



KÁTIA PITON SERRA

**SUBTIPOS CLÍNICO-PATOLÓGICOS DE CARCINOMA DE
MAMA E SUA RELAÇÃO COM A EXPRESSÃO DA COX2 E
DA p53**

***(CLINICO-PATHOLOGICAL SUBTYPES OF BREAST
CANCER RELATED TO COX2 AND p53)***

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***CLINICO-PATHOLOGICAL SUBTYPES OF BREAST CANCER RELATED TO
COX2 AND p53***

Tese de Doutorado apresentada à Pós-Graduação da
Faculdade de Ciências Médicas da Universidade Estadual
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Dedico este trabalho...

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Símbolos, Siglas e Abreviaturas

- AC** – Antraciclina e taxano
- BRCA1** – Gene do câncer de mama 1 (breast cancer 1)
- CAISM** – Hospital da Mulher Prof. Dr. José Aristodemo Pinotti - Centro de Atenção Integral à Saúde da Mulher
- CCNB1** – Gene CCNB1, codificador da proteína ciclina-B1
- CEP** – Comitê de Ética em Pesquisa
- CMF** – Ciclofosfamida, metotrexate, fluoracil
- Coxib** – Inibidor seletivo da COX2
- DNA** – Ácido desoxirribonucleico
- ERBB2/Erb-B2/HER2** – Receptor do fator de crescimento epidérmico humano 2 (epidermal growth factor receptor 2)
- FAPESP** – Fundação de Amparo à Pesquisa do Estado de São Paulo
- FGFR4** – Gene FGFR4 (fibroblast growth factor receptor)
- FISH** – Hibridização fluorescente in situ (fluorescent in situ hybridization)
- GATA3** – Gene GATA3, codifica a proteína GATA3
- GPR160** – Gene GPR160, codificador da proteína de acoplamento G160
- GRB7** – Gene GRB7 (Growth factor receptor-bound protein 7)

- IQ** – Imunoistoquímica
- ki67** – Proteína ki67
- LAPE** – Laboratório de Patologia Experimental
- MINDACT** – Estudo MINDACT
- MKI67*** – Gene MKI67, codificador da proteína ki67
- MYBL2*** – Gene MYBL2, codificador da proteína B
- OMS** – Organização Mundial da Saúde
- OX (1,2,3)** – Cicloxigenase (1,2,3)
- p53** – Proteína p53
- P53/TP53*** – Gene P53/TP53, codificador da proteína p53
- PAM50** – Assinatura genética PAM50
- R337H*** – Mutação do gene TP53
- RB1*** – Gene do Retinoblastoma (RB1)
- RE** – Receptor de estrógeno
- RP** – Receptor de progesterona
- RxPONDER** – Estudo RxPonder
- SUS** – Sistema Único de Saúde
- TAILOR x** – Estudo TAILORX
- TC** – Taxano e ciclofosfamida
- TMEM45B*** – Gene TMEM45B, codificador da proteína transmembrana 45B
- UNICAMP** – Universidade Estadual de Campinas
- VEGF** – Fator de crescimento vascular (vascular endothelial growth factor)

VEGFR2 – Receptor do fator de crescimento vascular 2 (vascular endothelial growth factor receptor 2)

Resumo

Introdução: Na última década, diferentes subtipos moleculares de câncer de mama foram propostos. A classificação clínico-patológica dos subtipos vem comprovando ser estratégica para prever sobrevida e resposta ao tratamento.. Embora exista associação apreciável com o prognóstico e indicação de terapia citotóxica e endócrina, os subtipos parecem falhar em explicar completamente o comportamento da doença e a resposta ao tratamento. Moléculas como as da família das cicloxigenases (COX), essencialmente a COX 2 vem demonstrando associação com a carcinogênese mamária, e a análise da expressão da p53 nos tumores de mama pode também oferecer informações adicionais para determinação do prognóstico. **Objetivos:** Foi avaliada a associação entre os subtipos clínico-patológicos do câncer de mama com o prognóstico em uma casuística de pacientes brasileiras com câncer de mama, que foram acompanhadas por cerca de quatro anos. Foram discutidas as vantagens e possíveis ressalvas relacionadas à nova classificação. Também foi mensurada a expressão da COX2 e da p53 em relação aos subtipos clínico-patológicos e avaliada se a

expressão destas proteínas poderia explicar a variabilidade no prognóstico ainda encontrada entre os subtipos clínico-patológicos do câncer de mama. **Metodologia:** O total de 183 amostras de câncer de mama foi obtido de mulheres tratadas no Hospital da Mulher Prof. Dr. José Aristodemo Pinotti - CAISM da Universidade Estadual de Campinas, Campinas, Brasil, entre junho de 2008 e janeiro de 2011. *Tissue microarrays* (TMA) foram construídos dos blocos originais de parafina para realização de imunohistoquímica (IQ) e hibridização fluorescente *in situ* (FISH). IQ foi realizada para detecção da expressão de RE, RP, ki67, COX2 e p53; o *status* do HER2 foi avaliado por FISH nas 183 amostras. Os tumores foram classificados em cinco categorias de acordo com a definição correspondente clínica-patológica dos subtipos intrínsecos do câncer de mama, definida durante a *13th St Gallen International Breast Cancer Conference (2013)*. As características clínicas e patológicas das pacientes e seus tumores e a sobrevida foram avaliadas em relação aos subtipos clínico-patológicos, a COX2 e a p53. O tempo médio de seguimento foi 2,94 anos (90% faixa central = 0,93 a 4,1 anos). **Resultados:** Aproximadamente 75% dos tumores foram classificados como luminais-*like*. OS HER2 positivos (não luminais) somaram 9,3% dos casos e os Triplos-negativos 13,1%. Os Luminais B-*like* e HER2 positivos (não luminais) foram associados a alto grau histológico quando comparados aos Luminais A-*like* ($p < 0,01$). Os Luminais A-*like* associaram-se significativamente com melhor sobrevida global e livre de doença quando comparados aos HER2 positivos (não luminais) e Triplos-negativos. Não houve tendência à expressão de COX2 relacionada aos subtipos de Luminal A-*like* a Triplo-negativo. Em contraste, a p53 se expressou

em cerca de 67% dos tumores Luminais *A-like*, 50% dos Luminais *B-like* HER2 positivos, 60,9% dos Luminais *B-like* HER2 negativos, 82% dos HER2 positivos (não-luminais) e 87% dos Triplos-negativos (p para tendências = 0,06). Houve uma significativa expressão de COX2 nos tumores (66,9%) quando a p53 era também positiva, comparada àqueles tumores que não expressavam p53 (em cujo caso apenas 18,0% dos tumores foram positivos para COX2; $p < 0,001$). Nem a COX2, nem a p53 se relacionaram à sobrevida das pacientes.

Conclusões: O critério mais estrito para definir os tumores Luminais *A-like* aumentou a acurácia da classificação para selecionar tumores que partilhem um bom prognóstico e respondam à terapia endócrina. Parece haver uma associação positiva entre a expressão da COX2 e da p53. Por outro lado, nem a expressão da COX2 nem a da p53 se associaram aos subtipos clínico-patológicos, características clínicas e do tumor e ao prognóstico.

Palavras-chave: câncer de mama, subtipos clínico-patológicos, Saint Gallen, COX2, p53.

Summary

Background: In the last decade, different molecular subtypes of breast cancer have been proposed. The clinico-pathological surrogate subtypes of breast cancer classification has been proven as straightforward strategy to predict patient survival and response to treatment. Although displaying appreciable association with disease prognosis value of cytotoxic and endocrine therapeutic modalities, the subtypes seem to fail at completely explaining disease behavior and response to treatment. Molecules such as those of the cyclooxygenase (COX) family, essentially COX2 have been shown to be associated with breast carcinogenesis, and the analysis of p53 expression in breast tumors may also offer some additional prognostic clues. **Objectives:** We tested the association of the current clinico-pathological surrogate subtypes of breast cancer with the main prognostic and predictive factors in a dataset of breast cancer Brazilian patients, which were followed up for almost four years. We discuss the advantages and possible caveats related to this new classification. Our study also assessed COX2 and p53 expression in these clinico-pathological subtypes, and evaluated whether the expression of these proteins could help further explain the variability in prognosis still found within the surrogate molecular groups of breast cancer. **Methods:** A total of 183 breast cancer samples were

obtained from women treated at the Women's Hospital of Campinas State University, Campinas, Brazil, between June 2008 and January 2011. Tissue microarrays (TMA) were constructed from the original paraffin blocks for immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) analyses. Immunohistochemistry was performed to detect the expression of ER, PR, ki67, COX2, and p53; the HER2 status of the 183 specimens was assessed using FISH. Tumors were subtyped into five distinct categories according to the Clinico-Pathological surrogate definitions of intrinsic subtypes of breast cancer defined during the 13th St Gallen International Breast Cancer Conference (2013). Clinical and pathological features of patients and their tumors, and patients' survival were assessed in relation to the surrogate subtypes, COX2 and p53. Mean follow-up time was 2.94 years (90% central range = 0.93 to 4.1 years). **Results:** Approximately 75% of the tumors were classified as luminal-type-like. HER2 positive (non-luminal) tumors accounted for 9.3% of the cases and Triple-negative tumors for the remainder 13.1%. Luminal B-like and HER2 positive (non-luminal) tumors were associated with higher histological grades when compared to Luminal A-like tumors ($p < 0.01$). Luminal A-like tumors were significantly associated with better disease free and overall survival when compared to HER2 positive (non-luminal) and Triple-negative tumors. There was no trend in COX2 overexpression from Luminal A to Triple-negative subtypes. By contrast, p53 was expressed in roughly 67% of the Luminal A-like tumors, 50% of the Luminal B-like HER2 positive tumors, 60.9% of the Luminal B-like HER2 negative, approximately 82% of the HER2 positive (non-luminal) and 87% of the Triple-negative tumors (p for trends = 0.06). There was a

significantly higher proportion of COX2 positive tumors (66.9%) when p53 was also positive compared to when the tumor was negative for p53 (in which case only 18.0% of the tumors were positive for COX2; $p < 0.001$). Neither COX2 nor p53 were found to be associated with patients' survival. **Conclusions:** The more strict criteria to define Luminal A-like tumors increased the accuracy of the classification by selecting tumors that share a good prognosis and response to endocrine therapy. There seems to be a positive association between the expressions of COX2 and p53. On the other hand, neither the expression of COX nor that of p53 was associated with clinic-pathological subtypes, tumor features and prognosis.

Key words: breast cancer, clinico-pathological surrogate subtypes, Saint Gallen, COX2, p53

1. Introdução

O câncer de mama é, atualmente, a principal neoplasia entre as mulheres, tanto em países desenvolvidos como em desenvolvimento^{1,2}. É a segunda causa de morte por câncer entre as mulheres dos países desenvolvidos, após o câncer de pulmão, e a principal causa de morte por câncer nos países em desenvolvimento^{1,2}. Assim sendo, observa-se que a sobrevida é maior entre mulheres nos países desenvolvidos¹. A maior mortalidade nos países em desenvolvimento se deve ao diagnóstico da doença em estádios mais avançados, dificuldade de acesso aos serviços de saúde e falta de tecnologia adequada para diagnóstico e tratamento^{1,2}. A Organização Mundial da Saúde (OMS), através do “GLOBOCAN Project”², divulgou em 2012 estimativas sobre incidência, prevalência, mortalidade e sobrevida para vários tipos de câncer no mundo. Em relação ao câncer de mama, foram estimados 1,67 milhão de novos casos e 522.000 mortes pela doença². Aproximadamente 57.120 novos casos de câncer de mama são estimados para ocorrer no Brasil no ano de 2014 e foram registradas 13.345 mortes pela doença em 2011¹.

Até o final da década de 1990 o carcinoma de mama era classificado essencialmente em função da sua morfologia. Entretanto, já se observava que tumores com os mesmos tipo e grau histológicos, diagnosticados no mesmo

estádio apresentavam prognósticos diferentes e respondiam diferentemente aos tratamentos. Assim, concluiu-se que a classificação histológica morfológica não era suficiente para explicar o comportamento desse câncer e, atualmente, considera-se que o carcinoma de mama não pode ser considerado uma doença única^{3,4,5}. Diferentes subtipos moleculares com diferentes respostas ao tratamento e prognóstico foram identificados^{6,7,8,9,10,11,12,13,14,15}. Observou-se também que cada subtipo poderia apresentar fatores de risco e evolução natural diferenciada^{16,17,18}. Hoje, é internacionalmente reconhecido que essas diferenças devem ser levadas em consideração desde o diagnóstico até a decisão do tratamento e comparação dos resultados^{4,5}.

Inicialmente, a classificação proposta para agrupar os diferentes subtipos de carcinoma de mama foi baseada em *microarrays* de DNA e padrões de expressão gênica resultando em quatro subtipos intrínsecos (Receptor de Estrógeno Positivo (*ER+*)/Luminal-*like*, Basal-*like*, Erb-B2+ e Normal *breast-like*)⁶. Posteriormente foram classificados em cinco diferentes subtipos intrínsecos (Luminal A, Luminal B, Erb-B2+, Basal-*like* e Normal *breast-like*)¹⁹. Esses subtipos tinham sua origem em dois grandes grupos: um grupo caracterizado por expressar o receptor de estrógeno (RE) e genes relacionados a ele, que corresponde ao grupo dos “Luminais” (Luminal A e Luminal B) e outro, marcado por não expressar o RE e seus genes associados, constituído pelos tumores Erb-B2+, Basal-*like* e Normal *breast-like*. O primeiro grupo foi caracterizado pela alta expressão de genes relacionados ao epitélio luminal e baixa expressão de genes do epitélio basal; já o segundo grupo apresentava

forte expressão de genes do epitélio basal e baixa expressão de genes do epitélio luminal^{6,19}.

Geneticamente, pode-se dizer que o subtipo intrínseco Luminal A apresenta alta expressão do gene RE alfa, *GATA 3*, proteína X-box entre outros genes relacionados^{6,19,20}. O Luminal B expressa o gene *RE alfa* embora também expresse genes relacionados ao *Human epidermal growth factor receptor 2 (HER2)*, o *ERBB2* e o *GRB7*, e à proliferação celular (*MKI67*, *CCNB1* e *MYBL2*)²¹. Ao grupo Erb-B2+ está relacionada a ausência de expressão do RE assim como a amplificação dos genes do cromossomo 17 *ERBB2* e *GRB7*, o gene *FGFR4* do cromossomo 5, o *TMEM45B* do cromossomo 11 e o *GPR160* do cromossomo 3⁷. O subtipo Basal-like é caracterizado pela ausência de expressão do RE associado à alta expressão de queratinas 5 e 17, laminina e ácidos graxos ligados à proteína 7^{6,19,20}, e também relacionado à mutação dos genes *BRCA1*, *P53* e *RB1* e alta taxa de proliferação celular, mensurada pelo ki67²². E, finalmente, o grupo Normal *breast-like* também não expressa o gene *RE* e apresenta alta expressão de genes relacionados ao tecido adiposo e tecidos não epiteliais^{6,19,20}.

No final da década de 2000, a heterogeneidade dos tumores já era amplamente conhecida e o valor da classificação em diferentes subtipos intrínsecos para a prática clínica era muito estudado. Entretanto, a classificação molecular através de *arrays* de DNA era difícil e cara e assim não era utilizada na rotina. Vários autores buscavam uma classificação correspondente através de imunistoquímica (IQ) e hibridização fluorescente *in situ* (FISH). Em 2009, Cheang et al.²¹ publicaram o trabalho que serviu de base para a classificação

clínico-patológica dos subtipos de câncer de mama baseada em IQ e FISH. Posteriormente vários autores validaram a classificação por IQ e FISH, mais fácil e barata para o uso na prática clínica, através da expressão dos RE e RP, HER2 e ki67^{23,24}, conforme proposto por Cheang et al.²¹. Os subtipos classificados através de critérios clínicos e patológicos são similares, mas não idênticos aos intrínsecos; ainda assim, representam uma aproximação conveniente^{21,23,24}.

Até o *11th St Gallen International Breast Cancer Conference Expert Panel*³, em 2009, as deliberações sobre o tratamento do câncer de mama se basearam na expressão dos receptores de estrógeno (RE) e receptores de progesterona (RP) determinados por IQ e do HER2, determinado por IQ e FISH. O termo “triplo negativo”, já era utilizado na definição dos carcinomas que não expressavam RE, RP e HER2, mas sem a conotação de definir um subtipo de câncer, apenas para designar os tumores com tripla negatividade para RE, RP e HER2. Foi durante a *12th St Gallen International Breast Cancer Conference Expert Panel, 2011*⁴ que a classificação em subtipos clinico-patológicos, realizada através da IQ e FISH conforme proposto por Cheang et al.²¹, foi adotada para as deliberações sobre estratégias terapêuticas. Nesta conferência foi sustentado o uso apenas dos RE, RP, HER2 e ki67 para a classificação dos subtipos e foi discutido o valor de corte desses marcadores a ser utilizado.

Ficou estabelecido que os RE e RP fossem considerados positivos a partir de 1% de núcleos de células coradas. Nos casos de discordância RE negativo e RP positivo, seria mantida a indicação de terapia hormonal, pois se considerava um falso negativo do RE²⁵. Em relação ao HER2, houve

recomendação para estudo inicial por IQ, sendo o FISH realizado nos casos de resultado “indeterminado” pela IQ²⁶. Após comparação da expressão dos valores de ki67 avaliados por IQ com os subtipos intrínsecos determinados pelo PAM50, foi estabelecido um valor de corte de positividade por IQ de 14% para o ki67 por ser este o mais aproximado aos padrões de expressão gênica²¹. Dessa forma, os subtipos clínico-patológicos foram assim classificados: Luminal A: RE e/ou RP positivos, HER2 negativo e ki64 baixo (<14%); Luminal B HER2 negativo: RE e/ou RP positivos, HER2 negativo e ki64 alto (>=14%); Luminal B HER2 positivo: RE e/ou RP positivos e HER2 positivo; HER2 superexpresso (não luminal): RE e RP negativos e HER2 positivo; Triplo-negativo (ductal): ausência de expressão de RE, RP e HER2. Neste consenso os Triplos-negativos passaram a denominar um subtipo clínico-patológico, com características peculiares de comportamento e pior prognóstico. Foi ressaltado que existia uma correspondência de aproximadamente 80% entre os Triplos-negativos (ductais) clínico-patológicos e os intrínsecos *Basal-like* e que entre os Triplos-negativos existiam tipos histológicos não ductais, como o medular e o adenóide cístico, que cursavam com melhor prognóstico.

Então, a partir de 2011, esta classificação teve grande importância na indicação do tratamento do câncer de mama. De maneira geral, ficou estabelecido tratamento com hormonioterapia para os tumores Luminais, quimioterapia e anticorpo monoclonal anti-HER2 (trastuzumab) para os tumores que expressavam HER2 e quimioterapia para os Triplos-negativos. Nesta classificação o ki67 teve papel fundamental na divisão entre os tumores Luminais A e Luminais B HER2 negativos em relação ao tratamento citotóxico:

foi afirmado o tratamento apenas com hormonioterapia para os Luminais A, na maioria dos casos, e a necessidade de adição de quimioterapia aos Luminais B HER2 negativos, além da hormonioterapia⁴.

Na *13th St Gallen International Breast Cancer Conference Expert Panel, 2013*⁵ a classificação em subtipos clínico-patológicos foi novamente modificada, considerando-se a avaliação semi-quantitativa da expressão dos RP na evolução clínica e resposta ao tratamento. Prat et al.²⁷ avaliaram o valor prognóstico da proporção de células que expressavam os genes e a proteína RP nos tumores classificados como Luminais A. Quando compararam a classificação intrínseca (molecular) e clínico-patológica (IQ) em mais de duas mil pacientes com carcinomas de mama Luminais A ou B observaram que: a grande maioria dos tumores Luminais A intrínsecos também eram Luminais A pela IQ. Entretanto, 35% a 52% dos tumores Luminais B intrínsecos na realidade eram classificados como Luminais A pela IQ. Observaram que os tumores Luminais A intrínsecos apresentavam um escore de positividade dos RP muito maior quando comparado com Luminais B intrínsecos. A fraca positividade dos RP (<20%) pela IQ passou a ser considerada um marcador para Luminal B. Os autores não observaram diferença na expressão dos RE. Assim, Prat et al.²⁷ propuseram uma nova definição para os Luminais A por IQ, adotada por St. Gallen em 2013. Tumores Luminais A passaram a ser definidos como tendo: RE positivo, HER2 negativo, ki-67 <14% e RP positivo com expressão > 20%^{5,27}. Entre os Luminais B HER2 negativos foram incluídos os tumores RE positivos com RP negativo ou RP expresso em menos de 20% dos núcleos corados, assim como os tumores RE positivos com RP positivo e ki-67

> 14%. Portanto, apenas os tumores de melhor prognóstico foram considerados Luminais A, reduzindo proporcionalmente os cânceres classificados nesta categoria. Parte dos tumores classificados em 2011 como Luminais A pela IQ, passaram a ser incluídos na classificação de Luminais B HER2 negativos, tendo indicação para tratamento citotóxico, mais condizente com o prognóstico desses cânceres. Também foi discutido em plenária o valor de corte do ki-67, pois existe uma variedade interlaboratorial na definição da coloração do ki-67 por IQ. Vários autores validaram o valor de corte de 14%^{21,28}. Esse valor de 14% foi mantido, embora seja sugerido que cada laboratório deva definir seu ponto de corte para que o ki-67 seja considerado alto⁵.

Neste consenso também foi incorporado o papel das assinaturas gênicas na classificação dos subtipos Luminais e orientação de tratamento para esses tumores. As assinaturas gênicas *70-gene (MammaPrint; Agendia, Amsterdam, The Netherlands)* e *21-gene (OncoType; Genomic Health, Redwood City, CA)* têm sido incorporadas à prática clínica para complementar a patologia e orientar o tratamento em carcinomas de mama iniciais, com expressão de RE, sem comprometimento de linfonodos axilares. As assinaturas gênicas têm um papel prognóstico e preditivo da resposta, indicando as pacientes que não irão se beneficiar com a quimioterapia¹⁰. As limitações para o uso rotineiro das assinaturas incluem o alto custo, o envio de amostras dos tumores a centros de referências e a dificuldade de obter material suficiente para a realização das assinaturas em tumores muito pequenos¹⁰. Existem estudos em andamento para validar o uso das assinaturas gênicas em outros casos, como indicação de tratamento em tumores iniciais com RE positivo e axila positiva, indicação de

tratamento hormonal isolado *versus* hormonioterapia e quimioterapia, entre outros, que são: *RxPONDER (SWOG S1007) trial*, *TAILORx trial* e *MINDACT trial*²⁹.

Assim sendo, a nova classificação dos subtipos clínico-patológicos segundo a *13th St Gallen International Breast Cancer Conference Expert Panel, 2013*⁵, utilizada atualmente, ficou assim estabelecida: Luminal A-like: expressa RE e RP, não expressa HER2 e tem baixa expressão de ki-67, além de baixo índice de recorrência calculado pelas assinaturas gênicas (*21-gene* e *70-gene*) quando disponíveis; Luminal B-like HER2 negativo: expressa RE, não expressa HER2 e pode ter uma das seguintes características: alta expressão de ki-67 ou baixa expressão/negatividade dos RP ou alto índice de recorrência calculado pelas assinaturas gênicas; Luminal B-like HER2 positivo: expressa RE e superexpressa/amplifica HER2, independentemente do estado do RP e do ki-67; HER2 positivo (não luminal): não expressa receptores hormonais (RE e RP) e superexpressa/amplifica o HER2; Triplo-negativo: não expressa RE e RP, nem HER2.

As recomendações referentes ao tratamento sistêmico em relação aos subtipos segundo a última Conferência de *St. Gallen* ficaram assim estabelecidas: os Luminais A-like são tumores que respondem muito bem à hormonioterapia, sendo este o principal e quase sempre único tratamento sistêmico indicado^{5,9}. Esses tumores são menos responsivos à quimioterapia³⁰, sendo esta indicada apenas em algumas situações, como: tumores Grau 3 e de grande volume, envolvimento de 4 ou mais linfonodos axilares pela doença, alto escore nos testes de avaliação gênica (*21-gene* e *70-gene*) e doença em

mulheres muito jovens, com 35 anos ou menos⁵. Não há esquema preferencial de tratamento quimioterápico para esse subtipo, podendo ser utilizado qualquer regime padrão, como ciclofosfamida-metotrexate-fluorouracil (CMF), antraciclina-ciclofosfamida (AC) ou taxano-ciclofosfamida (TC)^{5,30}. Os Luminais *B-like* HER2 negativos são tratados com hormonioterapia e quimioterapia (na maioria dos casos), com esquemas baseados em antraciclina e taxano⁵. Os Luminais *B-like* HER2 positivos são tratados com terapia endócrina, trastuzumab e quimioterapia, preferencialmente com esquemas que incluem antraciclina e taxano⁵. Este grupo também tem pior resposta ao tamoxifeno por expressar genes envolvidos com resistência a esta droga²⁸. O tratamento dos HER2 positivos (não luminais) baseia-se em quimioterapia (preferencialmente esquemas contendo antraciclina e taxano) e trastuzumab⁵. Os Triplos-negativos são tratados com quimioterapia, na maioria das vezes esquemas contendo antraciclina e taxano⁵.

Embora os fatores de risco, classificação, tratamento e prognóstico no câncer de mama sejam essencialmente definidos pelos subtipos clínico-patológicos, associados ou não às assinaturas genéticas, ainda existem dúvidas sobre o papel de outros marcadores, essencialmente aqueles relacionados à inflamação e supressão tumorais.

Na relação da inflamação com a carcinogênese, as ciclooxigenases, ou COXs têm um papel fundamental. As COXs são uma família de enzimas chamadas “mieloperoxidases”, sendo enzimas-chave que catalisam os dois primeiros passos da conversão de ácido aracônico em prostaglandinas e tromboxanos³¹. Atualmente existem três formas de COX identificadas, a COX1,

a COX2 e a COX3. A COX1 é constitutiva nos tecidos e está envolvida na homeostase. A COX2 é uma isoforma induzível da enzima, sintetizada no citoplasma de células de tecidos envolvidos em processos inflamatórios e neoplásicos. Porém, um estudo recente³² demonstrou sua expressão no tecido mamário normal durante o ciclo reprodutivo da mulher jovem, com uma ampla variabilidade de expressão. Uma possível explicação é porque sua síntese é regulada por fatores de crescimento e citocinas, sendo os principais a interleucina 1 beta e o fator necrose tumoral alfa³¹. A COX3 é uma variante da COX1, constitucional no cérebro e medula espinhal, e sua função ainda não está bem estabelecida³¹.

Há anos vem-se demonstrando associação entre a expressão da COX2 e algumas neoplasias, como câncer de cólon, reto, estômago e mama³³. Existem evidências de que a COX2 esteja relacionada à carcinogênese mamária atuando na promoção da angiogênese, migração e invasão celulares e modulando o sistema imunológico de modo que reduza a imunidade antitumoral^{34,35,36,37}. No tecido mamário existe indução da COX2 em carcinomas *in situ* e invasor, além de estar presente no tecido mamário normal peritumoral^{32,38,39}. Sua expressão também está associada com marcadores amplamente utilizados para determinar prognóstico e tratamento no câncer de mama, como os receptores hormonais (RE e RP)⁴⁰ e o HER2³⁷ e com os subtipos intrínsecos de câncer de mama (Luminal A, Luminal B, HER2 superexpresso e Basal-like)^{31,41,42}. A COX2 está relacionada a vários fatores de pior prognóstico, como: a expressão do HER2, negatividade para receptores hormonais (RE e RP), comprometimento dos linfonodos axilares, menor

sobrevida global e livre de doença, metástases ósseas, associação aos subtipos intrínsecos de pior prognóstico, que são o HER2 superexpresso e o Basal-*like* e resistência a QT^{31,34,40,41,42,43,44}.

A importância de se estudar a associação da COX2 com as neoplasias, em particular com o câncer de mama, vem do seu potencial como alvo de tratamento ou quimioprevenção^{33,36}. Os primeiros agentes a serem estudados foram os inibidores seletivos da COX2, também chamados de Coxibs. Recentemente observou-se que em mulheres com carcinoma de mama dos subtipos Luminais, os inibidores da COX parecem aumentar o efeito do tamoxifeno naquelas cujos tumores apresentam alta angiogênese, avaliada pela expressão do VEGF/VEGFR2. Considerando-se que tumores com alta angiogênese são resistentes ao tamoxifeno, os inibidores da COX exercem um efeito antiangiogênico, podendo reduzir a resistência ao tratamento³⁶. Entretanto sua eficácia tem sido limitada pelos efeitos colaterais, essencialmente cardiovasculares³³. Existem outras drogas em estudo, com resultados promissores.

A Bromelaína é uma substância derivada do abacaxi que vem demonstrando ter um importante efeito anticâncer, através de vias diferentes: inibição da iniciação tumoral; inibição da proliferação celular; indução da apoptose principalmente promovendo uma regulação “para cima” do sistema p53; prejudica a sobrevivência das células malignas; atenua danos no DNA e, por fim, inibe a produção de COX2^{45,46,47}. O Targretin (bexaroteno) é um agonista dos receptores retinóides X (RXR) aprovado clinicamente para o tratamento de linfoma cutâneo de células T e de câncer de pulmão de células

não pequenas. Os agonistas RXR vêm demonstrando ter um efeito inibidor sobre a proliferação celular no câncer de mama, além de inibir a expressão de COX2 nestes tumores^{48,49}. A Metformina, biguanida utilizada para o tratamento do Diabetes tipo 2, vem demonstrando ter também efeito anticâncer, principalmente no câncer de mama. Já existem alguns mecanismos de ação conhecidos para esta ação, sendo a inibição da proliferação e do crescimento tumoral os principais explicados pela inibição da angiogênese e da expressão de COX2, entre outros mecanismos; há também evidência de que, associada ao tamoxifeno, possa reduzir a resistência a esta droga^{50,51}.

Quanto à supressão tumoral, o principal gene a ela relacionado é o TP53. O gene TP53 foi descoberto em 1979, identificado como um gene supressor da replicação celular quando existe dano no DNA. É responsável pela manutenção da integridade do genoma através da indução da apoptose celular ou colocando o ciclo celular em repouso, agindo assim como “guardião do genoma”⁵². Esse gene codifica a proteína p53, que normal é praticamente indetectável pela IQ porque apresenta meia-vida curta, e está presente em pequena quantidade dentro da célula. Já a superexpressão da p53 detectada por IQ indica mutação do gene, que leva à produção de formas estáveis e não funcionais da proteína p53⁵³. Mutação em um alelo do gene TP53 pode resultar em alteração ou inativação de sua função, sendo que o TP53 está alterado em grande número de neoplasias em humanos, inclusive no câncer de mama, além de estar associado a carcinomas familiares (Síndrome de Li-Fraumeni)^{54,55,56}.

Esta alteração tem particular importância nas regiões Sul e Sudeste do Brasil, em que foi descrita uma mutação germinativa no códon 337 do gene

TP53 (arginina por histidina), a mutação R337H⁵⁷, com uma frequência na população de 0,3%, considerada muito mais alta que outras mutações desse alelo^{58,59}. Foi descrita uma prevalência de 0,5% de mutação R337H em mulheres com câncer de mama em São Paulo e Rio de Janeiro⁶⁰. A expressão da p53 no câncer de mama está associada a fatores de mau prognóstico: tumores de alto grau, com alta taxa de proliferação celular, ocorrência em mulheres jovens, associação com receptores hormonais negativos, principalmente tumores *Basal-like*^{22,54,55,61}. Também está relacionada ao maior índice de recorrência local e menor sobrevida geral^{54,56}. Devido à importância da R337H no Sul e Sudeste do Brasil e à relevância da p53 no câncer de mama, principalmente em mulheres jovens, já existem autores propondo que a pesquisa desta mutação entre nos testes genéticos para rastreamento de carcinomas hereditários nestas regiões do país^{60,62}.

Existe uma forte relação entre a mutação TP53, mecanismos inflamatórios e o câncer de mama. Já são conhecidas as relações entre a expressão de p53 e COX2 com os tipos histológicos de carcinomas de mama e também algumas relações com seus subtipos intrínsecos. Todavia, sua relação com os correspondentes subtipos classificados por IQ, conforme proposto na *13th St Gallen International Breast Cancer Conference Expert Panel, 2013*⁵ e a repercussão para o tratamento e prognóstico de acordo com estes subtipos ainda não é conhecida. Este trabalho poderá contribuir para estabelecer estas relações entre p53, COX2 e subtipos clínico-patológicos de câncer de mama, estimar prognóstico e possibilitar o planejamento de estratégias para melhores diagnóstico e tratamento.

2. Objetivos

2.1 Objetivo Geral

Classificar as mulheres com carcinoma de mama tratadas no Hospital da Mulher Prof. Dr. José Aristodemo Pinotti- CAISM/UNICAMP nos diferentes subtipos clínico-patológicos, avaliar a expressão da COX2 e p53 em cada subtipo, correlacionar com características clínicas e patológicas do câncer de mama e com a sobrevida livre de doença e global.

2.2 Objetivos Específicos

■ Artigo 1

1. Avaliar a distribuição dos subtipos clínico-patológicos Luminal *A-like*, Luminal *B-like* HER2 positivo, Luminal *B-like* HER2 negativo, HER2 positivo (não luminal) e Triplo-negativo nas mulheres com carcinoma invasivo de mama atendidas no CAISM/UNICAMP;
2. Avaliar a relação entre a expressão dos receptores hormonais, do HER2 e do ki67;

3. Correlacionar os subtipos clínico-patológicos com as características clínicas e patológicas da doença;
4. Avaliar a sobrevida global e a sobrevida livre de doença segundo os subtipos clínico-patológicos.

Artigo 2

1. Avaliar a relação entre a expressão da COX2 e da p53 nos subtipos clínico-patológicos Luminal A-*like*, Luminal B-*like* HER2 positivo, Luminal B-*like* HER2 negativo, HER2 positivo (não luminal) e Triplo-negativo nas mulheres com carcinoma invasivo de mama atendidas no CAISM/UNICAMP;
2. Relacionar a expressão da COX2 com a p53;
3. Relacionar a expressão da COX2 e da p53 com as características clínicas e patológicas da doença;
4. Avaliar a sobrevida global e livre de doença segundo a expressão da COX2 e da p53.

3. Publicações

3.1 Artigo 1

The screenshot shows a Gmail interface in a Firefox browser window. The address bar displays the URL: <https://mail.google.com/mail/u/0/#inbox/1456201ef74bb316>. The Gmail header includes the search bar, navigation icons, and a notification to enable desktop notifications. The main content area shows an email titled "Fwd: Assigned ms.no.: THEBREAST-D-14-162" from "Kátia Serra" to "me" at 5:51 PM. The email body contains a forwarded message from "The Breast" journal, dated 2014-04-12 1:53 GMT-03:00. The subject is "Assigned ms.no.: THEBREAST-D-14-162". The recipients are "sophia.derchain@gmail.com", "derchain@bcm.unicamp.br", and "katiasserra@gmail.com". The email text reads: "Re: Submission The Breast", "Dear Prof. Derchain,", "Your submission entitled 'Clinical implications of the new Saint Gallen 2013 clinico-pathological surrogate molecular classification of breast cancer' has been assigned the following manuscript reference number: THEBREAST-D-14-162. Your manuscript is now with the editor.", "Please quote the reference number in all future communications.", "Yours sincerely,", "Editorial Office", "The Breast". Below the email content is a text input field with a "Click here to Reply or Forward" link. On the right side of the email, there are advertisements for "Dental Cremer", "Instrumentos Usados", "Netflix de Graça", and "Compre Celular por R\$89". The left sidebar shows the Gmail navigation menu with categories like "Inbox", "Spam (296)", "Personal (47)", "Travel", and "Work (13)". The Windows taskbar at the bottom shows the system tray with the date "14/04/2014" and time "17:51".

Clinical implications of the new Saint Gallen 2013 clinico-pathological surrogate molecular classification of breast cancer

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Keywords: breast cancer; clinico-pathological surrogate subtypes; Saint Gallen.

Abstract:

Background: The intrinsic subtypes of breast cancer classification has been proven as straightforward strategy to predict patient survival and response to treatment.

Objectives: We tested the association of the current clinic-pathological surrogate subtypes of breast cancer with the main prognostic and predictive factors in a relatively large dataset of breast cancer Brazilian patients, which were followed up for almost four years. We discuss the advantages and possible caveats related to this new classification.

Results: Approximately 75% of the tumors were classified as luminal-type-like. HER2 positive (non-luminal) tumors accounted for 9.3% of the cases and Triple-negative tumors, 13.1%. Luminal B-like and HER2 positive (non-luminal) tumors were associated with higher histological grades. Luminal A-like tumors were significantly associated with better overall and disease free survival. **Conclusion:** The more strict criteria to define Luminal A-like tumors increased the accuracy of the classification by selecting tumors that share a good prognosis and response to endocrine therapy.

Introduction

The intrinsic subtypes of breast cancer classification has been proven as straightforward strategy to predict patient survival and response to treatment^{1,2,3,4,5}. Current lines of research on breast cancer almost invariably should be designed taking into account the biological and clinical knowledge related to that classification^{6,7,8}. In the clinical practice, the original classification, based on DNA arrays, has been proven unfeasible due to technical and economical constraints and was replaced with a surrogate version, based on the proteic expression of the key molecules. It was only in 2011 during the 12th St Gallen International Breast Cancer Conference Expert Panel that the surrogate classification has been validated and adopted as the standard way to classify tumors for therapeutic deliberations⁹. The surrogate subtypes were thus determined on the sole basis of the protein expression of steroid [estrogen (ER) and progesterone (PR)] receptors, HER2 (the status of this marker should be determined with Fluorescent *in situ* Hybridization when immunohistochemistry results are equivocal), and ki67. A residual debate persisted with regards to the thresholds to be adopted for each molecular marker.

In the 13th St Gallen International Breast Cancer Expert Panel Conference 2013¹ the classification of the surrogate subtypes was modified again. This time, the debaters considered the semi quantitative evaluation of the expression of PR in the clinical course and response to treatment. One of the key studies used for the review of the classification parameters was that of Prat et al.⁸, who evaluated the prognostic value of the proportion of cells that express PR genes and protein in tumors classified as Luminal A. When comparing the intrinsic (molecular) and clinico-pathological classification for over two thousand patients with Luminal A and B breast carcinomas, they observed that

Luminal A tumors had a score of positivity for PR much higher than Luminal B tumors, which prompted establishing a new positivity threshold of 20% stained nuclei for the progesterone receptor status. Using this new threshold, Prat et al⁸ proposed a new definition for Luminal A tumors, which was adopted by St. Gallen in 2013. Therefore, two clinico-pathological surrogate subtypes received new definitions: Luminal A-like was defined as tumors ER positive, HER2 negative, ki-67 <14% and PR expression in $\geq 20\%$ of the nuclei, and Luminal B-like HER2 negative tumors that are ER positive, HER2 negative and ki67 high ($\geq 14\%$) or PR negative ($< 20\%$)¹. Therefore, only tumors with the best prognosis were considered Luminal A-like, proportionally reducing the cancers in this category. Part of tumors previously classified as Luminal A were reclassified as Luminal B-like HER2 negative, with formal indication to receive cytotoxic treatment, more consistent with the prognosis of these cancers.

The molecular classification of breast cancer is in its infancy and many new lines of evidence emerge constantly. We tested the association of the current clinic-pathological surrogate subtypes of breast cancer with the main prognostic and predictive factors in a relatively large dataset of breast cancer Brazilian patients, which were followed up for four years. We discuss the advantages and possible caveats related to this new classification.

Methods

Selection of the patients

A total of 183 breast carcinoma samples were obtained from women treated at the Women's Hospital of Campinas State University, Campinas, Brazil, between June 2008 and January 2011. Tissue microarrays (TMA) were constructed from the original paraffin blocks for immunohistochemistry (IHC) and fluorescent *in situ* hybridization (FISH) analyses. Samples from patients who were pregnant at the time of diagnosis and from those who received neoadjuvant chemotherapy were not included. Cases for which TMA staining was not feasible due to technical constraints were assessed using slides derived from the original paraffin blocks.

Histology

Samples were retrieved from the Hospital's archives. Criteria from the World Health Organisation were used to classify the invasive breast tumors¹⁰.

TMA construction

One seasoned pathologist (JV) selected the areas for TMA sampling with specialized needles (Beecher Instruments Microarray Technology, Silver Spring, CA, USA). Perforations of 1.0mm were performed in the selected areas and transferred to the TMA block. Silanized slides were then produced for the subsequent experiments (IHC and FISH).

Immunohistochemistry (IHC)

Immunohistochemistry was performed to detect the expression of ER, PR and ki67 in 183 breast cancer samples. Briefly, sections were deparaffinized with xylene and dehydrated in alcohol series. Endogenous peroxidase activity was blocked by using 0.3% hydrogen peroxide, followed by washes with distilled water. For antigen retrieval, we used a commercially available pressure cooker (Pascal, supplied by Dako, Carpinteria, CA, USA), in which slides were immersed in citrate buffer pH 6 for 30 min. The slides were dried at room temperature and washed in distilled water. Then, the sections were incubated in a moist chamber, with the specific primary antibodies at 4 °C overnight (ER: clone 1D5 1:1000, Dako; PR: clone PgR 636 1:800, Dako; ki67: clone MIB1 1:500, Dako). The slides were then washed in PBS, pH 7.4, then incubated in Advance™ HRP Detection System (Dako) at 37 °C for 1 h, and washed in PBS. For detection, DAB chromogenic substrate (3'-diaminobenzidine, Sigma–Aldrich, St. Louis, MO, USA) was applied, at the proportion 0.06 g to 100 ml of PBS, 500 µl hydrogen 3% peroxide and 1 ml dimethylsulfoxide (DMSO) at 37 °C for 5 min. Finally, the slide was washed in tap water and counterstained with Harris' hematoxylin. After being dehydrated, slides were mounted in resin (Entellan[®], Merck, Darmstadt, Germany). Internal and external, positive and negative controls were used to validate the reactions. Stained cells in each tissue were counted under a light microscope by an experienced pathologist.

Image analysis

The IHC staining was assessed independently by two observers who were unaware of the clinical and pathological features of the disease. Two TMA sets of each tumor (and individual slides from the original paraffin blocs when TMA analyses were impossible) were used for each marker. In *post hoc* analysis, if scores differed in the two analyses, the higher staining score (see below) was considered. Nuclear IHC staining was considered for ER, PR and ki67. For ER, a “positive” result was rendered when >1% of the nuclei were stained. For PR, a “positive” result was rendered when $\geq 20\%$ of the nuclei were moderately to strongly stained. For ki67, the “positive” status was granted to cases with $\geq 14\%$ moderate/strongly stained nuclei.

Fluorescent *In Situ* Hybridization (FISH)

HER2 status of the 183 specimens was assessed using FISH. Gene-specific probes (17q12-SE17; Kreatech[®], Amsterdam, Netherlands) were labeled in red (Red dUTP; Abbott Molecular, Inc.; cat. no. 02N34-050) and were applied onto samples together with commercial probes for the corresponding chromosome centromere (CEN17, Kreatech Diagnostics, cat. no. SE17 D17Z1; CEN8, Kreatech Diagnostics, cat. no. SE8 D8Z2; CEN11, Kreatech Diagnostics, cat. no. SE11 D11Z1), labeled in green, as an internal control.

The gene/centromere statuses were assessed by a single-blinded observer. For each core in the tissue microarray slide, 40 signals were observed during analysis and were evaluated as $< 2/2$, $2/2$, or $> 2/2$, with regard to the aneuploidy status. Gene gain or loss was elucidated from these results.

Clinico-Pathological Surrogate subtypes of breast cancer

Tumors were subtyped into five distinct categories according to the Clinico-Pathological surrogate definitions of intrinsic subtypes of breast cancer defined during the 13th St Gallen International Breast Cancer Conference¹ and recently published.

Estrogen/progesterone receptor and ki67 statuses were determined using immunohistochemistry. All cases were tested for HER2 status using FISH. The following definitions were thus used to determine the tumor Clinico-Pathological surrogate subtypes:

Luminal A-like: ER and PR positive, HER2 negative and ki67 low (<14%); Luminal B-like HER2 negative: ER positive, HER2 negative and ki67 high ($\geq 14\%$) or PR negative (<20%); Luminal B-like HER2 positive: ER positive, HER2 over-expressed or amplified, any ki67 and any PR; HER2 positive (non-luminal): HER2 over-expressed or amplified, ER and PR absent; Triple-negative (ductal): ER and PR absent, HER2 negative.

Data analysis

Chi-squares were used to assess the associations between the combined steroid receptor statuses and ki67 expression. Next, pairwise comparisons of each combined status with the dichotomous ki67 status classification were performed using chi-squares or Fisher's exact test were appropriate. Chi-squares were also used to evaluate the associations between the clinical and pathological features of patients and their tumors with the surrogate subtypes. Differences in survival were assessed using log-ranks. $p < 0.05$ was considered significant. Mean follow-up time was 2.94 years (90% central

range = 0.93 to 4.1 years). Calculations were performed with the R Environment for statistical computing¹¹.

Results

Table 1 shows the distribution of the clinico-pathological surrogate subtypes across the 183 selected breast cancer specimens. Approximately 75% of the tumors were classified as luminal-type-like. Roughly half (49.7%) of the cases were classified as Luminal B-like (14.2% were HER2 positive). HER2 positive (non-luminal) tumors accounted for 9.3% of the cases and Triple-negative tumors for the remainder 13.1%.

Table 2 shows the expression of ki67 in groups of tumors with ER/PR/HER2 varying setups. In luminal tumors, ki67 positivity reached its peak in ER+ PR- /HER2+ (67%), contrasting to only 25% in ER+ PR+ /HER2+ tumors. For non-luminal tumors, ki67 positivity was higher in HER2- cases (63%) compared to HER2+ tumors (47%). However, these differences were not statistically different.

There was no association between age, menopausal status, tumor size, axillary lymph node status, and disease stage and clinico-pathological surrogate subtype (**Table 3**). In contrast, Luminal B-like and HER2 positive (non-luminal) tumors were associated with higher histological grades when compared to Luminal A-like tumors ($p < 0.01$) (**Table 4**).

Figure 1 shows the Kaplan Meier representation of disease-free survival according to the clinico-pathological surrogate subtype of the tumors. Luminal A-like tumors were significantly associated with better survival when compared to HER2 positive (non-luminal) ($p = 0.01$) and Triple-negative ($p = 0.01$) tumors. Comparing overall

survival (**Figure 2**), Luminal A-like tumors were associated with a better prognosis when compared to HER2 positive (non-luminal) and Triple-negative tumors.

Discussion

Using the most recent clinico-pathological surrogate subtype classification of breast tumors, we detected a 50% prevalence of Luminal B-like tumors in a series of Brazilian women living in a densely populated urban region. Luminal A-like tumors accounted for roughly 30% of the tumors whereas HER2 positive (non-luminal) and Triple-negative tumors accounted for the 20% remainder. Not unexpectedly, cell proliferation rate (ki67 expression) was associated with negative HER2 status and Triple-negative subtype. In general, Luminal A-like tumors were well differentiated compared to the other subtypes.

This is the first study to describe the molecular subtype prevalence in Brazilian women using the most recent classification. It is worth noting that the distribution of the Luminal tumors differs from that described in previous studies, in which Luminal A tumors accounted for approximately 40% of the tumors whereas the Luminal B subtype was described for only 10 to 20% of the tumors^{12,13,14,15}. This departure from the usual proportion is most likely associated to the recent classification modifications that took place during the Saint Gallens' 2013 Conference, after which the threshold for PR positivity was dramatically raised from 1% to 20% of stained nuclei. This single move swapped the relative position of Luminal A-like and B-like tumors in the prevalence rank.

Results from an important study recently indicated that PR is an important prognostic factor in order to properly define subgroups with different prognosis within

the Luminal B-like subtype, irrespective of HER2 overexpression or amplification. The prognostic and predictive value of PR has been for a long time ascribed to the dependence of PR expression on ER activity, with the absence of the PR reflecting a nonfunctional ER and resistance to hormonal therapy¹⁶. However, the decision to raise the PR threshold during the last Saint Gallen conference is related to the fact that the previous classification using the 1% threshold many tumors classified as Luminal A-like were intrinsic Luminal B tumors, and the new classification possibly corrects this defect. It is expected that the new classification may bear a better correlation with disease prognosis, since patients with early breast cancer with tumors that are ER positive and PR positive (ie, Luminal A-like) have lower risks of recurrence and mortality compared with women with ER positive and PR negative tumors. One pivotal study showed that women with ER-positive/PR-negative, ER-negative/PR-positive, or ER-negative/ PR-negative tumors experienced higher risks of mortality compared with women with ER-positive/PR-positive tumors, independent of the various demographic and clinical tumor characteristics¹⁷. These data led to the conclusion that IHC subtype-based definitions of genomically defined luminal A and B tumors are imperfect and may give room to misinterpretations regarding the prognosis of breast tumors. One way to overcome the apparent incongruence between the IHC-based and molecular based subtype classifications is therefore to define Luminal A-like tumors as those HR-positive/HER2-negative/ki-67 less than 14% and PR more than 20%.

This study also offers some prospective data on the prognostic value of the new classification. These data may aid in the clinical validation of the IHC classification using the more strict cutoff points for PR. Our analyses confirmed the strong prognostic value of the new classification, since Luminal A-like tumors were significantly better

positioned in terms of a favorable prognosis than their counterparts and Luminal B-like with overexpression of HER2 also displayed better disease-free survival than the HER2 positive (non-luminal) tumors.

The recent consensus also established that the clinic-pathological subtypes should dictate treatment options. For instance, it has been shown that Luminal A-like tumors benefit the most from endocrine therapy, whereas Luminal B-like tumors should also be treated with chemotherapy, endocrine therapy and trastuzumab if HER2 overexpressed. HER2 positive (non-luminal) tumors benefit from chemotherapy with anthracyclines and taxanes and Triple-negative tumors should be aggressively managed with chemotherapy, although target therapy is under testing (e.g PARP inhibitors)^{1,9,18}.

Our study also addressed whether a few epidemiological features of the women were associated with the new molecular subtypes of breast cancer. We found no association between age and menopausal status with the clinico-pathological surrogate subtypes. Recently, the association of the breast cancer subtypes with reproductive factors has been examined to in depth in a review of the literature¹⁹. That review showed significant heterogeneity in reproductive risk factors for the distinct subtypes of breast tumors, with variation in strength and consistency of the associations. The strongest evidence exists for hormone receptor positive breast cancers, with nulliparity, current use of hormonal therapy, and prolonged interval between menarche and age at first birth being the most robust. Increased age at first birth and increased age at menopause were consistently associated with hormone receptor positive cancers in a majority of studies; and though less consistent, younger age at menarche was also positively associated with these tumors, whereas, longer periods of lactation and oral contraceptive use were associated with lower risk. The higher prevalence of hormone negative breast cancers

among African, American, and Hispanic women has been hypothesized to be due to an interaction between genetic and reproductive risk factors such as increased parity^{20,21}. Findings from that review show that although risk of Triple-negative is associated with some reproductive factors, but the results were largely inconsistent¹⁹. Other factors such as obesity and low levels of physical activity seem to be associated with an augmented risk of Luminal and non-luminal tumors²², but nuliparity is associated with an increased risk for ER+ tumors. Other reproductive factors are also associated with increased risk for specific clinico-pathological types, such as having the first offspring later in life, which is associated with an increased risk of developing a HER2 positive tumor²².

Our study clearly corroborates the prognostic significance of the most recent classification of clinico-pathological surrogate subtypes of breast cancer. In fact, we believe that the more strict criteria (i.e. the 20% positivity threshold for progesterone receptors) to define Luminal A-like tumors increased the accuracy of the classification by selecting tumors that share a good prognosis and an excellent response to endocrine therapy. For this particular small subset of patients, the literature shows very small or no benefit derived from chemotherapy. In order to safely choose patients for a less aggressive treatment, we believe that the new classification must be adopted.

Ethics

The present study has been approved by the Ethics Committee, FCM/UNICAMP (CEP 1246/2009).

Acknowledgements

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Conflicts of interest

The authors have no conflict of interest to declare.

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Table 1. Clinico-pathological surrogate subtype classification of the breast tumors.

Subtype	N	%
Luminal A-like	51	27.8
Luminal B-like	91	49.7
Luminal B-like HER2 positive	26	14.2
Luminal B-like HER2 negative	65	35.5
HER2 positive (non-luminal)	17	9.3
Triple-negative	24	13,1
Total	183	100

Table 2.ki76 expression in Luminal and Non Luminal-like tumors with different steroid and HER2 expression patterns.

	ki67 <14%	ki67 ≥14%	Total	
Steroid receptors/HER2	n (%)	n (%)	n(100%)	p
Luminal tumors				
ER+ PR+ /HER2-	51(70)	22(30)	73	0.31
ER+ PR+ /HER2+	15(75)	5(25)	20	
ER+ PR- /HER2-	30(70)	13(30)	43	
ER+ PR- /HER2+	2(33)	4(67)	6	
Non-luminal tumors				
ER- PR- /HER2+	9(53)	8(47)	17	0.36
ER- PR- /HER2-	9(37)	15(63)	24	
Total	116(63)	67(37)	183(100)	

Table 3. Epidemiological and clinical features of the women and clinico-pathological surrogate subtype classification.

Characteristics	Total n(%)	Luminal A- like n(%)	Luminal B- like n(%)	HER2 positive (non- luminal) n(%)	Triple- negative n(%)	p
Age (years)						
<=35	7(4)	0(0)	3(3)	0(0)	4(17)	0.09
36-49	56(30)	17(33)	26(29)	5(29)	8(33)	
≥50	120(66)	34(67)	62(68)	12(71)	12(50)	
Menopausal						
No	63(34)	17(33)	31(34)	5(29)	10(42)	0.86
Yes	120(66)	34(67)	60(66)	12(71)	14(58)	
Tumor size						
T1-T2	139(76)	34(67)	72(79)	13(76)	20(83)	0.30
T3-T4	44(24)	17(33)	19(21)	4(24)	4(17)	
Axillary lymph nodes						
N0	90(49)	24(48)	49(54)	4(23)	13(54)	0.09
N1	40(22)	15(29)	13(14)	7(42)	5(21)	
N2-N3	53(29)	12(23)	29(32)	6(35)	6(25)	
Stage						
I-II	109(57)	29(59)	54(59)	10(59)	16(75)	0.89
III-IV	74(43)	22(41)	37(41)	7(41)	8(25)	

Table 4. Pathological features of the women and clinico-pathological surrogate subtype classification.

Pathological features	Total n(%)	Luminal A-like n(%)	Luminal B-like n(%)	HER2 positive (non- luminal)n(%)	Triple- negative n(%)	p
Grade±						
I-II	35(20)	19(37)	12(13)	0(0)	4(17)	<0.01*
III	147(80)	32(63)	78(87)	17(100)	20(83)	
Nuclear grade±						
1-2	56(31)	26(51)	26(29)	2(12)	2(8)	<0.01*
3	126(69)	25(49)	64(71)	15(88)	22(92)	
Peritumoral angiolymphtic invasion						
No	122(67)	36(71)	61(67)	7(41)	18(75)	0.10
Yes	61(33)	15(29)	30(33)	10(59)	6(25)	
Peritumoral perineural invasion						
No	160(87)	45(88)	80(88)	13(76)	22(92)	0.53
Yes	23(13)	6(12)	11(12)	4(24)	2(8)	
Dermal invasion±						
No	166(91)	43(84)	86(96)	14(82)	23(96)	0.05
Yes	16(9)	8(16)	4(4)	3(18)	1(4)	
In situ component						
No	63(34)	16(31)	35(38)	3(18)	9(37)	0.39
Yes	120(66)	35(69)	56(62)	14(82)	15(63)	
Histological type±						
Ductal	165(90)	47(89)	78(86)	17(100)	23(96)	0.48
Lobular	11(6)	2(9)	9(10)	0(0)	0(0)	
**Other	6(4)	1(2)	4(4)	0(0)	1(4)	

±One case missing; *Pairwise comparisons of the clinical features in the different surrogate subtypes:
***Grade:** Luminal A-like vs Luminal B-like (p<0.01); Luminal A-like vs HER2 positive (p<0.01).
Nuclear grade:** Luminal A-like vs Luminal B-like (p=0.01) *Other types:** 2 medular, 1 medular atypical, 1 pleomorphic apocrine, 1 metaplastic and 1 colloid.

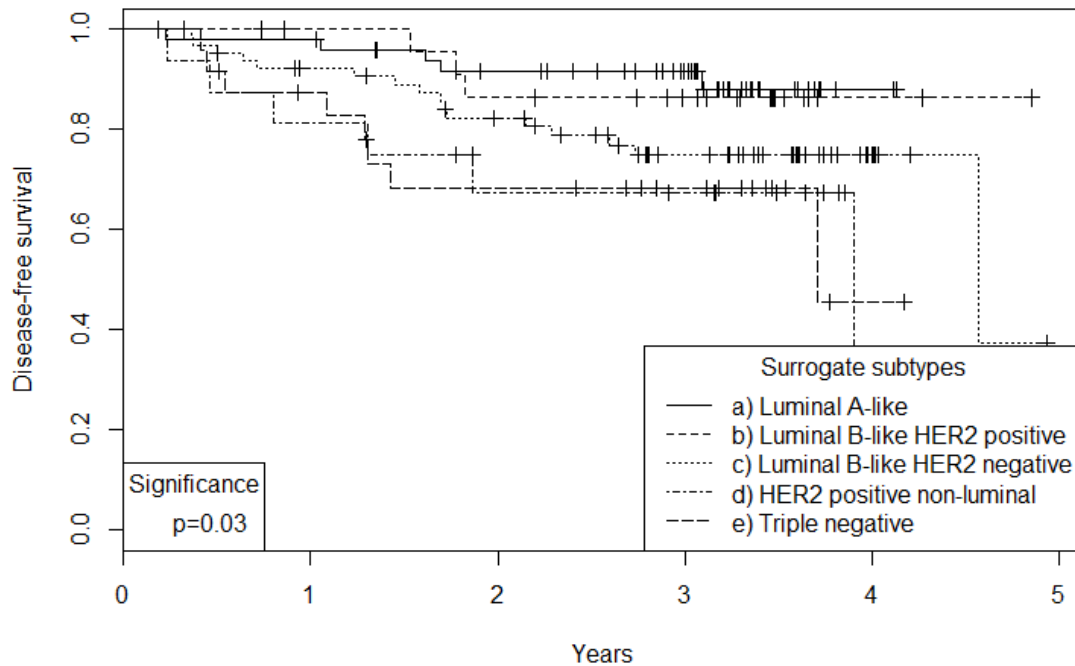


Figure 1. Disease free survival comparison of patients with different clinico-pathological surrogate subtypes of breast cancer (Saint Gallen 2013 classification).

Note: Significant pairwise log-rank comparisons of disease-free survival: Luminal A-like vs HER2 positive (non luminal) ($p=0.01$); Luminal A-like vs Triple-negative ($p=0.01$); Luminal B-like HER2 positive vs HER2 positive (non-luminal) ($p=0.01$); Luminal B-like HER2 positive vs Triple-negative ($p=0.02$).

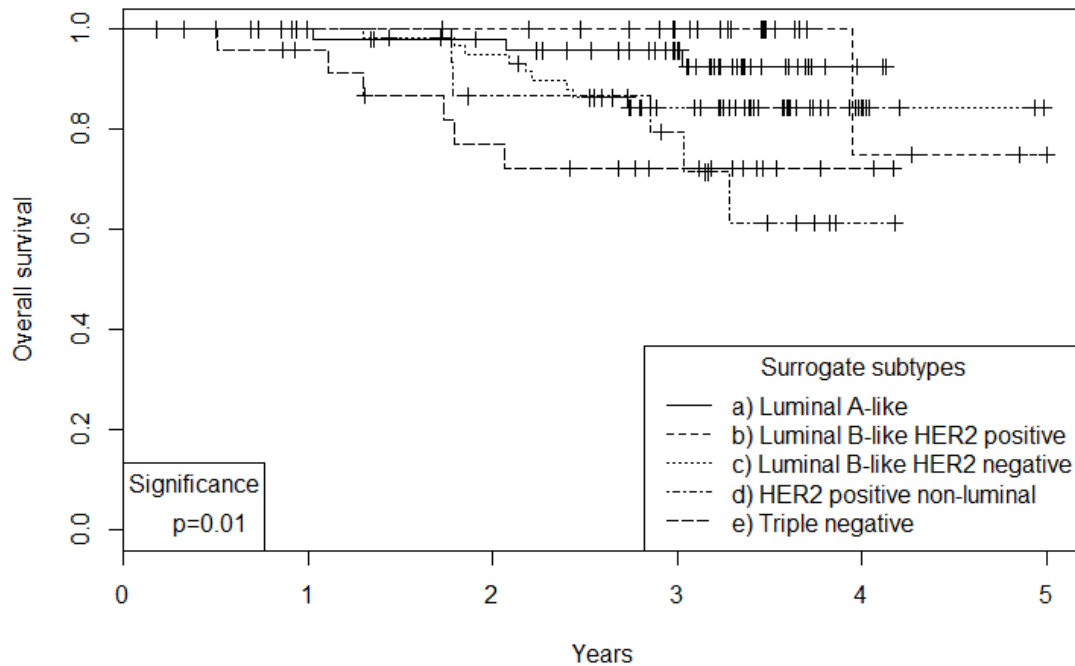


Figure 2. Overall survival comparison of patients with different clinico-pathological surrogate subtypes of breast cancer (Saint Gallen 2013 classification).

Note: Significant pairwise log-rank comparisons of overall survival: Luminal A-like vs HER2 positive (non-luminal) ($p=0.01$); Luminal A-like vs Triple-negative ($p=0.01$).

3.2 Artigo 2

The screenshot shows a Gmail interface in a browser window. The email is from BioMed Central Editorial, dated 4:05 PM (0 minutes ago). The subject line is "H04465211277041 Cyclooxygenase-2 (COX2) and p53 protein expression are interdependent in breast cancer but not associated with clinico-pathological surrogate subtypes, tumor aggressiveness and patient survival." The email body contains the following text:

Dear Prof Sarhan

Thank you for submitting your article. This acknowledgement and any queries below are for the contact author. This e-mail has also been copied to each author on the paper, as well as the person submitting. Please bear in mind that all queries regarding the paper should be made through the contact author.

A pdf file has been generated from your submitted manuscript and figures. We would be most grateful if you could check this file and let us know if any aspect is missing or incorrect. Any additional files you uploaded will also be sent in their original format for review:

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For your records, please find below link(s) to the correspondence you uploaded with this submission. Please note there may be a short delay in creating this file.

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In the meantime, if you have any queries about the manuscript you may contact us on editorial@biomedcentral.com. We would also welcome feedback about the online submission process.

Best wishes,

The BMC Cancer Editorial Team

The screenshot also shows a sidebar with a list of contacts under "Personal (47)" and "Work (13)", and a right-hand sidebar with advertisements for language learning, Netflix, musical instruments, and dental services.

Cyclooxygenase-2 (COX2) and p53 protein expression are interdependent in breast cancer but not associated with clinico-pathological surrogate subtypes, tumor aggressiveness and patient survival.

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Keywords: breast cancer, clinico-pathological surrogate subtypes, Saint Gallen, COX2, p53.

Abstract

Background: In the last decade, different molecular subtypes of breast cancer have been proposed. Although displaying appreciable association with disease prognosis and the prognostic value of cytotoxic and endocrine therapeutic modalities, the subtypes seem to fail at completely explaining disease behavior and response to treatment. Molecules such as those of the cyclooxygenase (COX) family, currently composed of three entities (COX 1, 2 and 3) have been shown to be associated with breast carcinogenesis, and the analysis of p53 expression in breast tumors may also offer some additional prognostic clues. Our study is aimed at assessing COX2 and p53 expression in these clinico-pathological surrogate subtypes, and to evaluate whether the expression of these molecules can help further explain the variability in prognosis still found within the clinico-pathological subtypes groups of breast cancer. **Methods:** A total of 183 breast cancer samples were obtained from women treated at the Women's Hospital of Campinas State University, Campinas, Brazil, between June 2008 and January 2011. Immunohistochemistry was performed to detect the expression of ER, PR, ki67, COX2, and p53 and the HER2 status of the 183 specimens was assessed using FISH. **Results:** There was no trend in COX2 overexpression from Luminal A-like to Triple-negative subtypes. By contrast, p53 was expressed in roughly 67% of the Luminal A-like tumors, 50% of the Luminal B-like HER2 positive tumors, 60.9% of the Luminal B-like HER2 negative, approximately 82% of the HER2 positive (non-luminal) and 87% of the Triple-negative tumors (p for trends = 0.06). There was a significantly higher proportion of COX2 positive tumors (66.9%) when p53 was also positive compared to when the tumor was negative for p53 (in which case only 18.0% of the tumors were positive for COX2; $p < 0.001$). Neither marker was found to be associated with patients' survival.

Conclusions: There seems to be a positive association between the expressions of COX2 and p53. On the other hand, neither the expression of COX nor that of p53 was associated with clinico-pathological subtypes, tumor features and prognosis. It seems to be too early to elect the detection of COX2 using IHC as prognostic or predictive tool, but incipient evidence points towards a possible role for the marker.

Introduction

In the last decade, different molecular subtypes of breast cancer have been proposed, essentially following the data observed by Perou et al.^{1,2,3,4}. Recently these molecular subtypes were redefined as surrogate clinico-pathological subtypes at the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013⁵. Although displaying appreciable association with disease prognosis and the prognostic value of cytotoxic and endocrine therapeutic modalities, the subtypes seem to fail at completely explaining disease behavior and response to treatment. The study of consolidated and novel molecules that are known to bear prognostic significance in other tumors seems to be still valid in breast pathology^{6,7,8}. One such group of novel molecules is the cyclooxygenase (COX) family, currently composed of three entities (COX 1, 2 and 3). COX2 is the inducible isoform of the enzyme. COX2 is synthesized in the cytoplasm of cells involved in inflammatory and neoplastic processes.

In a recent study, the expression of COX2 in normal breast tissues has been demonstrated to fluctuate during the menacme⁹. One possible explanation for this finding is that the synthesis of the enzyme is regulated by growth factors such as cytokines, chiefly among these interleukin 1 beta and tumor necrosis factor alpha⁶.

It has long been suggested that COX2 may be involved in carcinogenesis of colon, rectum, stomach and breast tumors¹⁰. In breast pathology, there is evidence suggesting that COX2 is associated with angiogenesis, tumor cells migration and invasion, and down-modulation of the immune system^{8,11,12,13}. There is COX2 induction in *in situ* and invasive carcinomas, in the tumor and surrounding tissues^{9,14,15}. Studies demonstrated an increased expression of COX2 in Triple-negative and HER2 positive (non-luminal) tumors^{6,7,8,16,17}, and the enzyme has also been linked to factors associated

with a worse prognosis such as positive axillary nodes, bone metastases, chemotherapy resistance and worse survival^{6,7,11,16,17,18,19}.

The analysis of p53 expression in breast tumors may also offer some additional prognostic clues. Non-functional forms of the protein can be detected by immunohistochemistry (IHC) when its incoding gene (TP53) is defective (mutated)²⁰. p53 expression in breast tumors is associated with high-grade, rapidly proliferating, Triple-negative disease and is relatively common in tumors that occur in young women^{4,21,22,23}. COX2 and p53 expression may be linked, since there is a strong relationship between TP53 mutation and inflammation^{15,24}. However, their relationship with the corresponding subtypes ranked by IHC as proposed in the 13th St Gallen International Breast Cancer Expert Panel Conference, 2013 and the repercussions for the treatment and prognosis according to these subtypes are not yet known.

Although scattered, there is substantial evidence suggesting a complimentary role for COX2 and p53 detection in the prognostic evaluation of breast tumors. These molecules may add clinical information to the now standard clinico-pathological surrogate molecular classification of breast tumors. Thus our study is aimed at assessing COX2 and p53 expression in these surrogate subtypes, and to evaluate whether the expression of these molecules can help further explain the variability in prognosis still found within the surrogate molecular groups of breast cancer.

Methods

Selection of the patients

A total of 183 breast cancer samples were obtained from women treated at the Women's Hospital of Campinas State University, Campinas, Brazil, between June 2008 and January 2011. Tissue microarrays (TMA) were constructed from the original paraffin blocks for immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) analyses. Samples from patients who were pregnant at the time of diagnosis and from those who received neoadjuvant chemotherapy were not included.

Histology

Samples were retrieved from the Hospital's archives. Criteria from the World Health Organisation were used to classify the invasive breast tumors²⁵.

TMA construction

One seasoned pathologist (JV) selected the areas for TMA sampling with specialized needles (Beecher Instruments Microarray Technology, Silver Spring, CA, USA). Perforations of 1.0mm were performed in the selected areas and transferred to the TMA block. Silanized slides were then produced for the subsequent experiments (immunohistochemistry and FISH).

Immunohistochemistry (IHC)

Immunohistochemistry was performed to detect the expression of ER, PR, ki67, COX2, and p53 in 183 breast cancer samples. Briefly, sections were deparaffinized with xylene and dehydrated in alcohol series. Endogenous peroxidase activity was blocked by using 0.3% hydrogen peroxide, followed by washes with distilled water. For antigen retrieval, we used a commercially available pressure cooker (Pascal, supplied by Dako, Carpinteria, CA, USA), in which slides were immersed in citrate buffer pH 6 for 30 min. The slides were dried at room temperature and washed in distilled water. Then, the sections were incubated in a moist chamber, with the specific primary antibodies at 4 °C overnight (ER: clone 1D5 1:1000, Dako; PR: clone PgR 636 1:800, Dako; ki67: clone MIB1 1:500, Dako; COX2: clone CX-294 1:100, Dako; p53: clone DO-7 1:1500, Dako). The slides were then washed in PBS, pH 7.4, then incubated in Advance™ HRP Detection System (Dako) at 37 °C for 1 h, and washed in PBS. For detection, DAB chromogenic substrate (3'-diaminobenzidine, Sigma–Aldrich, St. Louis, MO, USA) was applied, at the proportion 0.06 g to 100 ml of PBS, 500 µl hydrogen 3% peroxide and 1 ml dimethylsulfoxide (DMSO) at 37 °C for 5 min. Finally, the slide was washed in tap water and counterstained with Harris' hematoxylin. After being dehydrated, slides were mounted in resin (Entellan®, Merck, Darmstadt, Germany). Internal and external, positive and negative controls were used to validate the reactions. Stained cells in each tissue were counted under a light microscope by an experienced pathologist.

Image analysis

The IHC staining was assessed independently by three observers, blind to the clinical and pathological features of the disease. Two TMA sets of each tumor (and individual slides from the original paraffin blocs when TMA analyses were impossible) were used for each marker. In *post hoc* analysis, if scores differed in the two analyses, the higher staining score (see below) was considered. Nuclear IHC staining was considered for ER, PR, p53 and ki67. For ER, a “positive” result was granted when >1% of the nuclei were stained; for PR, a “positive” result was rendered when >20% of the nuclei were moderately to strongly stained. For ki67, the “positive” status was granted to cases with >14% moderate/strongly stained nuclei. For p53, a positive result was granted when a percentage of 1% of stained nuclei was found. The German Immunoreactive Score was used to grade COX2 expression. Cytoplasmic expression was graded from 0 – absent, 2 – moderate, and 3 – strong. Then, the percentage of positive cells was converted into five categories (0 – absent/ 1 – 1 to 10%/ 2 – 11 to 50%/ 3 – 51 to 80%, and 4- 81 to 100%). The final score was calculated multiplying the two scores. A “positive” result was ascertained for cases with a final score >4.

Fluorescent *In Situ* Hybridization (FISH)

HER2 status of the 183 specimens was assessed using FISH. Gene-specific probes (17q12-SE17; Kreatech[®], Amsterdam, Netherlands) were labeled in red (Red dUTP; Abbott Molecular, Inc.; cat. no. 02N34-050) and were applied onto samples together with commercial probes for the corresponding chromosome centromere (CEN17, Kreatech Diagnostics, cat. no. SE17 D17Z1; CEN8, Kreatech Diagnostics, cat. no. SE8 D8Z2; CEN11, Kreatech Diagnostics, cat. no. SE11 D11Z1), labeled in green, as an internal control.

The gene/centromere statuses were assessed by a single-blinded observer. For each core in the tissue microarray slide, 40 signals were observed during analysis and were evaluated as $<2/2$, $2/2$, or $>2/2$, with regard to the aneuploidy status. Gene gain or loss was elucidated from these results.

Clinico-pathological surrogate subtypes of breast cancer

Tumors were subtyped into five distinct categories according to the Surrogate definitions of molecular subtypes of breast cancer defined during the 13th St Gallen International Breast Cancer Conference (2013) and recently published⁵.

Estrogen/progesterone receptor and ki67 statuses were determined using IHC. All cases were tested for HER2 status using FISH. The following definitions were thus used to determine the tumor surrogate subtypes:

Luminal A-like: ER and PR positive, HER2 negative and ki67 low (<14);

Luminal B-like HER2 negative: ER positive, HER2 negative and ki67 high ($\geq 14\%$) and or PR negative ($<20\%$);

Luminal B-like HER2 positive: ER positive, HER2 over-expressed or amplified, any ki67 and any PR;

HER2 positive (non-luminal): HER2 over-expressed or amplified, ER and PR negative;

Triple-negative (ductal): ER and PR absent, HER2 negative.

Data analysis

Chi-squares were used to assess the associations between the combined steroid receptor statuses and ki67 expression. Next, pairwise comparisons of each combined status with the dichotomous ki67 status classification were performed using chi-squares or Fisher's exact test were appropriate. Chi-squares for trends were used to test whether p53 expression increased from Luminal A to Triple-negative tumors. Chi squares were also used to evaluate the associations between the clinical and pathological features of patients and their tumors with the surrogate subtypes. Kaplan-Meyer curves were produced to depict overall (OS) and disease-free survival (DFS) according to p53 status and COX2 status. Differences in survival were assessed using log-ranks. $p < 0.05$ was considered significant. Calculations were performed with the R Environment for statistical computing²⁶.

Results

Table 1 shows the proportions of positive COX2 and p53 cases as related to the surrogate subtypes of breast cancer. There was no trend in COX2 overexpression from Luminal A to Triple-negative subtypes. By contrast, p53 was expressed in roughly 67% of the Luminal A-like tumors, 50% of the Luminal B-like HER2 positive tumors, approximately 82% of the HER2 positive (non-luminal) and 87% of the Triple-negative tumors (p for trends = 0.06).

The cross tabulation of COX2 and p53 expression is shown in **Table 2**. There was a significantly higher proportion of COX2 positive tumors (66.9%) when p53 was also positive compared to when the tumor was negative for p53 (in which case only 18.0% of the tumors were positive for COX2; $p < 0.001$).

Table 3 shows the distribution of COX2 overexpression according to p53 status and the surrogate molecular subtypes of the tumors. In neither p53 positive nor p53 negative tumors the subtypes were related to COX2 status ($p = 0.49$ and 0.23 , respectively).

None of the clinical features of the tumors was found to be associated with COX2 or p53 expression (**Table 4**). p53 expression was associated with undifferentiated nuclear grade 3 ($p = 0.04$); none of the other pathological characteristics were associated with COX2 and p53 expression (**Table 5**).

The Kaplan Meyer representations of disease free survival and overall survival as related to p53 and COX2 statuses are depicted in **Figure 1**. Neither marker was found to be associated with patients' survival.

Discussion

COX2 expression has been demonstrated to be common in breast cancer. In our study, approximately 50% of the tumors were positive for COX2, a finding in close alignment with the results of a pooled analysis of 12 studies, which showed that approximately 42% of the tumors expressed that marker¹⁴. We also found that there was no trend in COX2 overexpression from Luminal A-like to Triple-negative clinico-pathological subtypes. By contrast, we observed that p53 was overexpressed essentially in HER2 positive (non-luminal) tumors and in Triple-negative tumors. We found a significantly higher proportion of COX2 positive tumors when p53 was also positive. Also of note, COX2 status was not associated with the clinico-pathological subtypes regardless of p53 status. None of the clinical or pathological features of the tumors was found to be associated with COX2 or p53 and neither marker was found to be associated with patients' survival.

Previous studies demonstrated that the high rates of COX2 expression in breast tumors is not replicated in normal breast tissues. This finding boosted the interest surrounding the molecule, since its high level of expression in abnormal tissues, contrasted to a much lower expression in the healthy breast epithelium, points to an obvious potential clinical target^{10,13}. COX2 inhibitors (Coxibs) were thus tested as coadjuvant drugs in some clinical scenarios. One relatively recent study showed that Luminal-type tumors may respond better to endocrine therapy when patients are also exposed to COX2 inhibition, especially if VEGF/VEGFR2 overexpression is also present¹³. This finding is especially meaningful since tumors with high VEGF/VEGFR2 expression have been shown to respond poorly to tamoxifen¹⁰.

There is, however, a substantial concern surrounding the adverse effects associated with the Coxibs. Other less harmful agents have now been tested, e.g. the pineapple-derived agent bromelain. This naturally occurring molecule has shown tumor initiation inhibition properties, alongside pro-apoptotic effects through upregulation of p53^{27,28,29}. Targretin, a retinoid receptor agonist (RXR), has been granted FDA approval for the treatment of cutaneous lymphoma and non-small-cell lung cancer. RXR agonists were shown to inhibit cell proliferation in breast cancer and to downregulate COX2 expression in these tumors^{30,31}. Metformin, a biguanide used to treat type-2 diabetes, has showed anticancer properties in breast cancer patients. These properties have not yet been fully explained, but antiangiogenic effects and COX2 inhibition are the best explanatory candidates so far^{32,33}.

COX2 overexpression has been associated with aggressive histological and clinical features of breast cancer. A recent study assessed COX2 expression in various subtypes of breast cancer in 66 primary tumors (18 Luminal A, 17 Luminal B, 15 HER2-overexpressing and 16 Triple-negative tumors). In that study, the mean COX2 level was higher (but not statistically different) in the HER2-overexpressing subtype than in the Luminal A, Luminal B or Triple-negative groups⁶. There is also some evidence suggesting that COX2 may be a marker of poor prognosis and resistance to chemotherapy in patients with Triple-negative tumors¹⁹. In our study, we found that HER2 positive (non-luminal) and Triple-negative tumors were associated with higher p53 expression, and that p53 expression was positively associated with that of COX2, but COX2 expression was not associated with the clinico-pathological subtypes. It is well known that carcinomas overexpressing HER2 have a worse prognosis than Luminal tumors. Those authors hypothesized that an elevated expression of COX2 in a

HER2-overexpressing subtype may contribute to a more aggressive behavior and be used as diagnostic and prognostic markers in breast cancer.

Some studies suggested that COX2 expression may correlate well with tumor size, histological grading, and increased number of metastatic lymph nodes^{18,34}. These associations may be imparted to the known role played by COX2 in promotion of tumor angiogenesis^{13,32}. However, our findings do not corroborate those assumptions, since the clinical and pathological features of the tumors were not associated to COX2 expression in our sample. Also, in our study, the expression of p53 was not associated with the clinical and pathological features of the tumors.

Conclusions

We found a positive association between the expressions of COX2 and p53. On the other hand, neither the expression of COX nor those of p53 were associated with clinico-pathological subtypes, tumor features and prognosis. It seems to be too early to elect the detection of COX2 using IHC as prognostic or predictive tool, but incipient evidence points towards a possible role for the marker if further study findings corroborate them.

Ethics

The present study was approved by the Ethics Committee, FCM/UNICAMP (CEP 1246/2009).

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Conflicts of interest

The authors have no conflict of interest to declare.

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Table 1. COX2 and p53 expression in the clinico-pathological subtypes of breast cancer.

	Total n(%)	Luminal A-like n(%)	Luminal B-like HER2 neg n(%)	Luminal B-like HER2 pos n(%)	HER2 positive (non-luminal) n(%)	Triple-negative n(%)	p
COX2							
Negative	91(49.7)	21(41.2)	34(52.3)	15(57.7)	9(52.9)	12(50)	
Positive	92(50.3)	30(58.8)	31(47.7)	11(42.3)	8(47.1)	12(50)	0.39
p53*							
Negative	61(33.5)	17(33.3)	25(39.1)	13(50)	3(17.6)	3(12.5)	
Positive	121(66.5)	34(66.7)	39(60.9)	13(50)	14(82.4)	21(87.5)	0.06
Total	183 (100)	51(27.8)	65(35.5)	26(14.2)	17(9.3)	24(13.1)	

*For one case p53 status was not available.

Table 2. Cross tabulation of COX2 and p53 expression.

	Total n(%)	*p53 Negative n(%)	*p53 Positive n(%)	P
COX2				
Negative	90(49,5)	50(82,0)	40(33,1)	<0.001
Positive	92(50,5)	11(18,0)	81(66,9)	
Total	182(100)	61(33,5)	121(66,5)	

*For one case p53 status was not available.

Table 3. COX2 expression in the clinico-pathological subtypes in p53 negative and positive tumors.

*p53	COX2		**p	
	Negative n(%)	Positive n(%)		
Negative	Luminal A-like	13 (76)	4(24)	0.49
	Luminal B-like HER2 negative	22(88)	3(12)	
	Luminal B-like HER2 positive	11(85)	2(15)	
	HER2 positive (non-luminal)	2(67)	1(33)	
	Triple-negative	2(67)	1(33)	
Positive	Luminal A-like	8(23)	26(77)	0.23
	Luminal B-like HER2 negative	11(28)	28(72)	
	Luminal B-like HER2 positive	4(31)	9(69)	
	HER2 positive (non-luminal)	7(50)	7(50)	
	Triple-negative	10(48)	11(52)	
Total(182)	90(49)	92(51)		

*For one case p53 status was not available. ** Chi-squares and Fisher's Exact Test.

Table 4. Clinical features of the women and COX2 and p53 expression.

Characteristics	Total n(%)	COX2 Negative n(%)	COX2 Positive n(%)	p	*p53 Negative n(%)	*p53 Positive n(%)	p
Age (years)							
<=35	7(3.8)	4(4.4)	3(3.3)	0.45	1(1.6)	6(5.0)	0.52
36-49	56(30.6)	24(26.4)	32(34.8)		20(32.8)	36(29.8)	
≥50	120(65.6)	63(69.2)	57(61.9)		40(65.6)	79(65.3)	
Menopause							
No	63(34.4)	30(33.0)	33(35.9)	0.79	21(34.4)	42(34.7)	1.00
Yes	120(65.6)	61(67.0)	59(64.1)		40(65.6)	79(65.3)	
Tumor size							
T1-T2	139(76.0)	65(71.4)	74(80.4)	0.21	42(68.9)	96(79.3)	0.17
T3-T4	44(24.0)	26(28.6)	18(19.6)		19(31.1)	25(20.7)	
Axillary lymph nodes							
N0	90(49.2)	43(47.2)	47(51.1)	0.56	30(49.2)	59(48.8)	0.29
N1	40(21.8)	23(25.3)	17(18.5)		17(27.9)	23(19.0)	
N2-N3	53(29.0)	25(27.5)	28(30.4)		14(23.9)	39(32.2)	
Stage							
I-II	109(59.6)	54(59.3)	55(59.8)	1.00	36(59)	72(59.5)	0.98
III-IV	74(40.4)	37(40.7)	37(40.2)		25(41)	49(40.5)	

*For one case p53 status was not available.

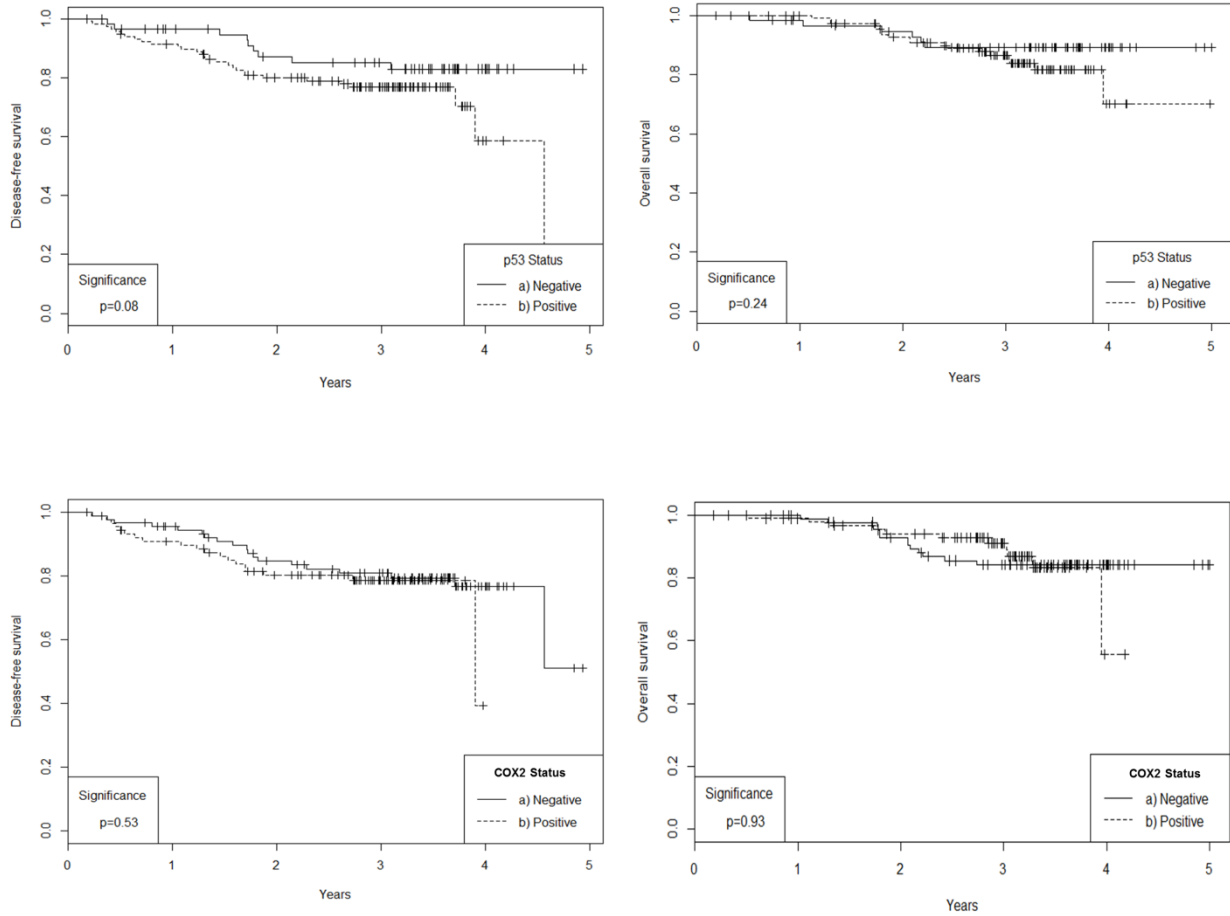
Table 5. Pathological features of the tumors and COX2 and p53 expression.

Characteristics	Total n(%)	COX2 Negative n(%)	COX2 Positive n(%)	p	*p53 Negative N(%)	*p53 Positive N(%)	p
Grade							
I-II	36(19.7)	17(18.7)	19(20.7)	1.00	13(21.3)	22(18.3)	0.69
III	147(80.3)	74(81.3)	73(79.3)		48(78.7)	98(81.7)	
Nuclear grade							
1-2	57(31.1)	25(28.3)	32(34.8)	0.42	25(41)	30(18.3)	0.04
3	126(68.9)	66(71.7)	60(65.2)		36(59)	90(81.7)	
Peritumoral angiolympathic invasion							
No	122(66.7)	58(63.7)	64(69.6)	0.49	41(67.2)	80(66.1)	1.00
Yes	61(33.3)	33(36.3)	28(30.4)		20(32.8)	41(33.9)	
Peritumoral perineural invasion							
No	160(87.4)	81(89.0)	79(85.9)	0.65	55(90.2)	104(86)	0.48
Yes	23(12.6)	10(11.0)	13(14.1)		6(9.8)	13(14)	
Dermal invasion							
No	167(91.3)	82(90.1)	85(92.4)	0.60	53(88.3)	112(92.6)	0.41
Yes	16(8.7)	9(9.9)	7(7.6)		7(11.7)	9(7.4)	
In situ component							
No	63(34.4)	32(35.2)	31(33.7)	0.87	23(37.7)	40(33.1)	0.62
Yes	120(65.6)	59(64.8)	61(66.3)		38(62.3)	81(66.9)	
Histological type							
Ductal	165(90.2)	84(92.3)	81(88.0)	0.49	56(91.8)	109(90.8)	0.70
Lobular	11(6.0)	4(4.4)	7(7.6)		4(6.6)	6(5.0)	
**Other	7(3.8)	3(3.3)	4(4.3)		1(1.6)	5(4.2)	

*For one case p53 status was not available. ** 2 medulary carcinomas, 1 atypical

medular carcinoma, 1 pleomorphic, 2 metaplastic and 1 colloid carcinoma.

Figure 1: Disease free survival and overall survival second COX2 and p53



4. Discussão

O câncer de mama é hoje a neoplasia que mais acomete as mulheres brasileiras, acompanhando a tendência mundial^{1,2}. Tem uma alta incidência e mortalidade, sendo considerado um problema de saúde pública: gera um grande impacto no Sistema Único de Saúde (SUS) em relação às estratégias de rastreamento, diagnóstico e tratamento. Excetuando-se os tumores de pele não-melanoma, é a neoplasia mais incidente nas Regiões Sudeste, Sul, Centro-Oeste e Nordeste e a segunda neoplasia mais incidente na Região Norte¹. Configura-se como a principal causa de morte por câncer em mulheres no Brasil, obedecendo à mesma distribuição Regional. Entretanto, para algumas cidades das Regiões Sudeste e Sul, há uma tendência de declínio nas taxas de mortalidade, semelhante ao observado em alguns países desenvolvidos. Nas demais regiões ainda se observa um aumento na incidência e mortalidade¹. Essas diferenças essencialmente socioeconômicas regionais se refletem no sistema de saúde: a população das regiões mais pobres encontra dificuldade de acesso aos serviços de saúde, falta de profissionais de saúde capacitados e inadequação tecnológica. Com isso a doença é diagnosticada em estádios mais avançados, o tratamento é inadequado e há um maior índice de mortalidade.

A implantação de estratégias de combate ao câncer de mama no Brasil é relativamente recente. O primeiro Documento de Consenso⁶³ que propôs diretrizes técnicas para o controle do câncer de mama foi elaborado em 2004. Nos anos seguintes as estratégias para rastreamento, diagnóstico e tratamento foram aprimoradas, resultando em melhora na assistência, porém ainda sem atingir os índices desejáveis como nos países desenvolvidos. Estas estratégias foram elaboradas a partir do conhecimento epidemiológico da doença no país, porém ainda hoje é escasso o conhecimento da biologia tumoral do câncer de mama no Brasil. Existem poucos trabalhos brasileiros que classificam a neoplasia de mama em seu perfil clínico-patológico e avaliam o comportamento desses tumores na população.

Esta tese trabalhou com a mais recente classificação clínico-patológica por IQ, definida segundo a *13thSt Gallen International Breast Cancer Conference Expert Panel, 2013*⁵, que levou em consideração a expressão do ki67 e a porcentagem de expressão dos RP, além do RE e do HER2, e encontrou 27,8% de Luminais *A-like*, 49,7% de Luminais *B-like*; destes 14,2% HER2 positivos e 35,5% HER2 negativos, 9,3% de tumores HER2 positivos (não luminais) e 13,1% de Triplos-negativos. Foi demonstrado que os tumores Luminais *A-like* são melhor diferenciados que os Luminais *B-like*, que os Triplos-negativos apresentam maior índice de proliferação celular que os Luminais, que os tumores Luminais têm melhor sobrevida livre de doença que os HER2 e Triplos-negativos e que os Luminais A têm melhor sobrevida global que os HER2 e Triplos-negativos.

Anteriormente, Matos et al.⁶⁴ utilizando a expressão IQ dos RE, RP e HER2 classificaram 168 carcinomas de mama em subtipos moleculares *likes*. Encontraram 56,3% de tumores na época classificados como Luminais A, 16,5% de Luminais B, 17,7% de HER2 superexpressos e 7,6% de Basal-*like*. De Carvalho et al.⁶⁵ utilizando os mesmo critérios, em uma série de 72 mulheres jovens (entre 19 e 40 anos), encontraram 55% de Luminais A, 11% de Luminais B, 13% de HER2 superexpressos e 18% de Basal-*like*, sendo este subtipo mais frequente em mulheres com menos de 35 anos de idade. Ainda apenas valorizando a expressão IQ dos RE, RP e HER2, Herrera et al.⁴¹, entre 53 carcinomas de mama, encontraram 66% de tumores Luminais (A e B), 20,8% de HER-2 superexpressos e 13,2% de Triplos-negativos. Neste trabalho, os HER2 superexpressos e os Triplos-negativos foram associados a pior prognóstico.

Um fator limitante desta tese foi a impossibilidade de avaliação do efeito do tratamento na sobrevida global e sobrevida livre de doença nas mulheres estudadas. Muitos países desenvolvidos seguem há anos a recomendação da *10th St Gallen International Breast Cancer Conference Expert Panel, 2007*⁶⁶, que já preconizava a utilização do trastuzumab para o tratamento dos tumores que expressam HER2 (Luminais e não-Luminais). Porém, o trastuzumab passou a ser incorporado pelo Ministério da Saúde do Brasil para tratamento do câncer de mama apenas em julho de 2012 (MS/GM Portaria nº 18 / 25 jul 2012)¹. Visto que, na presente casuística foram estudados casos coletados entre junho de 2008 a janeiro de 2011 e, nesta época, ainda não era fornecida terapia anti-HER2 pelo SUS, não foi possível a avaliação do tratamento para

essas pacientes. Hoje o tratamento do câncer de mama está assegurado por diretriz do Ministério da Saúde do Brasil, garantindo cirurgia de tratamento e reparadora, quimioterapia, hormonioterapia e trastuzumab conforme as indicações apropriadas a todas as mulheres com câncer de mama¹. Entretanto, ainda existe certa falta de acesso aos serviços de saúde para diagnóstico precoce, indisponibilidade de exames, profissionais qualificados e drogas para tratamento que assegurem a cobertura de todas as doentes.

A busca por novos marcadores associados ao câncer de mama e do melhor conhecimento do comportamento desta neoplasia no Brasil ainda é incipiente se comparada a países desenvolvidos, porém promissora. Há anos grupos de pesquisa vêm estudando inflamação e câncer e relacionando a expressão de COX2 ao carcinoma de mama e seus fatores de prognóstico e resposta ao tratamento^{39,70,71}. Nesta tese, aproximadamente 50% dos tumores expressaram COX2, porém não houve relação entre sua expressão e os subtipos clínico-patológicos de câncer de mama.

Os resultados demonstram que existe um potencial benefício da inibição da expressão da COX2 como quimioprevenção ou tratamento do câncer de mama. Os Coxibs foram as primeiras drogas estudadas para essa finalidade; entretanto, os efeitos colaterais cardiovasculares dos Coxibs atualmente disponíveis levaram a uma grande restrição da utilização desses medicamentos em pesquisas clínicas³³. Atualmente existem outras drogas em estudo para essa finalidade: a Bromelaína, o Targretin e a Metformina, que buscam a inibição da COX2 minimizando os efeitos colaterais, e apresentam resultados promissores^{45,46,47,48,49,50,51}.

Existe uma grande expressão da mutação germinativa do gene TP53, a R337H, no Sul e Sudeste do Brasil e essa mutação aumenta o risco de câncer de mama principalmente em mulheres muito jovens. Na presente casuística houve expressão de p53 em aproximadamente 66% dos tumores e uma tendência a maior expressão nos subtipos não-Luminais de câncer de mama, ou seja, naqueles que conhecidamente apresentam comportamento mais agressivo. Autores brasileiros estão propondo que a avaliação desta mutação seja incluída nos testes de rastreamento genéticos no Sul e Sudeste do país, em mulheres de alto risco: por história familiar de câncer de mama ou ovário e naquelas com Síndrome de Li-Fraumeni^{59,60,62}.

O Hospital da Mulher Prof. Dr. José Aristodemo Pinotti - CAISM desempenha importante papel Regional e Estadual como centro de referência em tratamento do câncer feminino no setor público, principalmente do câncer de mama. Por estar dentro da Universidade, também desempenha importante papel na produção de conhecimento e na integração entre os resultados das pesquisas com o tratamento das pacientes. Neste contexto, esta tese é fruto de uma linha de pesquisa que vem buscando delinear o perfil de pacientes e tumores tratados nesta instituição e identificar novos marcadores que definam tratamento e prognóstico. Paralelamente, observou-se na instituição o aperfeiçoamento de tecnologias que passaram a entrar na rotina do serviço, como os testes por IQ e FISH. Já foram desenvolvidos vários estudos que procuraram avaliar esses marcadores do câncer de mama na população aqui atendida^{39,67,68,69}.

A IQ é um método fácil e barato para realização de rotina e fornece resultados que não são idênticos, porém tem boa aproximação com os moleculares, o que torna seu uso conveniente no dia a dia. Um fator limitante da IQ é a falta de padronização de técnica em muitos laboratórios, que muitas vezes emitem resultados pouco confiáveis. Atualmente existem processos de acreditação e certificação dos serviços que avaliam, entre outras coisas, a padronização de técnicas, visando a diminuir a discordância entre os resultados. Ainda assim, a *13thSt Gallen International Breast Cancer Conference Expert Panel, 2013*⁵, sugere que os laboratórios possam estudar e estabelecer seus valores de corte de positividade dentro da metodologia utilizada, objetivando resultados consistentes.

5. Conclusões

5.1 Artigo 1

- Entre os 183 casos estudados, aproximadamente 75% dos carcinomas foram classificados como subtipos luminais-*like*. Destes, 27,8% foram Luminais A-*like* e 49,7% Luminais B-*like*. Esta distribuição entre os tumores luminais-*like* se deu principalmente pela mudança na positividade do RP (considerada alta a partir de 20%). Entre os subtipos não-luminais, houve 9,3% de HER2 positivos e 13,1% de Triplos-negativos;
- Não houve diferença na expressão do ki67 em relação aos receptores hormonais e ao HER2;
- Os subtipos Luminais B-*like* e HER2 positivos (não luminais) foram histologicamente menos diferenciados que os Luminais A-*like*. Não houve diferença entre os subtipos clínico-patológicos em relação às demais características clínicas e patológicas do câncer de mama;
- Os subtipos Luminais A-*like* tiveram melhor sobrevida global e livre de doença que os HER2 positivos (não luminais) e Triplos-negativos. Os Luminais B-*like* HER2 positivos tiveram melhor sobrevida livre de doença que os HER2 positivos (não luminais) e Triplos-negativos.

5.2 Artigo 2

- Não houve relação entre a expressão da COX2 e os subtipos clínico-patológicos de câncer de mama. Houve tendência a maior expressão da p53 nos subtipos não Luminais (HER2 e Triplo-negativo);
- Houve maior expressão de COX2 nos casos positivos para p53. Porém, não houve associação entre a expressão conjunta de COX2 e p53 com os subtipos clínico-patológicos;
- Houve maior expressão da p53 nos tumores grau nuclear 3. Não houve relação entre a expressão de COX2 nem de p53 com as demais características patológicas do câncer de mama, nem com as características clínicas;
- A COX2 e a p53 não se relacionaram à sobrevida livre de doença nem à sobrevida global.

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7. Anexos

7.1 Anexo 1 – Ficha de coleta de dados

Ficha |_|_|_|

Iniciais |_|_|_| HC |_|_|_|_|_|_|_|_|

Ficha |_|_|_|

1. Idade: |_|_|

2. Estado menstrual: 1) Menopausa |_| 2) Menacme |_| 3) Ignorado |_|

3. Estádio: I) |_| IIa) |_| IIb) |_| IIIa) |_| IIIb) |_| IIIc) |_| IV) |_|

4. Tempo de seguimento: |_|_|_| meses

5. Tratamento cirúrgico: 1) Não 2) Sim 3) Ignorado |_|

a) Mastectomia |_| b) Quadrantectomia |_| c) Outro |_|

6. Quimioterapia: 1) Não |_| 2) Sim |_| 3) ignorado

a) CMF |_| x |_|_| ciclos Linha |_| A |_| NA |_|

b) AC |_| x |_|_| ciclos Linha |_| A |_| NA |_|

c) FAC |_| x |_|_| ciclos Linha |_| A |_| NA |_|

d) FEC |_| x |_|_| ciclos Linha |_| A |_| NA |_|

e) Taxano |_| x |_|_| ciclos Linha |_| A |_| NA |_|

f) Outro |_| _____ x |_|_| ciclos Linha |_| A |_| NA |_|

7. Radioterapia: 1) Não |__| 2) Sim |__| 3) Ignorado |__|

a) Mama |__| b) Mama e FSC |__| c) Outro |__| _____

8. Hormonioterapia: 1) Não |__| 2) Sim |__| 3) Ignorado |__|

a) Tamoxifeno |__| por ____ anos

b) Inibidor da Aromatase |__| por ____ anos

9. Recidiva: a) Não |__| b) Sim |__| c) Ignorado

1) Local |__| após |__|__| meses

2) A distância |__| após |__|__| meses

a) Osso |__| b) Pulmão |__| c) Pleura |__| d) Fígado |__| e) Outro |__|_____

10. Progressão de doença: 1) Não |__| 2) Sim |__| 3) Ignorado |__|

1) Local |__| após |__|__| meses

2) A distância |__| após |__|__| meses

a) Osso |__| b) Pulmão |__| c) Pleura |__| d) Fígado |__| e) Outro |__|_____

11. Diagnóstico Histopatológico: Carcinoma Ductal invasivo

Número da biópsia: _____ Bloco selecionado: _____

Carcinoma *in situ* associado: 1) Não |__| 2) Sim |__| 3) Ignorado |__|

a) Ductal |__| b) Lobular

Grau Histológico: I) |__| II) |__| III) Ignorado |__|

Grau nuclear: 1) |__| 2) |__| 3) |__|

Invasão Linfática Peritumoral: 1) Sim |__| 2) Não |__| 3) Ignorado |__|

Invasão Linfática da Derme: 1) Sim |__| 2) Não |__| 3) Ignorado |__|

Invasão Vascular Peritumoral: 1) Sim |__| 2) Não |__| 3) Ignorado |__|

Comprometimento Axilar:

No. Linfonodos Dissecados |__|__|

No. Linfonodos Acometidos |__|__|

Invasão linfática extra-nodular: 1) Sim |__| 2) Não 3) Ignorado

12. Imunoistoquímica

Marcadores (escore final)

COX-2: |__|__|

p53: |__|__|

Receptor de estrógeno: |__|__|%

Receptor de progesterona: |__|__|%

ki67: pontuação final |__|__| %

13. Teste de FISH

Positivo |__| Negativo |__| Inconclusivo |__|

7.2 Anexo 2 - Parecer CEP



FACULDADE DE CIÊNCIAS MÉDICAS
COMITÊ DE ÉTICA EM PESQUISA

www.fcm.unicamp.br/pesquisa/etica/index.html

CEP, 15/12/09.
(Grupo III)

PARECER CEP: Nº 1246/2009 (Este nº deve ser citado nas correspondências referente a este projeto)
CAAE: 0967.0.146.000-09

I - IDENTIFICAÇÃO:

PROJETO: “EXPRESSÃO DA COX-2 E DA P53 NOS SUBTIPOS MOLECULARES DE CARCINOMA DUCTAL INVASIVO DE MAMA E AVALIAÇÃO DE SEU VALOR COMO FATOR PREDITIVO E PROGNÓSTICO”.

PESQUISADOR RESPONSÁVEL: Kátia Pítton Serra

INSTITUIÇÃO: CAISM/UNICAMP

APRESENTAÇÃO AO CEP: 14/12/2009

APRESENTAR RELATÓRIO EM: 15/12/10 (O formulário encontra-se no *site* acima)

II - OBJETIVOS

Correlacionar os subtipos moleculares de carcinoma ductal invasivo de mama com a expressão da COX-2 e da p53 e avaliar seu valor preditivo e prognóstico.

III - SUMÁRIO

Serão analisados 261 casos de mulheres com carcinoma ductal invasivo de mama, incluídos os blocos de parafina e preparadas lâminas de Tissue Microarray (TMA). A avaliação da expressão da COX-2 p53, e os marcadores que definem e classificam os subtipos moleculares de câncer de mama será realizada por imunohistoquímica e FISH. Análise estatística: Os dados serão analisados descritivamente por meio de frequência absoluta (n) e relativas (%), médias, desvio-padrão, mediana, primeiro e terceiro quartis, valores mínimos e máximos. O teste qui-quadrado de Pearson e exato de Fisher serão utilizados para avaliar a homogeneidade entre os grupos e as variáveis categóricas; serão empregados os testes t de Student ou Anova para as variáveis contínuas. Para avaliar o tempo de sobrevida serão construídas curvas de sobrevida usando o método Kaplan-Meier. O nível de significância adotada será de 5% e o software utilizado para análise o SAS.

IV - COMENTÁRIOS DOS RELATORES

Trata-se de um estudo de coorte retrospectivo, com análise de material anátomo-patológico no Laboratório de Patologia Experimental do CAISM/UNICAMP. O projeto apresenta-se bem redigido, com metodologia adequada. Os critérios de inclusão e exclusão dos sujeitos estão bem definidos; cálculo do tamanho amostral e análise estatística estão bem embasados por cálculos estatísticos. Os aspectos éticos estão bem discutidos no corpo do projeto e será solicitada a dispensa do Termo de Consentimento Livre e Esclarecido. O orçamento é detalhado.

Comitê de Ética em Pesquisa - UNICAMP
Rua: Tessália Vieira de Camargo, 126
Caixa Postal 6111
13083-887 Campinas – SP

FONE (019) 3521-8936
FAX (019) 3521-7187
cep@fcm.unicamp.br

- 1 -



V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e atendendo todos os dispositivos das Resoluções 196/96 e complementares, resolve aprovar sem restrições o Protocolo de Pesquisa, a dispensa do Termo do Consentimento Livre e Esclarecido, bem como todos os anexos incluídos na pesquisa supracitada.

O conteúdo e as conclusões aqui apresentados são de responsabilidade exclusiva do CEP/FCM/UNICAMP e não representam a opinião da Universidade Estadual de Campinas nem a comprometem.

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e)

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VII – DATA DA REUNIÃO

Homologado na XII Reunião Ordinária do CEP/FCM, em 15 de dezembro de 2009.


Prof. Dra. Carmen Silvia Bertuzzo
VICE-PRESIDENTE do COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP

7.3 Anexo 3 – Apresentação de pôster em Congressos Internacionais

17^o World Congress on Breast Diseases of the Senologic International Society (S.I.S)



Hospital Da Mulher Prof. Dr. José Aristodemo Pinotti - CAISM

Women treated at CAISM-UNICAMP: a great sample of Luminal B molecular subtype of breast cancer

Katia Piton Serra¹, Luis Otávio Sarian¹, José Vassallo^{2,3}, Glauce Aparecida Pinto², Fernando Augusto Soares³, Isabela Werneck da Cunha³, Adriano Mesquita Bento¹, Raquel Mary Rodrigues Peres¹, Juliana Espinola¹, Sophie Derchain¹

1. Department of Obstetrics and Gynecology, State University of Campinas – UNICAMP, Campinas, São Paulo, Brazil
2. Department of Pathology, State University of Campinas – UNICAMP, Campinas, São Paulo, Brazil
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Corresponding author: katiaserra@gmail.com

INTRODUCTION

Breast cancer is the second most common malignancy worldwide. In Brazil, approximately 52,608 new cases of breast cancer are predicted to occur in 2012. The morphologic classification is not sufficient to characterize breast carcinomas, once the tumors with the same grade, stage and histologic type can show different prognostic and therapeutic response. Limitations in the morphologic classification are probably caused by an incapacity to consider the biologic characteristics of the tumors. Classification in molecular subtypes is demonstrating a great variety between the tumors with histopathologic similarity.

OBJECTIVES

To classify breast invasive carcinomas treated at CAISM-UNICAMP in molecular subtypes of breast cancer and to assess the relationship between de subtypes and woman's diseases characteristics. To evaluate overall survival and disease free survival of this sample.

SUBJECTS AND METHODS

240 patients were included in the study. TMA (tissue microarray) was constructed and expressions of hormone receptors of estrogen (ER) and progesterone (PR) and Ki67 were assessed with immunohistochemistry. FISH were used to assess HER2 status. The tumors were classified by: Luminal A expressed ER and/or PR, not expressed HER2 and low expressed Ki67. Luminal B expressed ER and/or PR, not expressed HER2 with high expression of Ki67 or expressed ER and/or PR and expressed HER2. HER2 subtype was characterized by not express ER and PR and overexpress HER2. Basal-like was characterized by not express ER, PR and HER2. Chi-square and Fisher's exact test were calculated to assess the cross-tabulation of subtype expression and the several characteristics. Mean standard deviation (SD) and percentiles were calculated to estimate the woman's follow-up. Overall survival and disease free survival were evaluated, as well. Proportion trend test and Chi-square residual analysis were calculated to evaluate the progressive relationship between molecular subtypes and disease severity.

RESULTS

We found 21.6% of Luminal A, 54.2% Luminal B, 9.2% HER2 and 15% of Basal-like. There was a preponderance of Luminal B and Basal-like among women less than 50 years old. 47% of the tumors were T2. 52% of axillary nodes were affected (13.9% were pathologic N3). The most of tumors were undifferentiated grade. Only 23.1% of the included patients were pathologic stage I. The mean follow-up was 25 months. About 80% of the women are alive without metastatic disease; 6% are alive with metastatic disease and 8% died. 84.1% of Luminal A phenotype and 88.2% of Luminal B are alive without disease. 12.5% of subtype HER2 and 13.3% of Basal-like died. There was more significant expression of EGFR in the Luminal B (61.5%, $p < 0.01$). P53 was more expressed in HER-2 (81.8%) an Basal (80.0%) than Luminal A (67.4%) and Luminal B (53.1%) ($p = 0.02$); there was significant progression in the expression of p53 as related to molecular subtypes ($p < 0.01$).

Table 1: Women's distribution second molecular subtypes

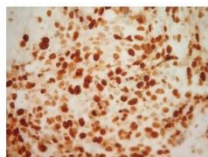
Molecular subtype	(N)	(%)
Luminal A	52	21,6
Luminal B	130	54,2
HER-2	22	9,2
Basal	36	15,0
Total	240	(100)

Table 2: Expression of EGFR and p53 as related to molecular subtypes

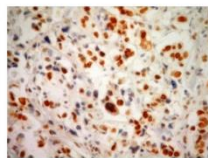
	Total n(%)	Luminal A n(%)	Luminal B n(%)	HER2 n(%)	Basal n(%)	p
EGFR (n=240)						
Positive	127(52,9)	16(30,8)	80(61,5)	13(59,1)	18(50,0)	
Negative	113(47,1)	36(69,2)	50(38,5)	9(40,9)	18(50,0)	<0,01
P53 (n=236)						
Positive	159(67,4)	26(53,1)	87(66,9)	18(81,8)	28(80,0)	
Negative	77(32,6)	23(46,9)	43(33,1)	4(18,2)	7(20,0)	0,02

Table 3: Evolution after treatment

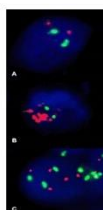
	Total n(%)	Luminal A n(%)	Luminal B n(%)	HER2 n(%)	Basal n(%)
Mean follow-up (months and SD)		28,8(29,3)	25,3(7,7)	24,9(7,7)	24,0(7,9)
Alive without disease (n=201)	169(84,1)	37(84,1)	97(88,2)	13(81,2)	22(71)
Alive with disease (n=202)	13(6,4)	1(2,3)	7(6,2)	1(5,9)	4(13,3)
Death (n=201)	17(8,5)	5(11,4)	6(5,4)	2(12,5)	4(13,3)



High expression of ki67 in a sample of invasive breast carcinoma (400x).



High expression of p53 in a sample of invasive breast carcinoma (400x).



Fluorescent in situ hybridization using a probe for Her-2/neu (red dots) and chromosome 17 centromere (green dots). (A) not amplified, (B) amplified, and (C) polysomy without amplification. All figures 2000x.

DISCUSSION

We found a different distribution of subtypes regarding the international literature. We obtained more Luminal B than the expected, maybe because our cases were more advanced and had a high proliferation index (expressed by Ki67).

CONCLUSION

Despite our short follow-up and advanced cases, the prognostic appears good at the moment, especially for Luminal subtypes. This work was funded by FAPESP 2009/17097-1.

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17th WORLD CONGRESS
ON BREAST DISEASES
of the Senologic International Society – SIS.
17º CONGRESSO MUNDIAL DE MASTOLOGIA

Salvador
BAHIA
2012
10 a 13 de Outubro - Brasil
October 10 - 13 - Brazil



CERTIFICATE

We certify that

KÁTIA PITON SERRA

attended 17th World Congress on Breast Diseases of the Senologic International Society (S.I.S.), that occurred from October 10th to 13th, 2012, in Salvador, at the Bahia Othon Palace Hotel
as Author of the Abstract *“Women treated at CAISM-UNICAMP: A great sample of Luminal B molecular subtype of breast cancer”*.

Salvador, October 10th, 2012.

Dr. Ezo Novais Dias
President of 17th SIS World Congress on Breast Diseases
President of S.I.S. - Senologic International Society /
The World Society of Breast Diseases

Dr. Carlos Alberto Ruiz
President of Brazilian Society of Mastology

18^o International meeting of the European Society of Gynecological Oncology (ESGO)



Clinicopathological features of breast cancer molecular subtypes in Brazilian women



S. Derchain¹, K.P. Serra¹, L.O. Sariani¹, J. Vassallo², G.A. Pinto², G. Paiva², F.A. Soares¹, L.W. da Cunha³, R.M.R. Pires¹, J. Espinola¹, A. M. Bento¹
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Background

Breast cancer is the second most common malignancy worldwide. In Brazil, approximately 52,608 new cases of breast cancer are predicted to occur in 2012. The morphologic classification is not sufficient to characterize breast carcinomas, once the tumors with the same grade, stage and histologic type can show different prognostic and therapeutic response. Limitations in the morphologic classification are probably caused by an incapacity of consider the biologic characteristics of the tumors. Classification in molecular subtypes is demonstrating a great variety between the tumors with histopathologic similarity.

Objectives

The aim of this study was to assess the relationship between molecular subtypes and clinical and pathological characteristics in a subset of Brazilian women with invasive breast carcinomas.

Table 1: Women's distribution second molecular subtypes

Molecular subtypes	N	%
Luminal A	84	39.8
Luminal B	69	32.7
HER2	22	10.4
Triple negative	36	17.1
Total	211	100

Subjects & Methods

211 patients were included in the study. TMA (tissue microarray) was constructed and expressions of hormone receptors of estrogen (ER) and progesterone (PR) and ki67 were assessed with immunohistochemistry. FISH were used to assess HER2 status. The tumors were classified by: Luminal A expressed ER and/or PR, not expressed HER2 and low expressed ki67. Luminal B expressed ER and/or PR, not expressed HER2 with high expression of ki67 or expressed ER and/or PR and expressed HER2. HER2 subtype was characterized by not express ER and PR and overexpress HER2. Triple-negative was characterized by not express ER, PR and HER2. Chi-square and Fisher's exact test were calculated to assess the cross-tabulation of subtype expression and the several characteristics. Mean standard deviation (SD) and percentiles were calculated to estimate the woman's follow-up.

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Table 2: Women's distribution second clinic characteristics and molecular subtypes

Clinic and pathologic characteristics	Total n(%)	Luminal A n(%)	Luminal B n(%)	HER2 n(%)	Triple-negative n(%)	P
Age (n=199)						
< 50 years	77 (38.7)	25(31.6)	28(43.8)	6(28.6)	18(51.4)	0.130
≥ 50 years	122(61.3)	54(68.4)	36(56.2)	15(71.4)	17(48.6)	
Menstrual status (n=195)						
Pre-menopause	69(35.4)	23(29.5)	25(40.3)	6(28.6)	15(44.1)	0.332
Menopause	126(64.6)	55(70.5)	37(59.7)	15(71.4)	19(55.9)	
Tumor size (n=195)						
T1	61(31.3)	24(31.1)	24(38.1)	8(40.0)	5(14.2)	0.012
T2	93(47.7)	30(39.0)	30(47.6)	7(35.0)	26(74.3)	
T3	4(2.0)	1(1.3)	2(3.1)	0	2(5.9)	
T4	37(19.0)	22(28.6)	7(11.1)	5(25.0)	3(8.6)	
Nodal status (n=195)						
N0	32(16.7)	35(46.0)	35(54.7)	4(20.0)	18(51.4)	0.081
N1	45(23.1)	16(21.1)	12(18.7)	8(40.0)	9(25.8)	
N2	33(16.9)	12(15.8)	11(17.2)	2(10.0)	6(17.1)	
N3	27(13.8)	13(17.1)	6(9.4)	6(30.0)	2(5.7)	
Final stage (n=195)						
I	46(23.5)	16(20.5)	22(34.4)	3(15.0)	5(14.7)	0.079
II	73(37.2)	23(29.5)	23(35.9)	8(40.0)	19(55.9)	
III	73(37.2)	37(47.4)	18(28.1)	9(45.0)	9(26.5)	
IV	4(2.1)	2(2.6)	1(1.6)	0	1(2.9)	

Results

Among the 211 tumors, 39.8% were classified as Luminal A, 32.7% as Luminal B, 10.4% as double negative HER2 overexpressed and 17.1% as Triple-negative. Triple-negative tumors were associated with younger age and higher tumor size. There was a predominance of undifferentiated tumors in all molecular subtypes. Peritumoral vascular and lymphatic invasion (observed in 33.9% of the entire cohort), peritumoral neural invasion (detected in 12.6%) and dermal lymphatic invasion (present in 10.3%) were not associated with the molecular subtype.

Table 4: Evolution after treatment

Evolution	Total n(%)	Luminal A n(%)	Luminal B n(%)	HER2 n(%)	Triple-negative n(%)
Mean follow-up (months and DP)	28.2(23.5)	24.9(7.8)	24.9(7.7)	23.9(7.9)	23.9(7.9)
Alive without disease (n=174)	144(82.8)	61(87.1)	48(84.2)	13(81.2)	22(71.0)
Alive with disease (n=175)	13(7.4)	6(8.6)	2(3.4)	1(5.9)	4(13.3)
Death (n=174)	15(8.6)	2(2.9)	7(12.1)	2(12.5)	4(13.3)

Table 3: Women's distribution second pathologic characteristics and molecular subtypes

Histologic characteristics	Total n(%)	Luminal A n(%)	Luminal B n(%)	HER2 n(%)	Triple-negative n(%)	P
Histologic grade (n=189)						
I	4(2.1)	1(1.4)	2(3.3)	0	1(3.0)	0.004
II	32(16.9)	21(29.2)	5(7.9)	0	6(18.2)	
III	153(81.0)	50(69.4)	56(88.9)	21(100)	26(78.8)	
Nuclear grade (n=190)						
1	2(1.1)	1(1.4)	0	0	1(3.0)	0.005
2	54(28.4)	30(41.1)	18(28.6)	2(9.5)	4(12.1)	
3	134(70.5)	42(57.5)	45(71.4)	19(90.5)	28(84.8)	
Peritumoral vascular and lymphatic invasion (n=183)						
No	121(66.1)	48(66.7)	40(66.7)	9(50.0)	24(72.7)	0.432
Yes	62(33.9)	24(33.3)	20(33.3)	9(50.0)	9(27.3)	
Peritumoral neural invasion (n=183)						
No	160(87.4)	62(86.1)	54(90.0)	16(88.9)	28(84.8)	0.885
Yes	23(12.6)	10(13.9)	6(10.0)	2(11.1)	5(15.2)	
Dermal lymphatic invasion (n=195)						
No	175(89.7)	69(88.5)	59(96.7)	17(81.0)	30(85.7)	0.076
Yes	20(10.3)	9(11.5)	2(3.3)	4(19.0)	5(14.3)	
In situ carcinoma related (n=134)						
No	60(30.9)	26(32.9)	19(30.6)	3(15.0)	12(36.4)	0.399
Yes	134(69.1)	53(67.1)	43(69.4)	17(85.0)	21(63.6)	
Invasive histologic types (n=181)						
Ductal	162(89.5)	60(84.5)	53(89.8)	18(100)	31(94.0)	0.768
Lobular	13(6.1)	6(8.5)	4(6.8)	0	1(3.0)	
Others	8(4.4)	5(7.0)	2(3.4)	0	1(3.0)	

Conclusion

In this series of Brazilian women with invasive breast carcinoma, more than 70% of the tumors were Luminal molecular type. Younger age and advanced stage were associated with triple negative tumors.

Acknowledgements

Grant research from FAPESP 2009/17097-1

Certificate of Attendance

This is to certify that

Kátia Serra

Participated in the



18th International Meeting of the
European Society of Gynaecological Oncology (ESGO)
held in Liverpool, UK, October 19-22, 2013

with an e-poster presentation entitled

**CLINICOPATHOLOGICAL FEATURES OF BREAST
CANCER MOLECULAR SUBTYPES IN
BRAZILIAN WOMEN**

A handwritten signature in blue ink, appearing to read 'David Cibula'.

Prof. Dr. David Cibula
Chair, Scientific Programme Committee

A handwritten signature in blue ink, appearing to read 'Nicoletta Colombo'.

Prof. Dr. Nicoletta Colombo
ESGO President