODS

Patients with a documented history of triple-negative breast cancer were identified from 2235 cases of invasive breast carcinomas submitted to surgical treatment from 1985 to 2006 at the A.C. Camargo Cancer Hospital (São Paulo, Brazil), and at the Federal University of Minas Gerais (UFMG) Clinic Hospital (Belo Horizonte, Brazil). This study was approved by the Research Ethics Committee of our institution under protocol number ETIC 466/06, on March 21st, 2007 (COEP/UFMG).

Patients and specimens

Cases were selected based on the availability of archival material; original diagnosis of invasive breast carcinoma; and a previous pathology report showing negativity for ER, PR and HER2 (triple-negative breast cancer). Amongst 2235 cases informative for the markers ER, PR and HER2, 252/2235 (11.3%) had a previous pathology report showing the triple-negative immunophenotype. Of these 252 cases, 133 had archival formalin-fixed, paraffin-embedded (FFPE) tumour blocks suitable for analysis and formed the basis of this study. Lack of expression for ER, PR, and HER2 was confirmed by a new immunohistochemical study.

Clinicopathological data

Patients' clinical history and tumour characteristics were obtained from medical records. Original hematoxylin-eosin (H&E) stained sections representative of tumour blocks were reviewed and histological type and grade were re-evaluated by a single pathologist (M. D. B.). Tumours were graded according to a modified Bloom–Richardson scoring system [45,46]. Our series consisted of 133 women with TNC (106 invasive ductal carcinomas of no special type [IDC NST], 5 carcinomas with medullary features, 6 metaplastic carcinomas, 6

micropapillary carcinomas, 5 papillary carcinomas, 3 apocrine carcinomas, 1 medullary carcinoma and 1 adenoid cystic carcinoma).

Construction of the tissue microarray

A tissue microarray (TMA) was constructed by extracting 1.0 mm diameter cores of histologically confirmed invasive breast carcinomas from the original paraffin blocks using a tissue core extractor (Beecher Instruments, Silver Springs, MD) and re-embedding these cores into a gridded paraffin receptor block. Two cores were taken from tumour dense areas for each one of the 133 cases.

Immunohistochemistry and Scoring

Sequential slides from the TMA were deparaffinized in xylene, rehydrated in graded alcohol and stained with commercially available antibodies: ER, PR, HER2, cytokeratin 5 (CK5), cytokeratin 14 (CK14), EGFR, p63 and p53. Antigen retrieval varied according to the primary antibody following supplier's specifications. Negative controls were produced by omitting the primary antibody. Immunohistochemistry (IHC) was performed on a Link 48 Autostainer (DAKO, Carpinteria, CA, USA) using the Flex Plus visualization system per manufacturer's specifications. Stains were visualized using diaminobenzidine and hematoxylin counterstain provided by DAKO, Carpinteria, CA, USA. Immunoreactivity was assessed according to previously published criteria [47-49]. Primary antibodies, clones, dilutions, suppliers and cutoff values are shown in Table 1.

Definition of basal phenotype

Basal-like breast cancers were defined by the expression of basal cytokeratins (cytokeratin 5 and/or cytokeratin 14) in our series of TNCs [49].

Assessment of lymphangiogenic and angiogenic characteristics

For visualisation of lymph vessels and blood vessels, 4µm-thick whole tissue sections from each specimen were stained with CD34 and D2-40, respectively. Specifications are shown in Table 2. IHC procedure was performed on a Link 48 Autostainer as described previously. Two characteristics were noted for lymph and blood vessels: distribution/ location within and around tumours; LVD, a surrogate marker of lymphangiogenesis; and MVD, a surrogate marker of angiogenesis.

Assessment of LVD and MVD was conducted in sections stained with D2-40 and CD34, respectively, using a computerised image analyser [50,51]. First, slides were scanned with *ScanScope® XT- Aperio Digital Pathology System*, which creates high-resolution digital slides by whole slide scanning [52]. Using *Aperio ImageScopeTM*, each digital slide was examined by a pathologist (M.D.B) at low magnification (x20) and divided into three areas (Fig. 1): the central core of the tumour (intratumoural area; IT); the outer 1/3 of the tumour tissue (peripheral tumoural area; PP); and normal breast parenchyma surrounding the tumour (peritumoural area; PT). Subsequently, each area was analysed separately by applying a microvessel density analysis algorithm (*Microvessel Density v1, Aperio Digital Pathology System*), Figure 2. This method provided the LVD/MVD in the peritumoural area (PT-LVD; PT-MVD), in the peripheral tumoural area (PP-LVD; PP-MVD) and in the intratumoural area (IT-LVD; IT-MVD). Total LVD/MVD for each specimen was the sum of LVDs/MVDs in the three areas examined.

For MVD evaluation, criteria by Weidner *et al* were applied and only blood vessels in the most vascularised areas (the so called “hot spots”) were counted. By screening the whole section at 40x magnification, 3 to 5 “hot spots” were identified and selected with millimetered drawing tools in each area (IT, PP, PT - surface area of 6.0 mm^2 each). Selections were made without knowledge of the patient’s clinical outcome.

Alternately, LVD was measured in a fashion akin to that described by Mohammed and colleagues. Instead of counting vessels in hot spots, we adopted a modified method by assessing all lymph vessels in each area of the specimen (IT, PP, PT - surface area of 34.0 mm^2 each). Although this method is more time consuming, it examines all lymphatics across the whole section and allows distribution information to be obtained.

Other parameters measured in each area are illustrated in Table 3.

Microvessel analysis algorithm performance was controlled by a set of input parameters: maximum vessel area and vessel wall thickness threshold (large vessels with tunica media are excluded); region joining parameter (stained endothelial cell clusters separate from adjacent vessels are joined into a single microvessel, even in the absence of vessel lumen); endothelial stain; and background stain. A dual-level thresholding process screened out non-specific staining, when present.

Vascular invasion (VI), encompassing both lymph-vascular invasion (LVI) and blood vascular invasion (BVI), was identified when tumour clusters were detected within CD34- or D2-40-positve vessels, respectively.

Statistical analysis

Statistical analysis was performed using the software package SPSS for windows, version 17 (SPSS Institute, Chicago, IL, USA). We examined the associations between LVD, MVD and other parameters by categorizing the specimens into groups (high LVD x low LVD; high MVD x low MVD) according to the median value of LVD and MVD. Correlation between LVD, MVD and clinicopathological criteria was evaluated in univariate analysis using chi-squared test, Mann Whitney test or *t*-test, as appropriate. Survival analysis was conducted using the Kaplan–Meier method and differences between survival curves were evaluated by the long-rank and Wilcoxon rank tests. Multivariate analysis with the Cox proportional hazards model was performed and a P value of < 0.05 was considered significant.

RESULTS

TNC clinicopathologic and immunohistochemical characteristics

The median age at time of diagnosis was 55 years (range, 32–86 years), 48 of 133 patients (36.1%) were younger than 50 years and 41 (30.8%) were premenopausal women. A positive family history was present in 27 of 140 patients (19.2%). Altogether, 16 (12.0%) of the specimens were < 2.0 cm in size, 113 (85.0%) were grade III, 18 (13.5%) were grade II and 2 were grade I (1.5%); a high mitotic rate was detected in 62/140 (44.3%) tumours. In all, 44 (33.1%) patients presented with advanced stage of disease (pathologic stages III and IV) at diagnosis. Also, 80 (60.2%) specimens were lymph node positive and 101 (75.9%) were found to have a basal-like phenotype. Expression of markers related to the basal phenotype in our series of TNCs is demonstrated in Table 4. The main types of adjuvant therapy were chemotherapy (8%), radiotherapy (7%), or both (52%). Clinicopathological characteristics of our cohort of triple-negative cancers are summarised in Table 4.

Follow-up, outcome and sites of metastases

Follow-up data were available for 123 patients and time ranged from 1 to 161 months (median, 40 months; mean, 53 months). Overall, 53 (39.1%) patients had developed a recurrence by the time of last follow-up and 45 (33.8%) had died from breast cancer. Local recurrence was detected in 11 (8.3%) patients and 49 (36.8%) women developed distant metastasis (non-regional lymphatic metastasis, 2/49[4.1%]; hematogenic metastasis, 41/49 [83.7%]; both, 6/49 [12.2%]). Lungs, brain and bones represented the main sites of metastases (51.0%, 18.4%, and 28.5%, respectively). Other sites were liver (8.2%), pleura (6.1%), meninges (2.0%) and mediastinum (2.0%). Overall survival (OS) ranged from 1 to 161 months (median=40 months; mean=53 months); median disease-free interval (DFI) was 37 months (mean=48 months; range 0-161 months); 5 (3.8%) patients presented with disseminated disease at diagnosis.

Distribution of lymph vessels and blood vessels in normal breast tissue and triple-negative breast cancers

The distribution of lymph vessels in areas of normal breast parenchyma surrounding the tumour as well as in tumoural areas was examined. In tumours, lymphatics were located largely in the peripheral tumoural area. Peritumoural lymphatics (PT-L) were detected in 98/133 (73.7%) tumours while peripheral lymphatics (PP-L) were detected in 116/133 tumours (87.2%) compared with 92/133 tumours (69.2%) having intratumoural lymphatics (IT-L), Figure 3. In the normal breast, lymphatics were frequently observed in the interlobular stroma, but completely absent from the intralobular compartment, as illustrated in Figure 4. Blood vessels were also located mainly in the peripheral tumoural area. Vascular invasion was mostly represented by blood vascular invasion, which was detected in 64/133 (48.1%) specimens. No significant association was found between high MVD and the presence of BVI, neither between high LVD and LVI.

Regarding vessel density, LVD in the peritumoural area (PT-LVD) ranged from 0.00 to 8.09/mm2 (median 0.25/mm^2; mean 0.76/mm^2); LVD in the peripheral tumoural area (PP-LVD) ranged from 0.00 to 27.38/mm^2 (median 0.56/mm^2; mean 1.42/mm^2); while LVD in the intratumoural area (IT-LVD) ranged from 0.00 to 6.80/mm^2 (median 0.08/mm^2; mean 0.44/mm^2). Total LVD for the specimen ranged from 0.00 to 28.33/mm^2 (median 253.67/mm^2; mean 261.30/mm^2). Distribution of lymph vessels in the three tumoural areas is illustrated in Figure 5.

As for MVD, MVD in the peritumoural area (PT-MVD) ranged from 8.21 to 318.65/mm2 (median 92.54/mm^2; mean 100.37/mm^2); MVD in the peripheral tumoural area (PP-MVD) ranged from 34.58 to 278.06/mm^2 (median 99.19/mm^2; mean 102.19/mm^2); MVD in the intratumoural area (IT-MVD) ranged from 10.63 to 232.66/mm^2 (median 53.05/mm^2; mean

58.74/mm^2); lastly, total MVD for the specimen ranged from 61.21 to 606.69/mm^2 (median 253.67/mm^2; mean 261.30/mm^2).

Relationship between LVD, clinicopathological characteristics and patient prognosis

No significant associations were observed between LVD in different tumoural areas and clinicopathological criteria. Our analysis included age and stage at diagnosis, axillary lymph node *status*, histological type and grade, tumour size and expression of basal-like markers. Plus, presence of recurrence and development of distant metastasis did not correlate with high LVD. However, there was a trend for patients whose tumours had a high total LVD to relapse (p=0.094) and develop distant metastasis (p=0.071). This trend, nonetheless, did not reach statistical significance. The relationships between LVD, other variables and the basal phenotype are demonstrated in Table 5.

Survival analysis showed no significant associations of LVD in different tumoural areas, overall survival and disease-free survival, as illustrated in Figures 6 and 7.

Angiogenic characteristics and relationship with lymphangiogenesis and survival

The relationship between MVD and LVD was evaluated and no statistically significant correlation was found between high MVD and high LVD. Some tumours with high MVDs had low LVDs and vice versa.

Tumours with high total MVD, high MVD at the peritumoural area (PT-MVD) and high MVD at the peripheral tumoural area (PP-MVD) were significantly associated with the development of distant metastasis (p=0.017; p=0.005; p=0.020). Also, high PT-MVD was associated with the presence of recurrence (p=0.010), advanced tumoural stage at diagnosis (p=0.028) and positive axillary LN *status* (p=0.048). Moreover, TNCs with a basal phenotype presented a higher PT-MVD when compared to all TNCs (p=0.017). No association was found between IT-MVD and any of the parameters analysed.

In survival curves analysis, total MVD and PT-MVD were significantly associated with shorter overall survival (p=0.003; p=0.016) and shorter disease-free interval (p=0.040; p=0.007). Cox regression analysis of covariates was developed by removing nonsignificant parameters in a step-wise manner; the final Cox hazard model included only statistically significant variables: stage at diagnosis (p=0.044), axillary lymph node *status* (p=0.030), absence of distant metastasis (0.000), total MVD (0.008), PT-MVD (0.020) and PP-MVD (0.020). In multivariate analysis for OS, only absence of distant metastasis (HR=0.01; CI 95% 0.00-0.08; p=0.000) and total MVD (HR=2.20; CI 95% 1.11-4.35; p=0.023) retained significance after adjustment for those variables. Multivariate analysis for DFI using the same parameters showed significance for absence of distant metastasis (HR=0.033; 0.01-0.09; p=0.000) and PT-MVD (HR=1.99; 1.03-3.87; p=0.040).

DISCUSSION

To be written.

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COMPETING INTERESTS

The authors declare that they have no conflicts of interest.

STATEMENT OF AUTHOR CONTRIBUTIONS

MDB and HG conceived the study, participated in its design and coordination, and drafted the manuscript. RMR performed immunoassays and was involved in revising the manuscript critically. FAS contributed to the acquisition of data and participated in the reviewing process of the manuscript. All authors have read and approved the final manuscript.

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TABLES

Table 1 – Immunohistochemical study for 133 triple-negative breast cancers represented on the tissue microarray: primary antibodies, dilutions, sources, antigen retrieval and cutoff values

Antibody	Clone	Dilution	Source	Antigen retrieval method and duration	Cutoff values
ER	6F11	1: 1000	Novocastra, Newcastle, UK	Steamer/Citrate pH 6.0 Steamer 25 min Cooling 30 min	< 1% (negative)
PR	PGr312	1: 1000	Novocastra, Newcastle, UK	Steamer/Citrate pH 6.0 Steamer 25 min Cooling 30 min	< 1% (negative)
HER2	CB11	1: 80	Novocastra, Newcastle, UK	No pre-treatment	0 or 1+ (negative)
CK5	XM26	1: 100	NeoMarkers, Freemont, CA, USA	Pressure pan/Citrate pH 6.0 Pressure pan 25 min Cooling 30 min	≥ 10% (positive)
CK14	LL002	1: 400	Biogenex, San Ramon, CA, USA	Pressure pan/Citrate pH 6.0 Pressure pan 25 min Cooling 30 min	≥ 10% (positive)
EGFR	31G7	1:200	Zymed, San Francisco, CA, USA	Enzymatic Digestion Proteinase K 7 min	≥ 10% (positive)
p63	4A4	1: 2000	DAKO, Carpinteria, CA, USA	Water bath EDTA/TRIS* pH 9.0 25 min	≥ 10% (positive)
p53	D0-7	1:2000	DAKO, Carpinteria, CA, USA	Pressure pan/Citrate pH 6.0 Pressure pan 25 min Cooling 30 min	> 5 % (positive)

* EDTA, Ethylenediaminetetraacetic Acid; Tris buffer, Tris(hydroxymethyl)aminomethane

Table 2 – Immunohistochemistry performed on paraffin-embedded 4µm-thick whole tissue sections representative of 133 triple-negative breast cancers: primary antibodies, dilutions, sources, antigen retrieval and pattern of staining

Antibody	Clone	Dilution	Source	Antigen retrieval method and duration	Pattern of Staining
D2-40	D2-40	1: 400	DAKO, Carpinteria, CA, USA	Water bath EDTA/TRIS* pH 9.0 25 min	Membranous and/or cytoplasmic positivity
CD34	QBEnd10	1: 200	Dakocitomation, Denmark	Steamer/Citrate pH 6.0 Steamer 25 min Cooling 30 min	

* EDTA, Ethylenediaminetetraacetic Acid; Tris buffer, Tris(hydroxymethyl)aminomethane

Table 3 – Blood vessel parameters assessed by the microvessel analysis algorithm applied on high-resolution digital slides representative of 133 triple-negative breast cancers; digital slides were obtained by whole slide scanning of tissue sections stained with CD34 and CD105

	D2-40			CD34		
Region	**IT**	**PP**	**PT**	**IT**	**PP**	**PT**
Total Surface Area	34mm^2	34mm^2	34mm^2	6mm^2	6mm^2	6mm^2
Median Number of Vessels	2.00	18.00	8.00	318.00	585.00	555.00
Median Vessel Density	0.08	0.56	0.25	53.05	99.19	92.54
Median Vascular Area*	86.50	92.00	79.00	135.00	139.00	108.00
Median Vessel Area**	55.00	54.50	44.00	70.00	77.00	56.00
Median Lumen Area	23.00	32.00	21.00	18.00	23.00	19.00

IT, intratumoural area; PP, peripheral tumoural area; PT, peritumoural area. *The vascular area represents the sum of all endothelial cells. **The vessel area equals vascular area/2 + lumen area.

Table 4 – Clinicopathological characteristics of patients with triple-negative breast cancers in our series

Clinicopathological Criteria	Frequency n (%)
Age	
≤50 years old	48 (36.1)
>50 years old	85 (63.9)
Menopausal status	
Premenopause	41(30.8)
Post menopause	92 (69.2)
Family History of Breast Cancer	
Present	35 (26.3)
Absent	98 (73.7)
Stage*	
I	13 (9.8)
II	47 (35.3)
III	65 (48.9)
IV	5 (3.8)
Tumour type	
IDC NST	106 (79.7)
Other types	27 (20.3)
Tumour grade	
1	2 (1.5)
2	18 (13.5)
3	113 (85.0)
Recurrence	
No	70 (52.6)
Local	4 (3.0)
Distant	42 (31.6)
Both	7 (5.3)
Lost to follow up	10 (7.5)
Follow up	
No evidence of disease	70 (52.6)
Alive with disease	10 (7.5)
Death from breast cancer	43 (32.3)
Lost to follow up	10 (7.5)

* For 3 patients, complete clinical data could not be retrieved from medical records. Follow-up data were available for 123 patients. For each variable, missing information was regarded as censored in univariate analyses. Cox multivariate analysis took into consideration the presence of censored data.

Table 5 – Expression of basal-like markers in a series of 133 triple-negative breast cancers

Marker	Positive n (%)	Negative n (%)	Total n (%)
CK5	101 (75.9)	32 (24.1)	133 (100)
CK14	41 (30.8)	92 (69.2)	133 (100)
EGFR	46 (34.6)	87 (65.4)	133 (100)
p63	38 (28.6)	95 (71.4)	133 (100)
p53	90 (67.7)	43 (32.3)	133 (100)

Table 6 – Association between LVD at the three tumour areas, total LVD and clinicopathological criteria.

	IT-LVD				**PP-LVD**				**PT-LVD**				**Total LVD**			
	Low	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**
Age																
< 50 yrs	22(46)	26(54)	48	0.469	21(44)	27(56)	48	0.278	23(48)	25(52)	48	0.717	21(44)	27(56)	48	0.278
≥ 50 yrs	44(52)	41(48)	85		45(53)	40(47)	85		43(51)	42(49)	85		46(52)	39(48)	85	
Total			133				133				133				133	
Size																
≤ 2cm	6(38)	10(62)	16	0.238	9(56)	7(44)	16	0.719	11(69)	5(31)	16	0.173	10(62)	6(38)	16	0.409
> 2 cm	59(52)	55(48)	114		57(50)	57(50)	114		55(48)	59(52)	114		56(49)	58(51)	114	
Total			130				130				130				130	
Stage																
I / II	33(55)	27(45)	60	0.935	35(58)	25(42)	60	0.129	29(48)	31(52)	60	0.663	33(55)	27(45)	60	0.416
III / IV	31(44)	39(56)	70		31(44)	39(56)	70		36(51)	34(49)	70		33(47)	37(53)	70	
Total			130				130				130				130	
LN *status*																
Positive	35(44)	45(56)	80	0.116	37(46)	43(54)	80	0.192	41(51)	39(49)	80	0.736	39(49)	41(51)	80	0.582
Negative	30(60)	20(40)	50		28(56)	22(44)	50		24(48)	26(52)	50		27(54)	23(46)	50	
Total			130				130				130				130	
Tumour type																
IDC NST	49(46)	57(54)	106	0.120	50(47)	56(53)	106	0.262	52(49)	54(51)	106	0.795	50(47)	56(53)	106	0.262
Other Types	17(63)	10(37)	27		16(59)	11(41)	27		14(50)	14(50)	27		16(59)	11(41)	27	

	IT-LVD				PP-LVD				PT-LVD				Total LVD			
	Low	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**
Total			133				133				133				133	
Grade																
I	1(50)	1(50)	2	0.658	1(50)	1(50)	2	0.809	1(50)	1(50)	2	0.658	1(50)	1(50)	2	0.809
II	11(61)	7(39)	18		10(55)	8(45)	18		11(61)	7(39)	18		10(55)	8(45)	18	
III	54(48)	59(52)	113		55(48)	58(52)	113		55(48)	58(52)	113		55(48)	58(52)	113	
Total			133				133				133				133	
VI																
Positive	31(52)	33(48)	64	0.729	33(52)	31(48)	64	0.667	26(41)	38(59)	64	**0.046**	32(50)	32(50)	64	0.933
Negative	35(51)	34(49)	69		33(48)	36(52)	69		40(58)	29(42)	69		34(49)	35(51)	69	
Total			133				133				133				133	
CK5																
Positive	53(52)	48(48)	101	0.446	49(48)	52(52)	101	0.649	49(48)	52(52)	101	0.649	49(48)	52(52)	101	0.649
Negative	13(41)	19(59)	32		17(53)	15(47)	32		17(53)	15(47)	32		17(53)	15(47)	32	
Total			133				133				133				133	
CK14																
Positive	19(46)	22(54)	41	0.613	20(49)	21(51)	41	0.897	21(49)	20(51)	41	0.806	19(46)	22(54)	41	0.613
Negative	47(51)	45(49)	92		46(50)	46(50)	92		45(51)	47(49)	92		47(51)	45(49)	92	
Total			133				133				133				133	
EGFR																
Positive	24(52)	22(48)	46	0.669	21(46)	25(54)	46	0.505	25(54)	21(46)	46	0.428	20(43)	26(57)	46	0.303
Negative	42(48)	45(52)	87		45(52)	42(48)	87		41(47)	46(53)	87		46(53)	41(47)	87	

	IT-LVD				PP-LVD				PT-LVD				Total LVD			
	Low	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**	**Low**	**High**	**Total**	***p* value**
Total			133				133				133				133	
p63																
Positive	19(50)	19(50)	38	0.946	16(42)	22(58)	38	0.273	17(45)	22(55)	38	0.273	14(37)	24(63)	38	0.062
Negative	47(49)	48(51)	95		50(53)	45(47)	95		50(53)	45(47)	95		52(55)	43(45)	95	
Total			133				133				133				133	
p53																
Positive	45(50)	45(50)	90	0.900	41(46)	49(54)	90	0.175	49(54)	41(46)	90	0.620	42(47)	48(53)	90	0.900
Negative	21(49)	22(51)	43		25(58)	18(42)	43		17(40)	26(60)	43		24(56)	19(44)	43	
Total			133				133				133				133	
Relapse																
Yes	24(45)	29(55)	53	0.374	25(47)	28(53)	53	0.601	24(45)	29(55)	53	0.464	21(40)	32(60)	53	0.094
No	38(54)	32(46)	70		37(53)	33(47)	70		37(53)	33(47)	70		39(56)	31(44)	70	
Total			123				123				123				123	
DM																
Yes	21(43)	28(57)	49	0.173	21(43)	28(57)	49	0.173	23(47)	26(53)	49	0.531	19(39)	30(61)	49	**0.071**
No	41(55)	33(45)	74		40(54)	34(46)	74		38(51)	36(49)	74		41(55)	33(45)	74	
Total			123				123				123				123	

IT-LVD, lymph vessel density in the intratumoural area; PP-LVD, lymph vessel density in the peripheral tumoural area; PT-LVD, lymph vessel density in the peripheral tumoural area; LN, lymph node; IDC NST, invasive ductal carcinoma of no special type; VI, vascular invasion; CK5, cytokeratin 5; CK14, cytokeratin 14; EGFR, epidermal

growth factor receptor; DM, distant metastasis. * For 3 patients, complete clinical data could not be retrieved from medical records. Follow-up data were available for 123 patients. For each variable, missing information was regarded as censored in survival analysis. Cox multivariate analysis took into consideration the presence of censored data.