RODRIGO PINTO GIMENEZ

EXPRESSÃO PROTEICA DA ADIPONECTINA, RECEPTORES DE ADIPONECTINA TIPOS 1 E 2 E DA *ADIPOCYTE FATTY ACID BINDING PROTEIN* NO CARCINOMA INVASOR, NAS SUAS LESÕES PRECURSORAS E NAS LESÕES BENIGNAS DA MAMA

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Área de concentração em Oncologia Ginecológica e Mamária

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Dedico este trabalho...

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além do amor incondicional.

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"Na vida cicatrizes vamos ter, rugas serão sulcadas,
saibamos tirar o melhor proveito delas
para o nosso aprendizado e crescimento pessoal."

Rodrigo Pinto Gimenez

Sumário

Símbolos, Siglas e Abreviaturas

AdipoR1 – Receptor de Adiponectina Tipo 1

AdipoR2 – Receptor de Adiponectina Tipo 2

AdipoR1/R2 – Receptores de Adiponectina Tipos 1 e 2

APN – Adiponectina

ADH – *Atypical ductal hiperplasia*

BE – Doença benigna da mama

CAISM – Hospital da Mulher Prof. Dr. José Aristodemo Pinotti, Centro de Atenção Integral à Saúde da Mulher

CAPSS – *Columnar alteration with prominent apical snouts and secretions* (alteração colunar epitelial com secreção do tipo decapitação)

CDI – Carcinoma ductal invasor

CDIS – Carcinoma ductal *in situ*

CEP – Comitê de Ética em Pesquisa

CLIS – Carcinoma lobular *in situ*

DM – Diabetes Melitus

DT – *Distant tissue*

Ecp – Estadiamento clínico-patológico

ET – *Epithelial tissue*

FABP – *Fatty Acid Binding-Protein*

FABP4 – *Adipocyte Fatty Acid Binding-Protein*

FCM – Faculdade de Ciências Médicas

GD – Gordura distante

GH – Grau histológico

GN – Grau nuclear

GP – Gordura próxima

HAS – Hipertensão Arterial Sistêmica

HDT – Hiperplasia ductal típica

HDA – Hiperplasia ductal atípica

HLA – Hiperplasia lobular atípica

IMC – Índice de Massa Corporal

INCA – Instituto Nacional do Câncer

LASER – *Light amplification by stimulated emission of radiation*

LCIS – *Lobular carcinoma in situ*

p – p valor, nível de significância estatística

RE – Receptor de estrógeno

RP – Receptor de progesterona

TDH – *Tipical ductal hiperplasia*

TMA – *Tissue Microarray*

TNFα – Fator de Necrose Tumoral α

UNICAMP – Universidade Estadual de Campinas

Resumo

Introdução: A obesidade tem se mostrado responsável pelo aumento de 30% a 50% dos casos novos de câncer de mama, em particular na pós-menopausa. A mais recente hipótese para explicar tal fato situa os adipócitos e suas funções autócrina, parácrina e endócrina no centro do cenário, através da relação das adipocinas, por ele secretadas, com a obesidade e o câncer de mama. **Objetivo: Artigo 1-** Comparar o padrão de expressão imunoistoquímica da adiponectina (APN) e dos seus receptores tipos 1 e 2 (adipoR1/R2) no carcinoma invasor (CDI), carcinoma ductal *in situ* (CDIS) e lesões benignas da mama (BE) e correlacioná-los com parâmetros clínicos e histológicos. **Artigo 2-** Avaliar a expressão proteica da FABP4 nos tecidos epitelial e adiposo mamário de portadoras de CDI, CDIS e lesões benignas da mama. **Material e Métodos:** Foram incluídos os blocos de parafina de 223 mulheres sendo 69 com CDI, 73 com CDIS e 81 com biópsias negativas para câncer de mama, tratadas no CAISM/UNICAMP de janeiro de 2008 a dezembro de 2011, e preparadas lâminas de *Tissue Microarray* (TMA). A expressão de APN e Adipo R1/R2 foi avaliada no tecido tumoral nos casos CDI e CDIS e no tecido epitelial e nos casos benignos. A expressão de FABP4 foi avaliada no tecido tumoral, na gordura peritumoral (GP) e na gordura

mamária distante (GD) nos casos de CDI e CDIS, e no tecido epitelial e gordura mamários nos casos benignos. Para avaliar uma possível relação entre a expressão dos marcadores entre si e com parâmetros antropométricos, clínicos e histopatológicos, foram utilizados os testes qui-quadrado ou exato de Fisher, Mann-Whitney, Kruskal-Wallis e correlação de Spearman. As determinações foram calculadas considerando o valor de α=0,05 ($p<0,05$). **Resultados: Artigo 1 -** A APN mostrou-se expressa em 65% dos CDI, 48% dos CDIS e 12% das BE e AdipoR1 em 98%, 94% e 71%, respectivamente. Todos os casos de CDI e CDIS expressaram AdipoR2 contra 81% de BE. Nos CDI e CDIS observou-se associação entre maior expressão de APN e tumores RE negativo. No CDIS esta associação foi também observada com RP negativo. **Artigo 2 -** Observou-se expressão proteica da FABP4 no tecido epitelial em 90% dos CDI, 40% dos CDIS e 28% em BE. Considerando-se a GP e GD esta expressão foi maior nas BE que nos CDI, diferenças consideradas significativas. Nas pacientes com CDI a expressão da FABP4 foi maior quando o diagnóstico ocorreu até 50 anos de idade. A totalidade dos casos expressou moderada a intensamente este marcador no tecido gorduroso periepitelial e distante. **Conclusões:** As diferenças de expressões proteicas da adiponectina e dos seus receptores AdipoR1/R2 observadas em diferentes diagnósticos mamários sugerem sua participação no complexo mecanismo etiológico destas diferentes condições. Os resultados deste estudo indicam, ainda, que existe uma correlação direta entre expressão proteica da FABP4, câncer de mama e obesidade.

Summary

Introduction: Obesity has been shown to be responsible for a 30 to 50% increase in new breast cancer cases, in particular in the postmenopausal period. The most recent hypothesis that explains this fact places adipocytes and its autocrine, paracrine and endocrine functions at center stage, linking adipokines secreted by adipocytes to obesity and breast cancer. **Objective: Article 1-** to compare immunohistochemistry expression pattern of adiponectin (APN) and its receptors types 1 and 2 (adipoR1/R2) in invasive carcinoma (IDC), ductal carcinoma *in situ* (CDIS) and benign breast lesions (BE), correlated with clinical and histological parameters. **Article 2-** To assess FABP4 protein expression in epithelial and adipose breast tissue in women diagnosed with IDC, DCIS and benign breast lesions. **Material and Methods:** Paraffin-embedded blocks from 223 women were included. Of the total number of women, 69 had IDC CDI, 73 had CDIS and 81 had biopsies negative for breast cancer. The patients has been treated at CAISM/Unicamp from January 2008 to December 2011 and Tissue Microarray (TMA) slides were constructed. Expression of APN and Adipo R1/R2 was assessed in tumor tissue in cases of IDC and DCIS and in epithelial tissue in benign cases. FABP4 expression was evaluated in tumor tissue,

peritumoral fat tissue (PF) and distant fat breast tissue (DF) in cases of IDC and DCIS and in the epithelial tissue and breast fat tissue in benign cases. To assess a possible relationship between marker expression and anthropometric, clinical and histopathological parameters, the chi-square test or Fisher's exact test, Mann-Whitney test, Kruskal-Wallis test and Spearman's correlation were used. Determinations were calculated, considering a value α=0.05 ($p<0.05$) as significant. **Results: Article 1 -** APN was shown to be expressed in 65% of IDC, 48% of DCIS and 12% of BE and AdipoR1 in 98%, 94% and 71%, respectively. All IDC and DCIS cases expressed AdipoR2 versus 81% of BE. In IDC and DCIS, an association between a higher level of APN expression and ER-negative tumors was observed. In DCIS, this association was also observed with PR-negative tumors. **Article 2 -** FABP4 protein expression was observed in epithelial tissue in 90% of CDI, 40% of DCIS and 28% of BE. Considering PF and DF, FABP4 expression had a higher level in BE than in IDC, a difference that was considered significant. In patients with IDC, FABP4 expression was higher when diagnosis was made in patients aged up to 50 years. In all cases, this marker was moderately to intensely expressed in the peri-epithelial and distant fat tissue. **Conclusions:** Discrepancies in protein expression of adiponectin and its receptors AdipoR1/R2 observed in different breast diagnoses suggest its participation in the complex etiologic mechanism of these different conditions. Our results indicate that there is a direct correlation between FABP4 protein expression, breast cancer and obesity.

1. Introdução

O câncer de mama consiste na neoplasia mais diagnosticada e a segunda causa mais comum de morte por câncer no sexo feminino, tanto em países desenvolvidos como nos em desenvolvimento, representando um grave problema de saúde pública (1). Em 2008, segundo dados do Globocan ("*International Agency for Research on Cancer" of World Health Organization)* foram diagnosticados 1,38 milhão de novos casos no mundo, correspondendo a 23% de todos os casos de câncer na mulher (2).

A taxa anual de incidência do câncer de mama é maior nas regiões mais desenvolvidas do mundo, em populações urbanas e em caucasianos, variando de 19,3 por 100.000 mulheres no oriente africano a 89,9 por 100.000 mulheres no oeste europeu, sendo maior (80 por 100.000) em regiões mais desenvolvidas do mundo, exceto o Japão, e baixa (menos de 40 por 10.000) na maioria das regiões em desenvolvimento (3).

No Brasil, no ano de 2012 são esperados 52.680 casos novos de câncer da mama, com um risco estimado de 52 casos a cada 100 mil mulheres. Sem

considerar os tumores da pele não melanoma, esse tipo de câncer também é o mais frequente nas mulheres das regiões Sudeste (69/100 mil), Sul (65/100 mil), Centro-Oeste (48/100 mil) e Nordeste (32/100 mil). Na região Norte, é o segundo tumor mais incidente (19/100 mil). As taxas de mortalidade por câncer de mama são elevadas provavelmente porque a doença ainda é diagnosticada em estádios avançados. O número de mortes em 2010 foi de 12.852, sendo 147 homens e 12.705 mulheres (4).

Apesar da etiologia do câncer de mama não estar definida, diversos fatores foram descritos como relacionados ao seu elevado risco de ocorrência, como menarca precoce, menopausa tardia, ausência de gestações, idade avançada quando da primeira gestação a termo, não amamentar, obesidade no período pós-menopausa, utilização de terapia hormonal pós-menopausa, sedentarismo, consumo frequente de bebidas alcoólicas, exposição à radiação, uso de contraceptivo oral e histórico familiar positivo para o câncer de mama (4,5,6).

A obesidade está relacionada a diversos distúrbios de ordem metabólica como diabetes mellitus tipo 2, doença coronariana, hipertensão e está associada ao desenvolvimento de câncer em diferentes tecidos como cólon, próstata e mama (7). Está claramente demonstrado que a obesidade consiste em um fator de risco para o desenvolvimento de câncer de mama na pós-menopausa, além de favorecer o desenvolvimento de metástases e recorrência e, também, estar associada ao aumento da mortalidade (8). Além disso, mulheres acima do peso ou obesas portadoras de câncer de mama têm 2,5 vezes maiores chances de

morrer devido à doença nos cinco anos que sucedem ao seu diagnóstico, quando comparadas a mulheres com peso normal (9).

Inúmeros fatores têm sido sugeridos para explicar a relação entre obesidade e câncer de mama. A hiperinsulinemia da obesidade e os níveis elevados de estrógeno circulantes podem explicar a relação entre adipócitos e as células cancerígenas da mama (10,11). Em mulheres na pós-menopausa, o tecido adiposo é a principal fonte de aromatase, enzima que converte andrógenos em estrógenos, e nessas pacientes a capacidade de aromatização está aumentada devido ao número ou volume dos adipócitos. Além disso, o excesso de tecido adiposo está relacionado com níveis plasmáticos aumentados de insulina e *insulin-like growth factor-1* (IGF-1), que apresenta atividades mitogênicas estando envolvido na progressão do tumor mamário (12). Apesar de todas as hipóteses citadas, ainda não está definida a verdadeira relação entre obesidade e câncer de mama.

As evidências sugerem que o tecido adiposo, como órgão endócrino, produz e secreta vários fatores que interferem no desenvolvimento do câncer de mama (13). Estes fatores, chamados adipocinas, polipeptídeos com importante função regulatória do metabolismo energético, incluem fatores angiogênicos, mitogênicos (leptina) e antimitogênicos (adiponectina), fatores de crescimento e citocinas pró-inflamatórias (IL-1, TNF-alpha, IL-6) e estão envolvidos na mediação ou na coordenação de doenças inflamatórias e obesidade (14,15). Adipocinas são produzidas por diferentes sítios de depósitos gordurosos, incluindo gordura subcutânea, visceral e mamária. Assim, condições que modifiquem a biologia do tecido adiposo, como a obesidade, alteram a produção de adipocinas. Estas

podem ser detectadas no sangue e ter sua expressão mensurada através de imunoistoquímica em tecidos específicos (16).

A Síndrome Metabólica consiste na combinação de características e sintomas como dislipidemia, hipertensão arterial, alteração na tolerância à glicose, resistência à insulina, lipodistrofia centrípeta, condições pró-inflamatórias generalizadas e propensão a diabetes tipo 2 e doença cardiovascular. A maioria dos pacientes obesos apresenta alterações no metabolismo de tecido adiposo causadas por interações genéticas e ambientais, que levam à hipertrofia adipocitária, hipóxia, alterações na homeostase e processos inflamatórios diversos com aumento da produção de leptina, fator de necrose tumoral-α, interleucina 6 e diminuição da produção de adiponectina pelo tecido adiposo (17).

Acredita-se que as adipocinas, incluindo a adiponectina, agem no tecido mamário por mecanismo endócrino, através de depósitos de gordura externos, por via parácrina, através do tecido adiposo mamário e fontes não adiposas como células estromais e células inflamatórias, e por uma ação autócrina através do próprio tumor mamário. A estrutura anatômica da mama favorece a interação do tecido adiposo mamário com o tecido glandular, o que sugere que as adipocinas produzidas pelo tecido adiposo mamário e pelas células tumorais podem ser o principal fator de associação entre obesidade, progressão do câncer mamário e metástases (18,19,20,21).

O tecido adiposo produz mais de 50 tipos de adipocinas; dentre elas, a adiponectina (APN) parece ter papel fundamental na etiologia do câncer de mama. A adiponectina, também conhecida como Acrp30 (*adipocyte complement-*

related protein of 30kDa), adipoQ, ApM1 (*adipose most abundant gene transcript 1*) e GBP28 (*28kDa gelatin binding protein*), é codificada por um gene localizado no cromossomo 3q27. É a proteína de produção adipocitária de maior ocorrência na corrente sanguínea (22).

Miyoshi et al. (23) foram os primeiros a descrever a relação entre baixos níveis séricos de APN e elevado risco de câncer de mama . A APN pertence à família 1q do sistema complemento e pode ser encontrada em cinco diferentes configurações: a adiponectina globular (gAPN), adiponectina de cadeia longa (fAPN), adiponectina de baixo peso molecular (LMW), adiponectina de médio peso molecular (MMW) e adiponectina de alto peso molecular (HMW) (22). Alterações genéticas nas vias metabólicas de produção da APN podem afetar o risco do aparecimento do câncer de mama. Os polimorfismos rs1501299 TG e GG, responsáveis pela diminuição da APN circulante, estão associados ao aumento no risco de câncer de mama em 59% e 80%, respectivamente. O polimorfismo rs2241766, que acarreta aumento dos níveis séricos de APN, está associado à diminuição de 39% no risco de câncer de mama (24). No entanto, outros estudos não mostraram tais correlações (25).

As adipocinas, incluindo a APN, em geral agem via seus receptores nas células tumorais mamárias, influenciando a proliferação celular, migração e invasão tumoral, regulam a produção de proteínas epiteliais, proteínas angiogênicas e fatores de crescimento (26).

Os receptores de adiponectina R1 (AdipoR1) e R2 (AdipoR2) são codificados por genes localizados nos cromossomo 1p36.13-q41 e 12p13.31

respectivamente. O receptor AdipoR1 tem expressão mais abundante na musculatura esquelética, enquanto o AdipoR2 predomina no fígado. Estes dois receptores são proteínas de membrana, sendo que o AdipoR1 tem elevada afinidade pela gAPN enquanto o AdipoR2 reconhece, predominantemente, a fAPN (22). A T-caderina tem sido proposta como um receptor de APN para proteínas de alto peso molecular (HMW) (27). Tanto a expressão gênica (mRNA), quanto a proteica dos receptores de APN têm sido caracterizadas em diferentes linhagens celulares do câncer de mama, incluindo MCF-7, MDA-MB-231, SKBR3 e T47D através de imunoistoquímica e *tissue microarray* (28,29,30,31). Através da interação com estes receptores, tem sido demonstrada a atividade da APN no crescimento celular e seu potencial antiproliferativo em diversas linhagens celulares do câncer de mama (32).

Os mecanismos moleculares responsáveis pela síntese de adipocinas no tecido adiposo e pela ação da APN na carcinogênese e progressão tumoral ainda são desconhecidos, mas inúmeros estudos têm sido desenvolvidos neste sentido (33). Sabe-se que a APN age em conjunção com os receptores AdipoR1 e AdipoR2 promovendo a diminuição da resistência à insulina e da ação de citocinas inflamatórias, inibição da proliferação celular, indução à apoptose e diferenciação celular, diminuição da neovascularização e motilidade celular, diminuição da migração e invasão celular e diminuição da atividade da aromatase, configurando sua ação anticarcinogênica (13).

Estudos *in vitro* demonstram que a ação da APN na carcinogênese ocorre através de mecanismos complexos, utilizando diversas vias metabólicas que

inúmeras vezes se intercruzam (13,20,22,33,34,35,36,37). A APN, assim que sintetizada no tecido adiposo, é regulada por inibição pelo Fator de Necrose Tumoral α (TNFα). A APN bloqueia a ativação do Fator Nuclear kB pelas adipocinas e TNFα, levando à diminuição da produção de cininas inflamatórias e diminuindo a resistência à insulina (33). Através de regulação positiva com o receptor *peroxisome-proliferator-activated*γ, que forma heterodímeros com AdipoR, a APN promove apoptose e diferenciação celular por mecanismos diversos dependentes da p53, BAX, Bcl-2, c-myc e, principalmente, da *cyclinD1*. Neste mecanismo também estão envolvidos os bloqueios da *p42/p44 mitogen activated protein kinase* (p42/p44 MAPK) e da STAT3 (*Activation of Transcription 3*) (33,34,35,36,37).

A ação inibitória da APN sobre a neovascularização e motilidade celular deve-se à estimulação do fator supressor tumoral LKB1 e da AMP *kinase,* inibição da leptina, TNFα, IL-6, *Hepatocyte Growth Factor* (HGF) e b-GFG, levando ao bloqueio da ativação da via mTOR (*mammalian target of rapamycin*) (33,34,35,36,37). A APN, através de sua ação antagônica à leptina, reduz a atividade da aromatase e, consequentemente, a produção local de estrógeno via *fhosphatidylinositol-3-kinase*. Verifica-se também que a APN tem efeito inibitório na migração e invasão celular tumoral. Isso se deve à ação no AMPK através da ativação da PI3k via mTOR, com diminuição da fosforilação da AKT na presença do gene supressor tumoral LKB1 (34) (Figura 1 - Anexo).

A quantificação da APN mRNA no tecido mamário com câncer e no tecido adiposo adjacente revelou expressão muito baixa nestes tecidos, sendo 3,3 vezes maior no tecido mamário de mulheres saudáveis (38).

O estudo da expressão tecidual da APN através da imunoistoquímica demonstra que apenas 15% dos tumores ductais invasivos são positivos (29). Observa-se também que os receptores AdipoR1 e AdipoR2 são expressados simultaneamente em apenas 15% (7/45) dos casos de carcinoma ductal invasivo, e o receptor AdipoR2 foi o receptor de APN predominante no tecido cancerígeno mamário (82% dos casos de câncer invasivo) (28). Em contraste, 75% do tecido normal adjacente ao tumor expressaram APN, principalmente em células mioepiteliais, conhecidas como supressoras tumorais naturais (39). Alguns estudos não demonstram a expressão de APN em algumas linhagens de células tumorais da mama, mas no tecido adiposo ao redor (30).

Korner et al. (38) observaram a expressão dos AdipoR1 e Adipo R2 em aproximadamente 25% a 30% dos tecidos mamários com câncer. Através da imunoistoquímica demonstrou-se que o AdipoR1 se expressa mais no tecido mamário tumoral que no tecido mamário normal, com manifestação mais intensa nas células ductais. Não foram observadas diferenças na expressão do AdipoR2.

Em outro estudo demonstrou-se a expressão dos receptores AdipoR1 em células estromais e ausência de expressão dos receptores AdipoR2, sugerindo que a APN afeta este tecido via receptores AdipoR1 (29). Em estudo recente, observou-se que a expressão do receptor AdipoR2 é significativamente maior em células malígnas que no tecido mamário normal (28). Alguns estudos demonstram que a expressão do receptor AdipoR1 é 2,7- 4,2 vezes maior que do receptor AdipoR2 em células mamárias tumorais (30,38).

A utilização da microdissecção a LASER possibilitou a detecção dos dois receptores mRNA de APN no tecido adiposo mamário, no tecido tumoral, em células epiteliais normais e células estromais. Neste estudo verificou-se que a expressão do AdipoR1 foi maior nos tecidos tumoral e adiposo adjacente quando comparados ao tecido normal. Entretanto, o AdipoR2 não mostrou diferença em sua expressão quando comparados os tecidos tumorais e normais (38).

Além das adipocinas, outra família de proteínas do tecido adiposo que parece estar relacionada com o câncer de mama são as chaperonas lipídicas da família das proteínas transportadoras de ácidos graxos (*Fatty Acid-Binding Protein* -FABP)(40). Estas proteínas apresentam expressão tecidual específica e estão envolvidas no transporte de ácidos graxos (*fatty acids*) no interior das células, modulando o metabolismo lipídico intracelular e regulando a expressão gênica (41,42).

A *Adipocyte Fatty Acid Binding-Protein* (A-FABP, FABP4) ocorre predominantemente no citosol de adipócitos maduros e foi recentemente descrita como associada a marcadores da obesidade e patologias relacionadas. Esta proteína altera a sensibilidade à insulina, o metabolismo lipídico e a resposta inflamatória associada à aterosclerose (41), sendo que a FABP4 sérica pode estar envolvida com a patogênese do câncer de mama (43). Alguns estudos demonstram a presença da FABP4 nos macrófagos, onde estaria relacionada à modulação da produção de citocinas inflamatórias e ao armazenamento de éster de colesterol (44).

A FABP4 tem sido sugerida como participante do transporte de lipídios a compartimentos específicos na célula como mitocôndrias - para oxidação nos peroxissomos e regulação do processo de transcrição nuclear dependente de lipídeos - para o retículo endoplasmático - síntese de membrana, regulação da função de enzimas citoplasmáticas - e para vesículas lipídicas citoplasmáticas de reserva. Entretanto, estes mecanismos regulatórios em tecidos específicos precisam ser elucidados (40).

Além de encontrada no citosol celular, a FABP4 é uma proteína secretada e sua concentração sérica é elevada em pacientes com obesidade e outras alterações metabólicas (17,40,45,46,47). Participa da interação entre as vias metabólica e imunológica, sendo também relacionada ao estado de inflamação crônica associada a vários distúrbios metabólicos, como obesidade, resistência à insulina e diabetes tipo 2 (48,49).

A obesidade é altamente associada ao maior risco de se desenvolver diversas doenças, tais como diabetes tipo 2, doenças cardiovasculares e vários tipos de tumores malignos, como os cânceres de colo, endométrio, fígado e mama (46,50,51,52). A liberação contínua de mediadores inflamatórios, bem como a produção desregulada de adipocinas pelo tecido adiposo têm sido propostas como mecanismos importantes no desenvolvimento do câncer mamário (53,54).

A compreensão do papel do tecido adiposo na ocorrência do câncer de mama é de suma importância, principalmente em pacientes obesas. A despeito dos avanços terapêuticos e diagnósticos, apenas cerca de 50% das pacientes

tem câncer de mama diagnosticado em suas fases iniciais, o que justifica a busca de maior conhecimento sobre a sua etiologia e fisiopatologia.

Tendo em vista a possível correlação entre os níveis de FABP4, de adiponectina e da expressão de receptores de adiponectina AdipoR1/R2 com o câncer de mama, a falta de estudos agrupando estes fatores e, associado à falta de dados em relação a estes fatores e a mama normal ou patologias benignas, torna-se de elevada importância a realização de estudos com esta abordagem.

O estudo da relação entre obesidade, resposta inflamatória e doenças da mama, em especial o câncer de mama, é ainda incipiente e necessita maior investigação, com vistas a contribuir para a melhor compreensão dos mecanismos envolvidos na história natural desta doença.

2. Objetivos

2.1. Objetivo Geral

Comparar o padrão de expressão da Adiponectina, dos receptores de Adiponectina tipos 1 e 2 (Adipo R1/R2) e da *Adipocyte Fatty Acid Binding Protein* (FABP4) no carcinoma invasor, nas lesões precursoras e nas alterações benignas da mama e correlacioná-lo com parâmetros clínicos e histológicos.

2.2. Objetivos Específicos

- **Artigo 1:** Comparar o padrão de expressão imunoistoquímica da adiponectina (APN) e dos seus receptores tipos 1 e 2 (adipoR1/R2) no carcinoma invasor (CDI), carcinoma ductal *in situ* (CDIS) e lesões benignas da mama (BE) e correlacioná-lo com parâmetros clínicos e histológicos.

- **Artigo 2:** Avaliar o padrão de expressão proteica da FABP4 nos tecidos epitelial e adiposo mamário de portadoras de CDI, CDIS e lesões benignas da mama.

3. Publicações

Artigo 1 – Protein expression of adiponectin and adipoR1/R2 receptors in benign breast lesions, ductal carcinoma *in situ* and invasive breast câncer.

Artigo 2 – FABP4 protein expression in women with breast cancer, ductal carcinoma *in situ* and benign breast lesions – association with obesity.

3.1. Artigo 1

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Protein expression of adiponectin and adipoR1/R2 receptors in benign breast lesions, ductal carcinoma *in situ* and invasive breast cancer

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ABSTRACT

Obesity is responsible for a 30 to 50% increase in breast cancer incidence in the postmenopause. A possible explanation for this might be an association of adipokines secreted by adipose tissue with obesity and breast cancer. *Objective:* to compare the immunohistochemical expression pattern of adiponectin (APN) and its receptors type 1 and 2 (AdipoR1/R2) in invasive ductal carcinoma (IDC), ductal carcinoma *in situ* (DCIS) and benign breast lesions (BE), correlated with clinical and histological parameters. *Material and Methods:* Tissue blocks and slides from 223 women (69 IDC, 73 DCIS and 81 BE) treated at CAISM/UNICAMP from 2008 to 2011 were included and Tissue Microarray (TMA) slides were prepared. Marker expression was evaluated in tumor tissue in IDC and DCIS and in epithelial tissue in BE. To assess the correlation between marker expression and clinical/histological parameters, the chi-square test, Mann-Whitney test, Kruskal-Wallis test and Spearman correlation were used. Values of $p<0.05$ were considered significant. *Results:* APN was expressed in 65% of IDC, 48% of DCIS and 12% of BE cases and AdipoR1 was expressed in 98% of IDC, 94% of DCIS and 71% of BE. All IDC and DCIS cases expressed AdipoR2 versus 81% of BE cases. In IDC and DCIS, an association between a higher APN expression and ER-negative tumors was observed. In DCIS, this association was also observed with PR-negative tumors. *Conclusions:* Differences in protein marker expression observed in different breast diagnoses, suggest that these markers participate in the complex etiologic mechanism of these different conditions.

Keywords: adiponectin, breast cancer, DCIS**,** benign breast alterations, adiponectin R1 and R2 receptors, TMA**,** Immunohistochemistry.

INTRODUCTION

It has been suggested that a number of factors may explain the link between obesity and breast cancer. Hypersinsulinemia of obesity and high estrogen levels [1,2], adipose tissue as the main source of aromatase, converting androgens into estrogens in postmenopausal women are among the probable hypotheses. However, the actual relationship between obesity and breast cancer has still not been elucidated.

Evidence suggests that adipose tissue, recognized as an endocrine organ, produces and secretes a variety of mediators that interfere with the development of breast cancer [3]. These polypeptides termed adipokines have an important function in energy homeostasis. Adipokines include factors that act by stimulating or inhibiting angiogenesis and cell proliferation, growth factors and proinflammatory/antiinflammatory factors involved in the mediation of inflammatory diseases related to obesity [4,5]. These adipokines may be detected by gene expression, protein expression (tissue) or blood measurements [6].

It is believed that adipokines act on breast tissue in an endocrine fashion, by external fat deposit, via paracrine pathway, through the breast fat tissue and via autocrine action through the breast tumor itself. The anatomic structure of the breast promotes an interaction between the breast adipose and glandular tissue, suggesting that adipokines produced by breast adipose tissue and tumor cells may be the major link between obesity, breast cancer progression and metastases [7-10].

Over 50 types of adipokines have been described. Among them, adiponectin (APN) is the most abundant protein produced by adipocytes that is present in the bloodstream [11]. In general, adipokines act on breast tumor cells through their receptors, influencing cell proliferation, migration and tumor invasion, regulating the production of epithelial proteins, angiogenic proteins and growth factors [12].

The mechanism of action of APN, along with its receptors type 1 (AdipoR1) and type 2 (AdipoR2), ranges from inhibition of cell proliferation, to apoptosis and alterations in cell cycle and cell survival dependent on the tumor cell line, representing its anticarcinogenic action [3].

APN and receptors AdipoR1 and AdipoR2 have been characterized as different breast cancer cell lines by immunohistochemistry and tissue microarray (TMA) [13-16], demonstrating the activity of APN in cell growth and its potential antiproliferative action in breast cancer [17].

Although still controversial, the role of adipokines (particularly APN) in breast cancer development and progression, including noninvasive tumors, has been the reason for many studies [17-19]. Nevertheless, there are few studies on APN expression in normal breast tissue and in benign breast disorders.

Knowledge of the role of adipokines in breast tissue in different stages of epithelial proliferation until breast cancer may contribute to a better understanding about mammary gland carcinogenesis, contributing to potential interventions for primary and secondary prevention, or even tailored therapeutic interventions.

Thus, the aim of this study is to compare the immunohistochemical expression pattern of APN and receptors AdipoR1/AdipoR2 in benign epithelial tissue, DCIS and invasive breast carcinoma, correlated with clinical and histological parameters.

MATERIAL AND METHODS

This is a cross-sectional study evaluating slides and paraffin-embedded blocks obtained from 223 women treated in the Division of Gynecologic Oncology and Breast Disease at CAISM/UNICAMP from January 2008 to December 2011, undergoing

biopsy, quadrantectomy or mastectomy. Of these lesions, 69 were invasive ductal carcinomas (IDC), 73 were DCIS and 81 were negative for breast cancer (BE). All materials were submitted to TMA construction in triplicate and subsequent immunohistochemical analysis of each marker.

Clinical data, e.g. age, body mass index (BMI) and menopausal status and histopathological data, e.g. postoperative staging, estrogen receptor (ER), progesterone receptor (PR), histological and nuclear grade were collected from the respective patient medical charts.

Tissue microarray construction (TMA)

Representative tumor regions were marked on H&E-stained slides and regions corresponding to paraffin-embedded blocks. TMA (Beecher Instruments Microarray Technology, Silver Spring, CA, USA) slides were then constructed in triplicate. Three 1.0-mm thick sections of donor tissue block were collected and TMA cores were spaced 0.2 mm apart on the recipient paraffin block. After processing, the blocks were cut (5μm thick) and slides were constructed for immunohistochemical study.

Immunohistochemical study

TMA slides were hydrated in decreasing concentrations of ethanol (100, 80, 50% ethanol) and washed in distilled running water. Endogeneous peroxidase activity was blocked in three baths (3 minutes each) in 10 volumes of hydrogen peroxide, followed by washing in running distilled water. For antigen retrieval, a Pascal pressure cooker by Dako was used, with the purpose of unmasking the antigens. The slides were immersed

in sodium citrate buffer solution, pH 6.0 (10mM) at 95ºC for 30 minutes. Subsequently, these slides were cooled for 20 minutes at room temperature and washed in distilled running water. After this step, the histological sections were incubated in a humidified chamber with specific primary antibody in specific dilutions, as recommended by the manufacturer, at 4ºC overnight. After incubation, the slides were washed three times in PBS (phosphate buffered saline solution, pH 7.4 to 7.6), tapping off the excess buffer and dried. For detection, the slides were incubated with ADVANCE™ HRP Detection System (Dako) at 37ºC for 1 hour and then submitted to three washings in PBS, tapping off the excess. After incubation, DAB chromogen substrate (3´-diaminobenzidine, SIGMA, code D5637) was used for color development at a ratio of 0.06g to 100ml in PBS, 500µl of 3% hydrogen peroxide and 1ml of dimethyl sulphoxide (DMSO) at 37ºC for 5 minutes. Finally, the slides were washed in running water and counterstained with Harris' hematoxylin for 30 to 60 seconds. The sections were dehydrated in increasing concentrations of ethanol baths and tissue was cleared in three xylene baths. These sections were then mounted, applying coverslips and resin (Entellan). Internal and external positive and negative controls were used to validate the immunohistochemical reactions. All antibodies used were from Abcam: monoclonal anti-adiponectin (ab22554) antibody, at a dilution of 1:800; polyclonal anti-AdipoR1 (ab53398) antibody, at a dilution of 1:100; and polyclonal anti-AdipoR2 (ab53399) antibody, at a dilution of 1:100.

Interpretation of immunohistochemical study

Interpretation of the immunohistochemical study was performed by analyzing two parameters in the epithelial component: intensity [16,20] of marker expression, categorized into 0 (negative), 1 (mild), 2 (moderate) and 3 (strong); and percentage

[18,21] of stained cells, categorized into 0 (0%), 1 (< 30%), 2 (30 to 60%) and 3 (> 60%) (Figure 1).

Each patient was given two scores (positivity and intensity) for each marker. Both scores were added to obtain an intermediate score of marker expression. Since all TMA were constructed in triplicate, a mean score was calculated in the end. Final scores obtained were 0, 2, 3, 4, 5 and 6 (Table 1). When any spot was not assessable, it was considered lost and a mean value was determined among the most reliable spots. If only one spot was viable for analysis, it was considered for the final result.

Data were descriptively analyzed to evaluate the correlation between the marker expression and between clinical/histopathological parameters, using the chi-square tests, Mann-Whitney, Kruskal-Wallis and Spearman's correlation. Determinations were calculated, considering values of $\alpha = 0.05$ ($p<0.05$) as significant.

The study was approved by the Research Ethics Committee of the UNICAMP Medical School (nº 824/2011, CAAE: 0740.0.146.000-11), which dispensed with the use of a written informed consent term.

RESULTS

Of the 223 patients, 69 were diagnosed with IDC and 73% of these had histological grade III and 62% nuclear 3 tumors. Stages I and II tumors (36% and 54%, respectively) predominated and 45% had axillary lymph node compromise at the time of surgery.

Among the DCIS, 75% were multifocal and 47% had comedonecrosis. Only 7 cases exhibited a single histological type and the remaining cases showed combinations of 2 to 5 non-comedo subtypes. The most frequent subtypes were cribriform, solid, micropapilary and adherent.

Of the 81 BE cases, 56% had two or more histologic changes combined: 62% proliferative epithelial alterations without atypias (HDT, CAPSS, papilloma and sclerosing adenosis); 19.8% nonproliferative alterations (fibroadenoma, fibrosis/fibrosclerosis, apocrine metaplasia and normal breast tissue); and 19% proliferative epithelial alterations with atypias (HDA, CLIS, HLA and CAPSS with atypias).

Patient age of the total sample at diagnosis, ranged from 27 to 87 years. In addition, the groups were similar, with a mean age of 54.41±9.89 years in IDC, 54.53±10.39 years in DCIS and 51.74±11.76 years in benign cases (p=0.1178). BMI of the sample ranged from 18.4 to 52.1 Kg/m^2 (mean: 28.3±5.6 Kg/m^2). No difference in BMI was observed among the groups studied (p=0.8492) (data not shown), although about 70% of patients were overweight/obese. The groups were also similar in terms of menstrual status (p=0.1605) (Table 2).

There was APN immunohistochemical expression in the epithelial component in 65% of IDC, 48% of DCIS and 12% of BE. In contrast, AdipoR1 expression was detected in 98% of IDC, 94% of DCIS and 71% of BE. All cancer cases and DCIS expressed AdipoR2 versus 81% of benign lesions.

The mean score of APN immunohistochemical expression observed was higher in IDC but the difference was statistically significant between IDC and BE and between DCIS and BE. Regarding AdipoR1/R2, both were more highly expressed in IDC than in DCIS and BE and all differences were statistically significant (Figure 2).

No difference in the immunohistochemical expression of APN and adipoR1/R2 was observed regarding age, menopausal status and BMI in the three groups studied. Only a tendency towards a more highly positive APN (p=0.0707) and AdipoR1 (p=0.0614) expression was observed in women up to 50 years of age in the BE group (data not shown).

In IDC and DCIS cases, there was an association between a higher level of APN expression and ER-negative tumors. In DCIS, this association was also observed in PR-negative tumors. No association between AdipoR1/AdipoR2 expression and hormone receptor expression in the tumor was observed (Table 3).

In IDC, there was no association between immunohistochemical marker expression and postoperative staging, histological and nuclear tumor grades. In DCIS, an association between the expression of any marker and the presence of comedonecrosis, multifocality and histologic type was not observed. Furthermore, in benign breast lesions, there was no association between the expression of markers studied and epithelial proliferation or cell atypias (data not shown).

In terms of correlation between markers, in IDC it was observed that APN expression had a tendency to be positively correlated with AdipoR1 expression ($p=0.0754$) and there was no correlation with AdipoR2 expression. AdipoR1 expression was positively associated with AdipoR2 expression (Figure 3A). In DCIS, APN was positively correlated with AdipoR2 expression, showing that similar to IDC, there was a trend towards a positive association with AdipoR1 expression (Figure 3B). Finally, in benign cases APN expression was positively correlated with AdipoR1 and AdipoR2 expression (Figure 3C).

DISCUSSION

Our findings demonstrate that immunohistochemical expression of APN had a higher level in IDC and in DCIS than in the epithelial component of benign lesions and AdipoR1 and R2 were more highly expressed in IDC than in DCIS and BE. These findings may suggest that aggravation of epithelial alterations could result in an increase

in APN and its receptors. Thus, the breast tissue would make use of protective mechanisms against cancer development or progression.

APN protein expression in the epithelial component of breast cancer and in DCIS tumor cells was 65% and 48%, respectively, while in the literature it is reported that positivity values range from 54% to 33%, respectively [18]. There were also much lower values (15%) in IDC and no APN protein expression in DCIS [19]. We highlight that the studies cited differed in the methodology used. The positivity criteria and number of cases analyzed were different. Therefore, even when tissue protein expression is measured, discrepancies in the results can be found.

In a study by Korner et al. (2007) [20], a significant level of APN expression was not observed in breast cancer cells, apart from a probable localized autocrine mechanism, demonstrating a high level of expression in peritumoral tissue compared to controls. Immunohistochemical analysis in 96 samples of breast cancer tissue and 25 samples of normal breast tissue, showed that AdipoR1 receptors had a strongly positive protein expression in epithelial and ductal nonmalignant cells. AdipoR1 expression was marginal, more pronounced in malignant cells, and positive in 30% of cases, with a higher level of expression than in normal peritumoral breast tissue (13%). AdipoR2 receptor expression was similar in malignant cells (26%) and normal peritumoral breast tissue (29%).

These data are not in agreement with the results from our study, which showed a positive AdipoR1 receptor expression in 99% of tumor cases and 72% of benign cases ($p<0.0001$). AdipoR2 expression was shown to be positive in 100% of cancer cases and 82% of benign cases.

Our results are consistent with those observed by another study reporting a positive AdipoR1 expression in about 32% of DCIS and moderate to strong expression in 71% of tumors [16].

Concerning menstrual status, recent studies have demonstrated that there is no difference in expression between APN and AdipoR (unidentified type of receptor), which is in agreement with findings observed by our study [22].

Despite the involvement of estrogens in the etiology and development of breast cancer, approximately 30% of these tumors do not express ER. Therefore, these tumors are refractory to antiestrogen therapies. The remaining 40% of tumors express ER and do not respond to hormone therapy [9].

There are several mechanisms involved in the interaction of adipokines with ER and breast cancer, ranging from ER activation without estradiol involvement in the MCF-7 cancer cell line to an antiestrogen action, leading to suppression of cell proliferation [9]. An *in vitro* study showed that APN attenuated tumor cell growth in MDA-MB-231 ER-negative lines, inhibiting cell proliferation and inducing apoptosis. APN may also inhibit the proliferation induced by insulin and other growth factors in T47D, ER-positive tumor cell lines [23]. A negative correlation between APN and ER expression observed in our study may suggest a nonexistent or weak action of this hypothetical anti-tumor factor in ER-positive breast cancer.

APN is a protein primarily structured in monomers, which are organized into five different configurations: globular (gAPN), long-chain (fAPN), high molecular weight (HMW), intermediate molecular weight (MMW) and low molecular weight (LMW). In addition to structural differences, evidence indicates that different biological effects are produced in the body by different types of APN (11). It was demonstrated that HMW

concentration and the ratio between relative HMW and total APN concentrations are related to favorable metabolic effects [24].

This study investigated the expression pattern of APN and its receptors AdipoR1 and AdipoR2 in breast cancer, carcinoma *in situ* and benign breast lesions. However, despite the high positivity rates observed, it was not possible to make inferences about the action or functionality of these markers. There is a need for further studies that correlate these tissue findings with serum marker levels, with other clinical/laboratory parameters of obesity/metabolic syndrome and also with gene expression of these adipokines.

Additional knowledge of the physiology of adipose tissue, its actual functions and substances produced is crucial and opens a wide field of research. It has been suggested that adipose tissue, currently described as an endocrine organ, may have a causal role or may be directly related to several types of neoplasms.

This study demonstrated tissue expression of different markers related to adipocyte function in breast cancer, DCIS and patients with benign breast lesions. A better understanding of the expression of APN and its receptors AdipoR1 and AdipoR2 in these different breast conditions may contribute to the description of tumor markers related to breast cancer, or even aid in the development of APN agonist medication or receptor activators that may promote potential breast protection.

Dozens of markers secreted by adipose tissue linked to obesity have been described and are largely unknown. Studies into this subject are paramount not only for a greater understanding of the pathophysiology of breast cancer, but also of adipose tissue.

CONCLUSION

Positivity of APN immunohistochemical expression was higher in IDC and in DCIS cases than in benign cases. Concerning AdipoR1/R2, it was observed a stronger expression in IDC than in DCIS and benign.

AdipoR2 was positively expressed in all IDC and DCIS cases and 3/4 of BE cases.

APN tended to be more highly expressed in patients younger than age 50 in IDC and DCIS.

In IDC and DCIS, an association between APN expression and ER-negative tumors was observed. In DCIS, this association was observed in ER- and PR-tumors.

ETHICAL STANDARDS

The study was designed and conducted in accordance with the Declaration of Helsinki and with the recommendations of Resolution 196/96 of the Ministry of Health, Brazil. The study was approved by the Research Ethics Committee at UNICAMP School of Medicine (N^{o} 824/2011, CAAE: 0740.0.146.000-11).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Table 1 – Final scores obtained by analyzing parameters including intensity of marker expression and percentage of stained cells

	Intensity of reaction			
% stained cells	**0 (negative)**	**1 (mild)**	**2 (moderate)**	**3 (strong)**
0	0			
1 (< 30)		2	3	4
2 (30 to 60)		3	4	5
3 (> 60)		4	5	6

Table 2 – Clinical and histological characteristics, according to histopathological diagnosis

	IDC (N=69)	**DCIS (N=73)**	**BENIGN (N=81)**	
CHARACTERISTICS	**n (%)**	**n (%)**	**n (%)**	***p***
Age (years)				*0.3010*
≤ 50	27 (39.1)	28 (38.3)	40 (49.4)	
> 50	42 (60.9)	45 (61.7)	41 (50.6)	
BMI				*0.2936*
≤ 20 - 25	18 (26.5)	22 (30.1)	25 (31.3)	
> 25 - 30	30 (44.1)	31 (42.5)	24 (30.0)	
> 30	19 (29.4)	20 (27.4)	31 (38.8)	
Menstrual status				*0.1605*
Premenopause	25 (36.2)	25 (34.2)	39 (48.1)	
Postmenopause	44 (63.8)	48 (65.8)	42 (51.9)	
ER (N=142)				*0.1062*
Positive	56 (81.2)	32 (68.1)		
Negative	13 (18.8)	15 (31.9)		
PR (N=142)				*0.6989*
Positive	45 (65.2)	29 (61.7)		
Negative	24 (34.8)	18 (38.3)		

Chi-square; BMI=body mass index; IDC= invasive ductal carcinoma; DCIS= ductal carcinoma in situ; ER=estrogen receptor; PR=progesterone receptor

Table 3 – Adipokine marker expression scores in the epithelial component of IDC and DCIS, according to hormone receptor expression (ER and PR) and histopathological diagnosis (N=142)

	Adiponectin	**AdipoR1**	**AdipoR2**	
	Median (mean±SD)	**Median (mean±SD)**	**Median (mean±SD)**	**n**
IDC				
ER				68
Negative	4 (3,7 ± 1.4)	6 (5.3 ± 1.0)	6 (5.5 ± 0.9)	13
Positive	3 (2.6 ± 2.0)	6 (5.3 ± 1.0)	6 (5.6 ± 0.7)	55
p	*0.0302*	*1.0000*	*0.7848*	
PR				68
Negative	4 (3.2 ± 1.9)	6 (5.3 ± 0,9)	6 (5.5 ± 0.9)	24
Positive	3 (2.5 ± 1.9)	5 (5.3 ± 1.1)	6 (5.6 ± 0.7)	44
p	*0.1730*	*0.8186*	*0.7848*	
DCIS				
ER				47
Negative	4 (3.1 ± 2.1)	4 (4.2 ± 1.3)	5 (4.9 ± 0.8)	15
Positive	1 (1.8 ± 1.9)	5 (4.4 ± 1.4)	5 (4.9 ± 0.9)	32
p	*0.0321*	*0.3800*	*0.8162*	
PR				47
Negative	4 (3.2 ± 2.0)	5 (4.3 ± 1.2)	5 (4.9 ± 0.8)	18
Positive	0 (1.6 ± 1.9)	5 (4.3 ± 1.4)	5 (4.9 ± 0.9)	29
p	*0.0115*	*0.8396*	*0.7605*	

For each marker, two scores (positivity and intensity of the reaction) were given and the sum of both scores resulted in an intermediate score of marker expression. A mean final score was calculated; therefore, final scores were 0, 2, 3, 4, 5 and 6. Data were expressed as Medians and Means ± SD. Mann-Whitney test.

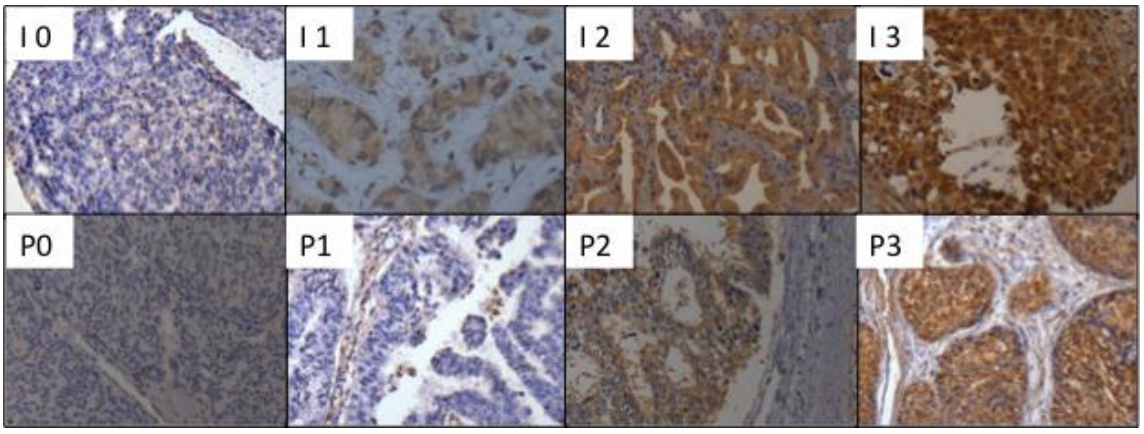

Figure 1. Intensity (I) and percentage (P) of immunohistochemical reaction: I0 - intensity 0 – APN in IDC (magnification of 100x); I1 – intensity 1 - APN in IDC (400x); I2 - Intensity 2 – APN in IDC (400x); I3 - Intensity 3 – APN in IDC (400x); P0 - percentage 0 – APN in IDC (100x); P1 - percentage 1– APN in IDC (100x); P2 - percentage 2 – APN in IDC (100x); P3 - percentage 3 – APN in IDC (100x).

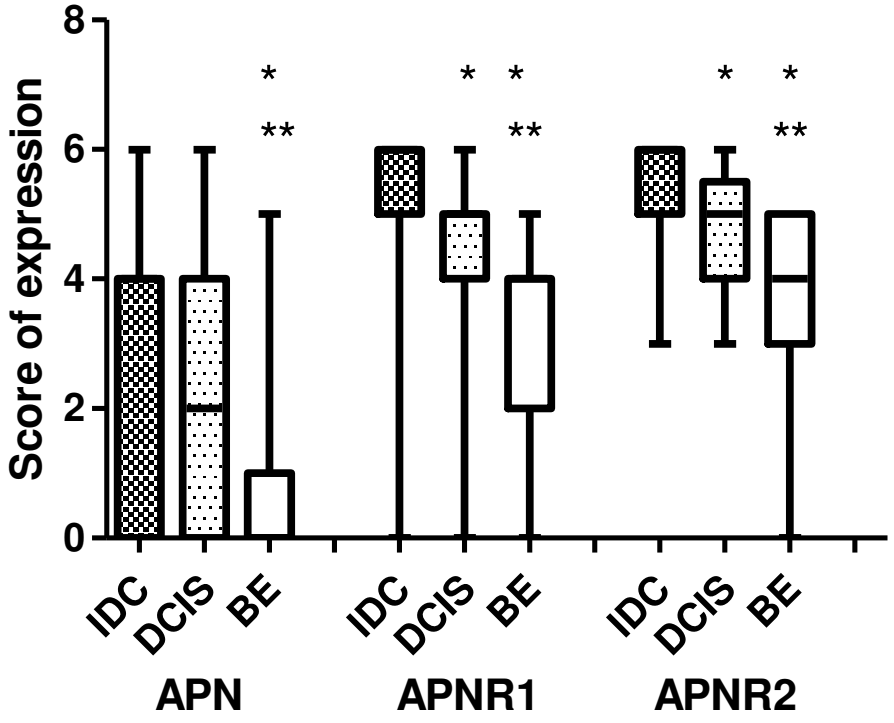

Figure 2. Marker expression scores in the epithelial component, according to histopathological diagnosis (N=223). For each marker, two scores (positivity and intensity of the reaction) were given and both were added to obtain an intermediate score of marker expression. A mean final score was calculated; thus, the final scores were 0, 2, 3, 4, 5 and 6. Data are expressed as Median, IQR, Min and Max. Kruskal-Wallis with Dunn's Multiple Comparison Test, * $p < 0.05$ between IDC and DCIS or IDC and BE; ** $p < 0.05$ between DCIS and BE.

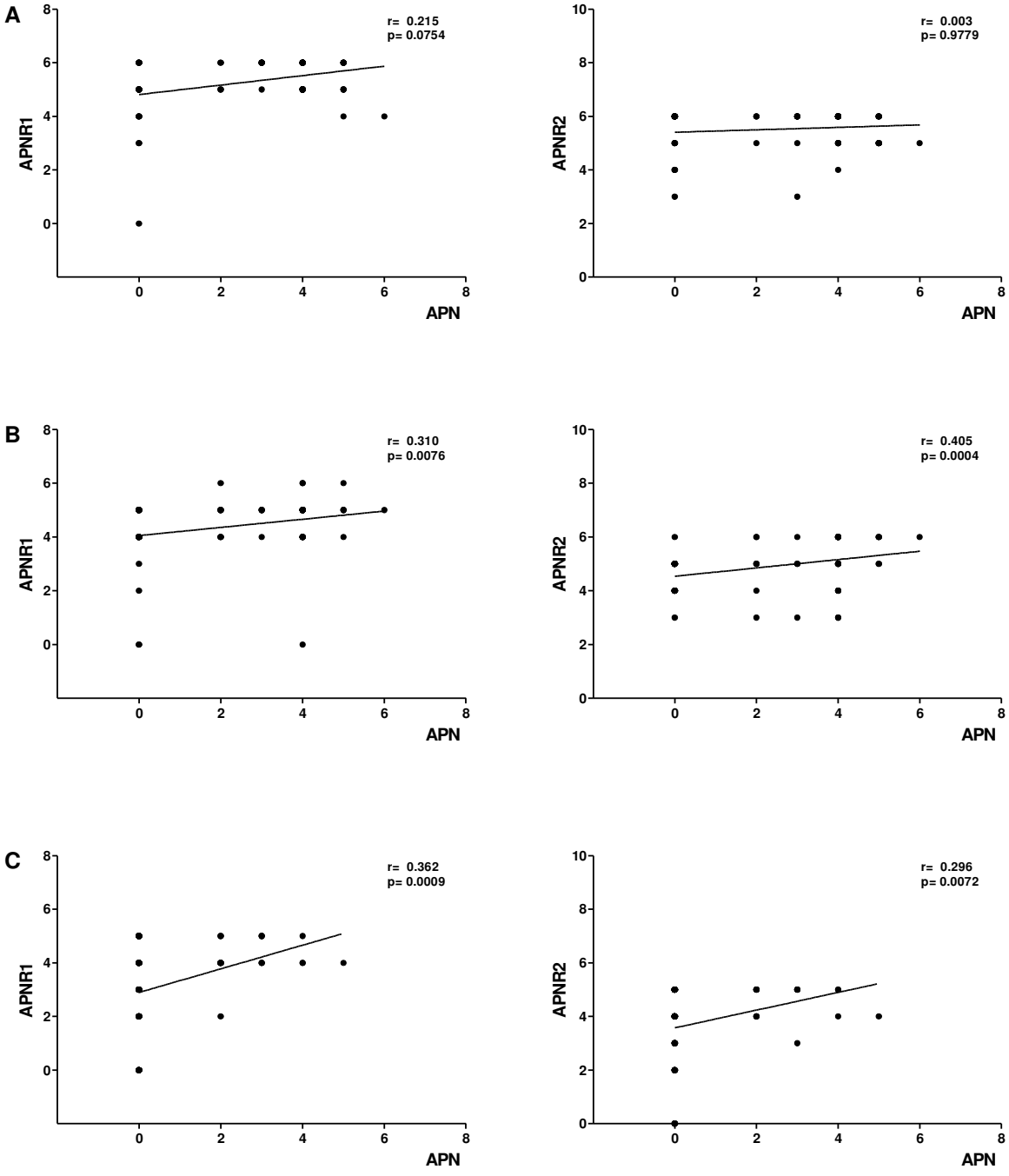

Figure 3. Correlation between APN expression and adipoR1/R2 expression, according to histopathological diagnosis.

3.2. Artigo 2

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Assunto: Fwd: BREA - Acknowledgement of Receipt
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FABP4 protein expression in women with breast cancer, ductal carcinoma *in situ* and benign breast lesions – association with obesity

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ABSTRACT

Fatty acid binding protein (FABP4) is one of the most abundant intracellular binding proteins present in mature adipocytes. High concentrations of FABP4 are found in obese individuals, who are at high risk for developing several types of cancer, including breast cancer. *Objective:* to assess FABP4 protein expression in breast epithelial and adipose tissue in women with breast cancer (BC), ductal carcinoma *in situ* (DCIS) and benign breast lesions (BE). *Material and Methods:* Paraffin-embedded blocks from 223 women (69 BC, 73 DCIS and 81 BE) treated at CAISM/Unicamp from 2008 to 2011 were included and Tissue Microarray (TMA) slides were prepared. FABP4 expression was assessed in the tumor/epithelial tissue, proximal fat (PF) and distant fat (DF) tissue in BC, DCIS and BE. To evaluate the correlation between marker expression at different sites and clinical/histological parameters, the Chi-square, Fisher exact, Mann-Whitney, Kruskal-Wallis tests and Spearman correlation were used. Values of $p<0.05$ were considered significant. *Results:* FABP4 protein expression was observed in epithelial tissue in 90% of BC, 40% of DCIS and 28% in BE. In PF and DF, FABP4 was more highly expressed in patients with BE than in patients with BC, a difference that was considered significant. In patients with BC, there was a higher level of FABP4 expression in patients aged 50 years or younger. All cases expressed this marker moderately or intensely in the peri-epithelial and distant adipose tissue. *Conclusion:* our results indicate that there is a direct correlation between FABP4 protein expression, breast cancer and obesity.

Keywords: breast cancer, A-FABP, FABP4, fatty acid binding proteins, obesity.

INTRODUCTION

Breast cancer is the most common malignancy in females and the main cause of cancer-related deaths worldwide [1,2]. Obesity is a well-known risk factor for the development of breast cancer in the postmenopausal period. Recent studies have linked obesity to advanced disease stages and poor prognosis [3]. Adipose tissue has been studied more intensively, since obesity emerged as a severe public health issue and adipose tissue is known to play a major role in several metabolic pathways that are vital to the human being [4].

Adipose tissue is currently recognized as an endocrine organ, producing and secreting several factors that interfere with the development of breast cancer [5]. These factors may be related to an important function in the homeostasis of energy metabolism, acting by stimulating or inhibiting angiogenesis and cell proliferation, growth factors and proinflammatory and antiinflammatory factors involved in the mediation of inflammatory diseases linked to obesity [6,7]. These proteins may be detected by gene expression, tissue expression or blood measurements [8]. Among these molecules are the lipid chaperones of the family of fatty acid-binding proteins (FABP) [9-14].

In mammals, at least nine different subtypes of FABP abundantly expressed in specific tissues and related to local lipid metabolism have been described. The family contains the following subtypes: liver (L-FABP/FABP1), intestinal (I-FABP/FABP2), heart (H-FABP/FABP3), adipose (A-FABP/FABP4/aP2), epidermal (E-FABP/FABP5/mal1), ileal (Il-FABP/FABP6), brain (B-FABP/FABP7), myelin (M-FABP/FABP8), and testicular (T-FABP/FABP9) [15,16].

FABP4 is expressed by adipocytes and macrophages and is involved in insulin and glucose metabolism. Many studies have also reported that this protein may have a causal role in breast cancer [14,17]. Is one of the most abundant fatty acid binding

intracellular proteins present in mature adipocytes [18,19]. It has been suggested that this protein participates in lipid trafficking to specific cell compartments, such as the mitochondria, for oxidation in peroxisomes, regulation of the lipid-dependent nuclear transcription process, to the endoplasmic reticulum, membrane synthesis, regulation of cytoplasmic enzyme function and to cytoplasmic lipid reserve vesicles. Nevertheless, these regulatory mechanisms in specific tissues need to be elucidated [9].

In addition to its location in the cell cytosol, FABP4 is a protein that is secreted. Its serum concentrations are elevated in obese patients and those with other metabolic alterations [9,20-23]. FABP4 participates in the interaction between the metabolic and immunologic pathways. Furthermore, it is also related to the chronic inflammatory state associated with various metabolic disorders, e.g. obesity, insulin resistance and type 2 diabetes [15,18].

Obesity is strongly associated with an increased risk of developing a diversity of diseases, e.g. type 2 diabetes, cardiovascular disease and various types of malignancies, e.g. cancers of the cervix, endometrium, liver and breast [22,24-26]. The continuous release of inflammatory mediators, as well as the dysregulated production of adipokines by adipose tissue, has been proposed as important mechanisms in the development of breast cancer [27,28].

There are several studies demonstrating associations between breast cancer and obesity, FABP4 and obesity and breast cancer and FABP4. To date, however the combined relationship between FABP4, obesity and breast cancer risk, has been explored by only one study [17]. Thus, the purpose of the present investigation was to assess FABP4 protein expression in breast epithelial and adipose tissue in women with breast cancer, DCIS and benign breast lesions and its correlation with BMI, in an attempt to investigate its potential role as a diagnostic marker and/or therapeutic target in breast cancer associated with obesity.

MATERIAL AND METHODS

This is a cross-sectional study evaluating slides and paraffin-embedded blocks obtained from 223 women treated in the Division of Gynecologic Oncology and Breast Disorders at CAISM/Unicamp from January 2008 to December 2011, undergoing biopsy, setorectomy or mastectomy. Of the total number of cases, 69 were breast cancer (BC), 73 DCIS and 81 biopsies were negative for breast cancer (BE). These biopsies had the following diagnoses: proliferative epithelial alterations without atypia, including typical ductal hyperplasia (TDH), columnar epithelial alteration with decapitation secretion or columnar alteration with prominent apical snouts and secretions (CAPSS), papilloma and sclerosing adenosis; nonproliferative alterations encompassing fibroadenoma, fibrosis/fibroesclerosis, apocrine metaplasia and normal breast tissue; and proliferative epithelial alterations with atypia, including atypical ductal hyperplasia (ADH), lobular carcinoma in situ (LCIS), atypical lobular hyperplasia (ALH) and CAPSS with atypia. All materials were sent to the laboratory for TMA construction in triplicate and subsequent immunohistochemistry analysis.

Clinical data, e.g. age, body mass index (BMI) and menopausal status; and histopathological data, e.g. postoperative staging, estrogen receptor (ER), progesterone receptor (PR) and histological and nuclear grade were collected from the respective patient medical charts.

Tissue microarray construction (TMA)

Representative tumor regions were marked on hematoxylin-eosin (H&E)-stained slides and corresponding regions in paraffin-embedded blocks (epithelial tissue (ET), peri-epithelial fatty tissue (PF) and distant fatty tissue (DF)). TMA slides were prepared (Beecher Instruments Microarray Technology, Silver Spring, CA, USA) in triplicate.

Three sections (1.0mm- thick) of donor block tissue were collected and spaced 0.2mm part on the recipient paraffin block. After processing, the blocks were sectioned (5µm thick) and slides were prepared for immunohistochemical study.

Immunohistochemical Study

TMA slides were hydrated in decreasing concentrations of ethanol (100, 80, 50% ethanol) and washed in running distilled water. Endogenous peroxidase activity was blocked with three baths (3 minutes each), in 10 volumes hydrogen peroxide, followed by washing in distilled running water. For antigen retrieval, a Pascal pressure cooker by Dako was used, with the purpose of unmasking the antigens. The slides were immersed in sodium citrate buffer solution, pH 6.0 (10mM) at 95ºC during 30 minutes. Subsequently, the slides were cooled at room temperature for 20 minutes and washed in distilled running water. After this step, the histological sections were incubated in a humidified chamber with specific primary antibody in specific dilutions, as recommended by the manufacturer at 4ºC overnight. After incubation, the slides were washed three times in PBS (phosphate buffered saline solution pH 7.4 to 7.6), tapping off the excess buffer and dried. For detection, the slides were incubated with ADVANCE™ HRP Detection System (Dako) at 37ºC for 1 hour and then submitted to three washings in PBS, tapping off the excess. After incubation, chromogen substrate DAB (3´-diaminobenzidine, SIGMA, code D5637) was used for color development, at a ratio of 0.06g to 100ml PBS, 500µl of 3% hydrogen peroxide and 1ml of dimethyl sulphoxide (DMSO) at 37ºC during 5 minutes. Finally, the slides were washed in running water and counterstained with Harris' hematoxylin for 30 to 60 seconds. Sections were dehydrated in increasing concentrations of ethanol baths and tissue was cleared in three baths of xylene. These sections were

then mounted, applying coverslips and resin (Entellan). Positive and negative, internal and external controls were used to validate the immunohistochemical reactions. The antibody used was anti-FABP4 (ab13979), from Abcam, at a dilution of 1:300.

Interpretation of immunohistochemical study

Interpretation of immunohistochemical study was performed by analyzing two parameters: intensity [29,30] of marker expression, categorized into 0 (negative), 1 (mild), 2 (moderate) and 3 (strong); percentage [31,32] of stained cells, categorized into 0 (0%), 1 (<30%), 2 (30 to 60%) and 3 (> 60%) (Figure 1).

Each patient was given two scores (positivity and intensity) for the marker evaluated. These scores were added, obtaining an intermediate marker expression score. In the end, a mean score was calculated from these two scores, since all TMAs were constructed in triplicate. Thus, the final scores obtained were 0, 2, 3, 4, 5 and 6 (Table 1). When any TMA spot was not assessable, it was considered lost and a mean value was determined among the most reliable spots. If only one TMA spot was viable for analysis, it was considered for final result.

A descriptive analysis of data was made. To assess a possible relationship between marker expression in different tissues and between clinical/histopathological parameters, the chi-square test or Fisher's exact test, Mann-Whitney test, Kruskal-Wallis test and Spearman correlation were used. Determinations were calculated, considering values of $\alpha=0.05$ ($p<0.05$) as significant.

The study was approved by the Research Ethics Committee at UNICAMP School of Medicine (N^{o} 824/2011, CAAE: 0740.0.146.000-11), dispensing with the use of a written informed consent term.

RESULTS

Of the 223 patients, 69 received a diagnosis of BC (72.5% of these were histological grade III and 62.3% were nuclear grade 3 tumors). There was a predominance of stages I and II (36.2% and 53.6%, respectively) and axillary lymph nodes were compromised in 45% of the patients at the time of surgery. Of the 73 DCIS, 75.4% were multifocal, 46.6% had comedonecrosis, 7 cases had a single histological type and the remaining patients exhibited a combination of 2 to 5 different non-comedo subtypes. The most frequent lesions were cribriform, solid, micropapillary and adherent. Of the 81 women with BE, 55.6% had two or more associated histological alterations. The most frequent were proliferative epithelial alterations without atypia (61.7%), nonproliferative alterations (19.8%) and proliferative epithelial alterations with atypia (18.5%).

Age of the sample at diagnosis ranged from 27 to 87 years (median: 53 years and mean: 53.5 + 10.8 years). The groups were similar and mean age was 54.41 + 9.89 years in BC, 54.53 + 10.39 years in DCIS and 51.74 + 11.76 years in BE (p=0.1178) (data not shown).

BMI of the sample ranged from 18.4 to 52.1 Kg/m^2 (mean: 28.3 $\pm$ 5.6 Kg/m2). There was no difference in BMI between the groups studied (p=0.8492), however, about 70% of the cases were overweight/obese. As seen in Table 2, there was no significant difference among groups regarding clinical or histopathological characteristics. The groups were also similar in terms of menstrual status (p=0.1605) and hormone receptor status, in cases of BC and DCIS (Table 2).

There was FABP4 protein expression in epithelial tissue in 90% of BC, 40% of DCIS and 28% of benign lesions. All cases showed a moderate to strong FABP4 expression in the PF and DF. The mean positivity scores of FABP4 protein expression differed significantly in different diagnoses and in different tissues. In the epithelial

component, it was more highly expressed in BC and less in BE, while in the DF and PF, there was an inverse relationship (Table 3).

FABP4 immunohistochemical expression was shown to have a higher intensity and percentage of tumor cells in patients with BC and DCIS who were diagnosed until 50 years of age. No difference in FABP4 expression was observed in PF and DF, regarding age in the three groups studied (Table 4).

FABP4 was more positively expressed in the epithelial component of patients with BC and DCIS diagnosed in the premenopausal period. No difference in FABP4 expression was observed in PF and DF, in terms of menstrual status in the three groups studied (Table 5).

FABP4 expression demonstrated a positive correlation with BMI in BC group (Figure 2), although this association did not occur in DCIS and BE.

In BC and DCIS cases, no association was observed between FABP4 expression in tumor tissue and hormone receptor status in the tumor. In contrast, the expression of FABP4 in PF was associated with ER- and PR-negative tumors (Table 6).

In BC group, there was no association of FABP4 immunohistochemical expression with postoperative staging, histological and nuclear tumor grade. In DCIS, no association between marker expression and comedonecrosis, multifocality and histological type was observed. In benign breast lesions, there was no association between FABP4 expression and epithelial proliferation or cell atypia (data not shown).

DISCUSSION

The results of this study demonstrate that FABP4 protein expression is associated with breast cancer, and to a lesser degree, DCIS.

In our study, we chose to study FABP4 protein expression by immunohistochemistry, since data relative to the issue is lacking in the literature. To the best of our knowledge, this is actually the first study to analyze FABP4 protein expression in breast cancer tissue, DCIS and benign lesions, both in epithelial tissue and breast adipose tissue. It is difficult to make a comparison with the literature, due to this fact.

In our study, we observed that FABP protein expression in the epithelial component of breast cancer has a significantly higher level of expression than in DCIS and benign breast lesions. Regarding analysis of the fat tissue adjacent to the lesion and the fat tissue distant from the lesion, it was observed that FABP4 had a higher level of expression in the BE group and a lower level of expression in the BC group.

Several studies suggest that FABP4 expression influences urogenital tract cancer [33,34,35], although data concerning this marker and breast cancer are rare and controversial [17]. Hammamieh et al. [33] demonstrated that FABP4 gene expression is significantly reduced in women with breast cancer, when compared to those with normal breast tissue. Li et al. [36] failed to observe any difference between FABP4 gene expression in breast cancer patients, in comparison to patients with fibroadenoma.

A study by Hancke et al. (2010) showed that FABP4 serum levels are significantly higher in breast cancer patients than in controls, and appear to be increased in the postmenopause group. This finding should be viewed with caution since the case study was small. In BC and DCIS groups, we observed that FABP4 had a higher protein expression in the premenopausal period, and in patients DCIS up to 50 years of age [17].

Terra et al. (2011) analyzed plasma values and gene expression of FABP4 in subcutaneous and visceral fat in obese patients and healthy women [20]. This study demonstrated that plasma concentrations of FABP4 were significantly higher in morbidly

obese patients, in comparison to nonobese patients, decreasing up to 30% during weight loss in the postoperative period of 12 months. There was a positive correlation between FABP4 serum levels and metabolic syndrome. Visceral gene expression of FABP4 was correlated with circulating levels of FABP4 in morbidly obese women. FABP4 expression was higher in subcutaneous tissue than in visceral fat tissue in these morbidly obese women.

Corroborating the results of previous studies on gene expression and serum levels of FABP4, the present study shows a positive correlation between FABP4 protein expression in breast cancer tissue and BMI.

The higher positivity of FABP4 expression in fat tissue adjacent to benign lesions than in breast cancer tissue merits further investigation. Furthermore, a comparison with FABP4 serum levels should be made in the same patient, a study that has already been undertaken by our group.

Similarly, the association between a higher level of FABP4 expression and ER-/PR-negative tumors in DCIS requires attention to detail in these individual cases, particularly in relation to anthropometric data and other clinical parameters of obesity.

A comprehensive study of the correlation between breast tumor tissue, adipose tissue and FABP4 is required, in view of its link to diverse metabolic disorders and malignancies in various systems and organs. A greater knowledge of these relationships in the near future, may contribute to the development of drugs with actions that could improve the prognosis in women with breast cancer suffering from metabolic disorders linked to obesity.

CONCLUSION

In our study, we observed that FABP4 protein expression is related to breast cancer and obesity. However, further studies are necessary to confirm the widespread use of these data.

ETHICAL STANDARDS

The study was designed and conducted in accordance with the Declaration of Helsinki and with the recommendations of Resolution 196/96 of the Ministry of Health, Brazil. The study was approved by the Research Ethics Committee at UNICAMP School of Medicine (N^{o} 824/2011, CAAE: 0740.0.146.000-11).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Table 1 – Final scores obtained by analyzing parameters including intensity of marker expression and percentage of stained cells

	Intensity of reaction			
% stained cells	**0 (negative)**	**1 (mild)**	**2 (moderate)**	**3 (strong)**
0	0			
1 (< 30)		2	3	4
2 (30 to 60)		3	4	5
3 (> 60)		4	5	6

Table 2 – Clinical and histological characteristics, according to histopathological diagnosis

	IDC (N=69)	**DCIS (N=73)**	**BENIGN (N=81)**	
CHARACTERISTICS	**n (%)**	**n (%)**	**n (%)**	***p***
Age (years)				*0.3010*
≤ 50	27 (39.1)	28 (38.3)	40 (49.4)	
> 50	42 (60.9)	45 (61.7)	41 (50.6)	
BMI				*0.2936*
≤ 20 - 25	18 (26.5)	22 (30.1)	25 (31.3)	
> 25 - 30	30 (44.1)	31 (42.5)	24 (30.0)	
> 30	19 (29.4)	20 (27.4)	31 (38.8)	
Menstrual status				*0.1605*
Premenopause	25 (36.2)	25 (34.2)	39 (48.1)	
Postmenopause	44 (63.8)	48 (65.8)	42 (51.9)	
ER (N=142)				*0.1062*
Positive	56 (81.2)	32 (68.1)		
Negative	13 (18.8)	15 (31.9)		
PR (N=142)				*0.6989*
Positive	45 (65.2)	29 (61.7)		
Negative	24 (34.8)	18 (38.3)		

Chi-square; BMI=body mass index; BC=breast cancer; DCIS= ductal carcinoma *in situ*; ER=estrogen receptor; PR= progesterone receptor

Table 3 – Mean scores of FABP4 expression in the epithelial component (ET) of proximal fat tissue (PF) and distant fat tissue (DF), according to histopathological diagnosis (N=223)

	ET		PF		DF	
	n	Median (mean±SD)	n	Median (mean±SD)	n	Median (mean±SD)
BC	69	5 (4.5 ± 1.7)	68	5 (5.0 ± 0.7)	64	5 (5.0 ± 0.7)
DCIS	73	0 (1.9 ± 2.1)	73	5 (5.0 ± 0.3)	72	5 (5.0 ± 0.3)
BE	81	0 (1.4 ± 1.7)	81	6 (5.8 ± 0.4)	11	6 (5.8 ± 0.4)
p		*<0.0001*		*<0.0001*		*<0.0001*

Mann-Whitney; BC = breast cancer; DCIS = ductal carcinoma in situ; BE = benign breast alterations

Table 4 – FABP4 immunohistochemical expression in the epithelial component, PF and DF, according to age group and histopathological diagnosis (N=223)

	ET		PF		DF	
	n	Median (mean±SD)	n	Median (mean±SD)	n	Median (mean±SD)
BC						
Age (years)						
≤ 50	27	5 (5.2 ± 0.7)	26	5 (5.0 ± 0.7)	23	5 (4.7 ± 0.5)
> 50	42	5 (4.1 ± 1.9)	42	5 (5.0 ± 0.8)	41	5 (4.6 ± 0.5)
p		*0.0226*		*0.9723*		*0.5032*
DCIS						
Age (years)						
≤ 50	28	3.5 (2,7 ± 2.3)	28	5 (5.0 ± 0.3)	27	5 (5.1 ± 0.4)
> 50	45	0 (1.3 ± 1.9)	45	5 (5.1 ± 0.3)	45	5 (5.1 ± 0.3)
p		*NC*		*0.3033*		*0.5519*
BE						
Age (years)						
≤ 50	40	2 (1.6 ± 1.7)	40	6 (5.9 ± 0.4)	6	6 (6.0 ± 0.0)
> 50	41	0 (1.2 ± 1.7)	41	6 (5.8 ± 0.5)	5	6 (6.0 ± 0.0)
p		*0.3124*		*0.5709*		*1.0000*

Mann-Whitney; NC=not calculable; BC = breast cancer; DCIS = ductal carcinoma in situ; BE = benign breast alterations

Table 5 – Mean scores of FABP4 expression in the epithelial component (ET) in proximal fat tissue (PF) and distant fat tissue (DF), according to menopausal status and histopathological diagnosis (N=223)

	ET		PF		DF	
	n	Median (mean±SD)	n	Median (mean±SD)	n	Median (mean±SD)
BC						
Pre	25	5 (5.2 ± 0.8)	24	5 (5.2 ± 0.7)	22	5 (4.6 ± 0.5)
Post	44	5 (4.2 ± 1.9)	44	4.9 (4.2 ± 0.8)	42	5 (4.7 ± 0.5)
p		*0.0236*		*0.2516*		*0.5595*
DCIS						
Pre	25	3 (2.6 ± 2.4)	25	5 (5.0 ± 0.3)	24	5 (5.0 ± 0.4)
Post	48	0 (1.5 ± 1.9)	48	5 (5.1 ± 1,9)	48	5 (5.1 ± 0.3)
p		*0.0352*		*0.3489*		*0.4717*
BE						
Pre	25	3 (2.6 ± 2.4)	25	5 (5.0 ± 0.3)	24	5 (5.0 ± 0.4)
Post	48	0 (1.5 ± 1.9)	48	5 (5.1 ± 1,9)	48	5 (5.1 ± 0.3)
p		*0.0352*		*0.3489*		*0.4717*

Mann-Whitney; BC = breast cancer; DCIS = ductal carcinoma in situ; BE = benign breast alterations

Table 6 – Mean scores of FABP4 expression in the epithelial component (ET) in the proximal fat tissue (PF) and distant fat tissue (DF), according to hormone receptor expression (ER and PR) and histopathological diagnosis (N=142)

	ET		PF		DF	
	n	Median (mean±SD)	n	Median (mean±SD)	n	Median (mean±SD)
BC						
ER						
Negative	13	5 (3.9 ± 2.0)	13	5 (4.9 ± 0.9)	12	5 (4.6 ± 0.5)
Positive	56	5 (4.7 ± 1.5)	55	5 (5.1 ± 0.7)	52	5 (4.7 ± 0.5)
p		*0.1711*		*0.4610*		*0.6579*
PR						
Negative	24	5 (4.1 ± 1.8)	24	5 (5.0 + 0.8)	23	5 (4.6 ± 0.5)
Positive	45	5 (4.8 ± 1.5)	44	5 (5.1 + 0.7)	41	5 (4.7 ± 0.5)
p		*0.0841*		*0.6976*		*0.3580*
DCIS						
ER						
Negative	15	2 (2.1 ± 1.9)	15	5 (5.2 ± 0.4)	15	5 (5.3 ± 0.5)
Positive	32	2.5 (2.3 ± 2.4)	32	5 (5.0 ± 0.2)	31	5 (5.1 ± 0.3)
p		*0.6495*		*0.0151*		*0.0684*
PR						
Negative	18	3 (2.6 ± 1,9)	18	5 (5.2 ± 0.4)	18	5 (5.2 ± 0.4)
Positive	29	0 (2.1 ± 2.4)	29	5 (5.0 ± 0.2)	28	5 (5.1 ± 0.3)
p		*0.5138*		*0.0270*		*0.5787*

Mann-Whitney; BC = breast cancer; DCIS = ductal carcinoma in situ; BE = benign breast alterations

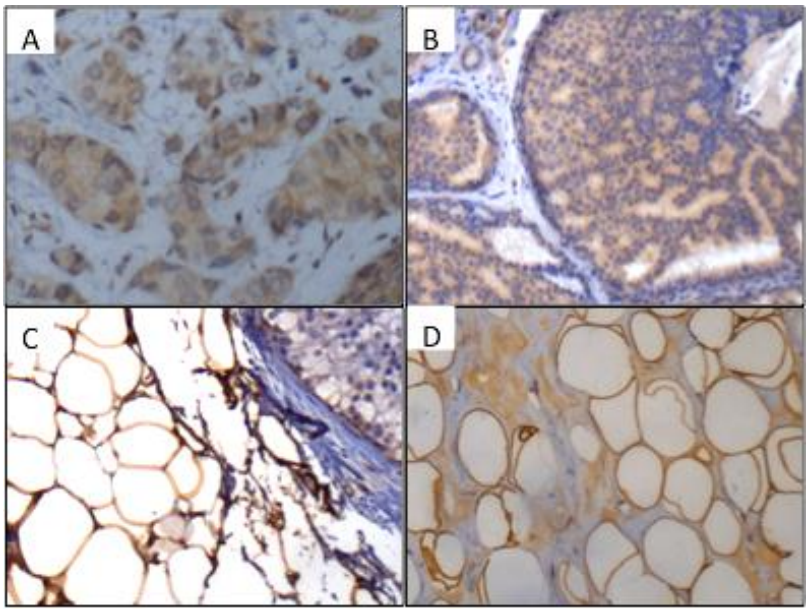

Figure 1. Intensity (I) and percentage (P) of the immunohistochemical reaction: (A) - I 2, P2 – FABP4 in BC (100x magnification); (B) - I 3, P3 – FABP4 in DCIS (100x); (C) - I 3, P 3 in the peritumoral fat tissue of DCIS (100x); (D) - I 3, P 3 in the distant fat issue of BC (100x).

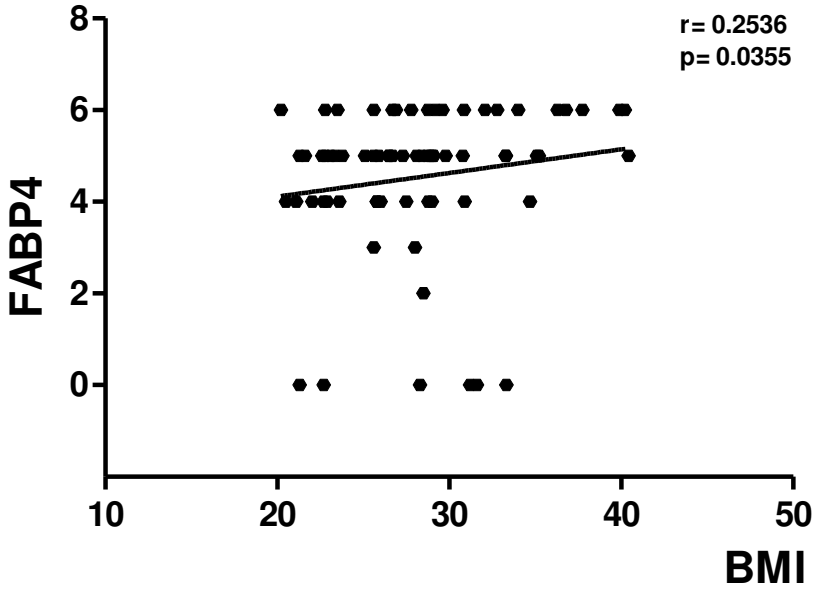

Figure 2. Correlation between FABP4 score expression and BMI in epithelial tissue from BC group.

4. Discussão

Os achados deste estudo demonstram que a adiponectina e AdipoR1 apresentam maior expressão proteica no epitélio tumoral que nos CDIS e nas lesões benignas, e a FABP4 está associada ao câncer de mama e ao CDIS em menor escala.

O câncer de mama é a neoplasia mais diagnosticada e a segunda causa de morte entre as mulheres, sendo que tal patologia parece ter forte relação com a obesidade (1). Verifica-se atualmente que a obesidade aumenta em proporções epidêmicas, principalmente no período pós-menopausa, associando-se a hábitos alimentares de elevada ingestão de carboidratos em pratos rápidos, realidade da falta de tempo do mundo moderno. Isto se agrava com a necessidade de a mulher ter gestações tardias ou não ter filhos devido à sua atuação no mercado de trabalho e às reposições hormonais no período pós-menopausa.

Na casuística deste estudo chama atenção o fato que o IMC médio foi de 28,3kg/m^2 e a maioria das pacientes realmente se situam próximas a esta faixa. Este índice de sobrepeso ainda parece piorar quando são analisadas somente

as mulheres na pós-menopausa. Fatores como estes, associados a alterações de hábitos e estilo de vida do mundo moderno, parecem fornecer um novo perfil de paciente que chega em número significativo para tratamento, que é a paciente jovem, com sobrepeso ou obesa e com câncer de mama. Analisando esta realidade é necessário direcionar mais esforços no sentido de realizar estudos relacionados não apenas à obesidade e ao câncer de mama, mas também para verificar se está sendo respeitada a fisiologia feminina com tais comportamentos e imposições da atualidade.

Neste novo contexto, o tecido gorduroso tem sido visto como um órgão endócrino produtor de substâncias cuja função é modular o metabolismo lipídico e se inter-relacionar com a etiologia de vários tipos de neoplasias. Estes fatores chamados adipocinas, polipeptídeos com importante função regulatória do metabolismo energético, incluem fatores angiogênicos, mitogênicos e anti-mitogênicos, fatores de crescimento e citocinas pró-inflamatórias e estão envolvidos na mediação de doenças inflamatórias e obesidade (14,15), Tais fatores funcionariam em equilíbrio e qualquer alteração pode levar ao desenvolvimento de doenças relacionadas ao metabolismo lipídico ou como consequência dele.

A maioria das pessoas obesas apresenta alterações no metabolismo de tecido adiposo causadas por interações genéticas e ambientais, que levam à hipertrofia adipocitária, hipóxia, alterações na homeostase e processos inflamatórios diversos com aumento da produção de leptina, fator de necrose tumoral-α, interleucina 6 e diminuição da produção de APN (17).

O tecido adiposo produz mais de 50 adipocinas já descritas e pouco conhecidas, produzidas por diferentes depósitos como gordura subcutânea, visceral e mamária. Acredita-se que as adipocinas agem no tecido mamário de forma endócrina, através de depósitos de gordura externos, por via parácrina e por uma ação autócrina (19,20,21,22). A estrutura anatômica da mama favorece a interação do tecido adiposo mamário com o tecido glandular, o que por si só justifica a necessidade de estudar-se a expressão das diferentes adipocinas no câncer de mama. A APN e seus receptores foram escolhidos para serem abordados neste estudo porque parecem estar diretamente relacionados à proteção da relação obesidade e câncer de mama. A FABP4 também foi incluída por ser uma proteína pouco conhecida e ter relação direta com o metabolismo lipídico.

Para que haja um maior entendimento da relação entre o metabolismo do tecido adiposo e câncer de mama é necessário que as informações se interliguem, de forma a montar um cenário mais claro. São realizados estudos em relação à concentração sérica, expressão de mRNA e expressão tecidual de algumas adipocinas, mas pouca correlação evidente tem sido obtida porque os resultados ainda são heterogêneos e pouco comparáveis.

Outra forma possível de utilização dos dados obtidos neste estudo seria a categorização da expressão dos marcadores em negativa e positiva objetivando sua utilização clínica. Assim, seria considerado negativo o exame com intensidade de coloração negativa ou leve e porcentagem de células coradas até 30% e positivo aquele com as demais combinações. Esta interpretação poderia ser uma sugestão de parâmetros para uso clínico ou em estudos futuros.

Nessa linha, no presente estudo foram avaliados 69 pacientes com câncer e 73 com CDIS, sendo que a expressão de APN seria positiva em 65,2% dos casos de câncer e 48% dos pacientes com CDIS. Em contrapartida, Jarde et al. (39), em estudo que avaliou 45 casos de carcinoma ductal invasivo, 14 casos de CDIS e 40 casos de tecido normal adjacente ao câncer, a expressão de APN em pacientes com carcinoma ductal invasor foi de 15%, nos casos de CDIS não se verificou expressão de APN e no tecido epitelial e mioepitelial normal peritumoral foi de 75%, sendo consideradas positivas quando havia coloração de mais de 5% das células no tecido estudado. Deve-se enfatizar que os estudos citados diferem quanto à metodologia empregada, sendo diferentes desde os parâmetros considerados de positivação da reação até o número de casos analisados. Portanto, mesmo mensurando a expressão proteica tecidual pode-se obter resultados diferentes. Quanto à expressão da adiponectina em pacientes com câncer observada neste estudo, uma vez que esta tem função protetora, esperava-se que esta expressão fosse menor, mas os dados de literatura a este respeito ainda são imprecisos.

Em estudo realizado por Korner et al. (38), não se verificou expressão significativa de APN em células de câncer de mama a não ser pelo mecanismo autócrino, localizado, mostrando expressão elevada em tecido adiposo peritumoral quando comparado com controles. A análise imunoistoquímica em 96 amostras de pacientes com câncer de mama e 25 de tecido mamário normal, revelou expressão fortemente positiva de receptores AdipoR1 em células epiteliais e ductais. A expressão de AdipoR1 foi marginal, mais pronunciada em células

malignas (34%) que em tecido mamário normal (13%) (p=0,09). O estudo da expressão do receptor AdipoR2 demonstrou que não houve diferença de expressão do mesmo em células malignas (26,3%) e tecido normal (29,2%).

Neste estudo verificou-se a expressão positiva do receptor AdipoR1 em 98,6% dos casos de câncer e 71,6% dos casos de lesões benignas (p<0,0001). O AdipoR2 revelou-se positivo em 100% dos casos de câncer e 81,5% dos casos de lesões benignas. Esta diferença importante dos achados nestes dois estudos está relacionada à diferença de metodologia, tanto na quantificação dos resultados como na realização da imunoistoquimica, pois no estudo anterior foi pesquisada a expressão gênica dos marcadores.

Quanto à expressão de FABP4, Hancke et al. (55), observaram que não houve diferença da expressão de FABP4 mRNA em pacientes com câncer de mama em relação a pacientes com fibroadenoma, mas como ambos os estudos pesquisaram a expressão do FABP4 mRNA dificilmente são comparáveis a outros. No presente estudo verificou-se que a expressão do FABP4 está aumentada nos casos de câncer, sendo positiva em cerca de 90% dos casos quando comparado com o grupo com CDIS (40%) e lesões benignas (28%) (p<0,0001).

As bases moleculares relacionadas a participação tanto da APN como da FABP4 na carcinogênese mamária ou até mesmo de outros órgãos, ainda são pouco conhecidas e têm sido alvo de inúmeros estudos recentes. São diversas as vias metabólicas que relacionam a APN à carcinogênese, e em todas estas vias estudos *in vitro* demostram resultados contraditórios (13,20,22,33,34,35,36,37).

Tal constatação leva-nos a crer que quaisquer que fossem os resultados deste estudo deveríamos relatá-los com cautela, pois quando as bases moleculares ainda não são descritas claramente, qualquer afirmação em relação a achados deve ser encarada como subsídio para estudos posteriores.

Este estudo demonstra a expressão proteica tecidual de diferentes marcadores relacionados à função adipocitária no câncer de mama, CDIS e em pacientes com lesões mamárias benignas. O melhor conhecimento da expressão da Adiponectina e seus receptores AdipoR1 e AdipoR2, assim como da FABP4 nestas diferentes condições mamárias pode colaborar para a descrição de mais um marcador tumoral do câncer de mama, ou até abrir espaço para o desenvolvimento de medicamentos agonistas da APN ou ativadores dos seus receptores de modo a promover possível proteção mamária. Estudos neste sentido já vêm sendo realizados em várias partes do mundo com resultados ainda muito contraditórios (22,34,35,36,37).

As dificuldades encontradas foram a pouca literatura sobre o tema, o que limita a comparação de resultados, e também o fato de ser um objeto de estudo muito recente. Apesar disso, acredito que este estudo contribuiu de forma significativa ao demonstrar a expressão destes marcadores no tecido dos diferentes tipos de diagnósticos histopatológicos, e abre caminho a novas pesquisas neste vasto e ainda pouco conhecido campo do conhecimento médico que é o câncer de mama.

5. Conclusões

Artigo 1

- A positividade da expressão imunoistoquímica da APN foi maior no CDI que no CDIS e nas BE. Quanto aos AdipoR1/R2, apesar de mais expressos no CDI que no CDIS e BE, essa diferença não foi tão acentuada.
- Os adipoR2 tiveram expressão positiva em todos os casos de CDI e CDIS e em 3/4 dos BE.
- Verificou-se tendência da APN expressar-se mais em pacientes abaixo de 50 anos nos CDI e CDIS.
- Nos CDI e CDIS observou-se associação entre a expressão de APN e tumores RE negativos. No CDIS esta associação foi observada com RE e RP.

Artigo 2

- Neste estudo verificou-se que a expressão proteica da FABP4 está relacionada ao câncer de mama e à obesidade. Entretanto, são necessários estudos adicionais para confirmação e ampla utilização destes dados.

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7. Anexos

7.1. Anexo 1 – Ficha de Coleta de Dados

Ficha I__I__I__I

Iniciais I__I__I__I **HC** I__I__I__I__I__I__I__I__I

- -

Ficha I__I__I__I

1. Idade: I__I__I (do diagnóstico de câncer ou da patologia)

2. Etnia: caucasiana I__I negra I__I asiática I__I mulata I__I

3. Estado menstrual: 1) Menopausa I__I 2) Menacme I__I 3) Ignorado I__I

4. Idade à menarca: ________

5. Idade à menopausa: ________

6. HAS: não I__I sim I__I

7. Cardiopatia: não I__I sim I__I

8. Tabagismo: nãoI__I sim I__I

9. DM: não I__I sim I__I

10. IMC: valor: ________
I__I abaixo do peso I__I peso ideal I__I sobrepeso
I__I obesidade moderada I__I obesidade severa I__I mórbida

11. Tratamento cirúrgico:
a) Mastectomia I__I b) Quadrantectomia I__I c) Outro I__I______________

12. Quimioterapia primária: 1) Não I__I 2) Sim I__I 3) ignorado

13. Radioterapia primária: 1) Não I__I 2) Sim I__I 3) Ignorado I__I

14. Hormonioterapia primária: 1) Não I__I 2) Sim I__I 3) Ignorado I__I

15. Diagnóstico Histopatológico:

Número da biópsia: _____ Bloco selecionado:_____

I__I Mama normal
I__I Hiperplasia ductal florida
I__I Papiloma ou papilomatose
I__I Adenose esclerosante
I__I Cicatriz radiada
I__I Hiperplasia ductal atípica
I__I Hiperplasia lobular atípica
I__I Alteração ou hiperplasia de células colunares com ou sem atipias – CAPSS
I__I Outros _____

I__I Carcinoma ductal *in situ*:

Carcinoma invasivo: a) Ductal I__I b) Lobular I__I c) outros I__I

Grau Histológico: I)I__I II) I__I III) Ignorado I__I

Grau nuclear: 1) I__I 2 I__I 3 I__I

Estadiamento patológico (TNM): _____

16. Imunoistoquímica

Marcadores

Receptor de estrógeno: I__I__I% de células coradas inconclusivo I__I

Receptor de progesterona: I__I__I% de células coradas inconclusivo I__I

HER2: I__I__I cruzes inconclusivo I__I

Intensidade (0= negativa; 1= leve; 2= moderada; 3= forte)

Porcentagem células coradas (0= 0%; 1= até 30%; 2= 30 a 60%; 3= >60%)

TMA 1
Lesão:

	Intensidade	**% células coradas**	**Soma 1**
Adiponectina			
AdipoR1			
AdipoR2			
FABP4			

Gordura Próxima:

	Intensidade	**% células coradas**	**Soma 1**
Adiponectina			
AdipoR1			
AdipoR2			
FABP4			

Gordura distante:

	Intensidade	**% células coradas**	**Soma 1**
Adiponectina			
AdipoR1			
AdipoR2			
FABP4			

TMA 2
Lesão:

	Intensidade	**% células coradas**	**Soma 2**
Adiponectina			
AdipoR1			
AdipoR2			
FABP4			

Gordura Próxima:

	Intensidade	% células coradas	Soma 2
Adiponectina			
AdipoR1			
AdipoR2			
FABP4			

Gordura distante:

	Intensidade	% células coradas	Soma 2
Adiponectina			
AdipoR1			
AdipoR2			
FABP4			

TMA 3

Lesão:

	Intensidade	% células coradas	Soma 3
Adiponectina			
AdipoR1			
AdipoR2			
FABP4			

Gordura Próxima:

	Intensidade	% células coradas	Soma 3
Adiponectina			
AdipoR1			
AdipoR2			
FABP4			

Gordura distante:

	Intensidade	% células coradas	Soma 3
Adiponectina			
AdipoR1			
AdipoR2			
FABP4			

TMA – MÉDIAS

Lesão:

	Soma 1	Soma 2	Soma 3	Média
Adiponectina				
AdipoR1				
AdipoR2				
FABP4				

Gordura Próxima:

	Soma 1	Soma 2	Soma 3	Média
Adiponectina				
AdipoR1				
AdipoR2				
FABP4				

Gordura distante:

	Soma 1	Soma 2	Soma 3	Média
Adiponectina				
AdipoR1				
AdipoR2				
FABP4				

7.2. Anexo 2 – Tabela 1

Distribuição dos escores de expressão dos marcadores de adipocinas no componente epitelial segundo o diagnóstico histopatológico

	Adiponectina n (%)	AdipoR1 n (%)	AdipoR2 n (%)	FABP4 n (%)
Câncer (N=69)				
0	20 (29,0)	1 (1,4)	0	6 (8,7)
2	4 (5,8)	0	0	1 (1,4)
3	9 (13,0)	2 (2,9)	2 (2,9)	2 (2,9)
4	25 (36,2)	6 (8,7)	4 (5,8)	14 (20,3)
5	10 (14,5)	24 (34,8)	18 (26,1)	27 (39,1)
6	1 (1,4)	36 (52,2)	45 (65,2)	19 (27,5)
CDIS (N=73)				
0	30 (41,1)	3 (4,1)	0	39 (53,4)
2	8 (11,0)	1 (1,4)	0	5 (6,8)
3	5 (6,8)	1 (1,4)	5 (6,8)	4 (5,5)
4	24 (32,9)	29 (39,7)	17 (23,3)	15 (20,5)
5	5 (6,8)	35 (47,9)	33 (45,2)	7 (9,6)
6	1 (1,4)	4 (5,5)	18 (24,7)	3 (4,1)
Benigno (N=81)				
0	61 (75,3)	12 (14,8)	7 (8,6)	45 (55,6)
2	10 (12,3)	11 (13,6)	8 (9,9)	13 (16,0)
3	6 (7,4)	13 (16,0)	8 (9,9)	9 (11,1)
4	2 (3,7)	27 (33,3)	22 (27,2)	11 (13,6)
5	1 (1,2)	18 (22,2)	36 (44,4)	3 (3,7)
6	0	0	0	0

7.3. Anexo 3 – Tabela 2

Expressão dos marcadores no componente epitelial segundo o diagnóstico histopatológico

	Adiponectina		***AdipoR1***		***AdipoR2***		***FABP4***		***Total***
	Negativo	***Positivo***	***Negativo***	***Positivo***	***Negativo***	***Positivo***	***Negativo***	***Positivo***	
	n (%)	***n (%)***	***n (%)***	***n (%)***	***n (%)***	***n (%)***	***n (%)***	***n (%)***	
Câncer	*24 (34,8)*	*45 (65,2)*	*1 (1,5)*	*68 (98,6)*	*0*	*69 (100)*	*7 (10,1)*	*62 (89,9)*	*69*
CDIS	*38 (52,1)*	*35 (48,0)*	*4 (5,5)*	*69 (94,5)*	*0*	*73 (100)*	*44 (60,3)*	*29 (39,7)*	*73*
Benigno	*71 (87,7)*	*10 (12,4)*	*23 (28,4)*	*58 (71,6)*	*15 (18,5)*	*66 (81,5)*	*58 (71,6)*	*23 (28,4)*	*81*
Total	*133*	*90*	*28*	*195*	*15*	*208*	*109*	*114*	*223*
p	*<0,0001*		*<0,0001*		*<0,0001*		*<0,0001*		

Qui-quadrado

7.4. Anexo 4 – Mecanismo sugerido da adiponectina na carcinogênese

(Fabian CJ. Adiponectin: a risk biomarker and attractive target for chemoprevention. J Clin Oncol. 2012 Jan 10;30(2):124-6).

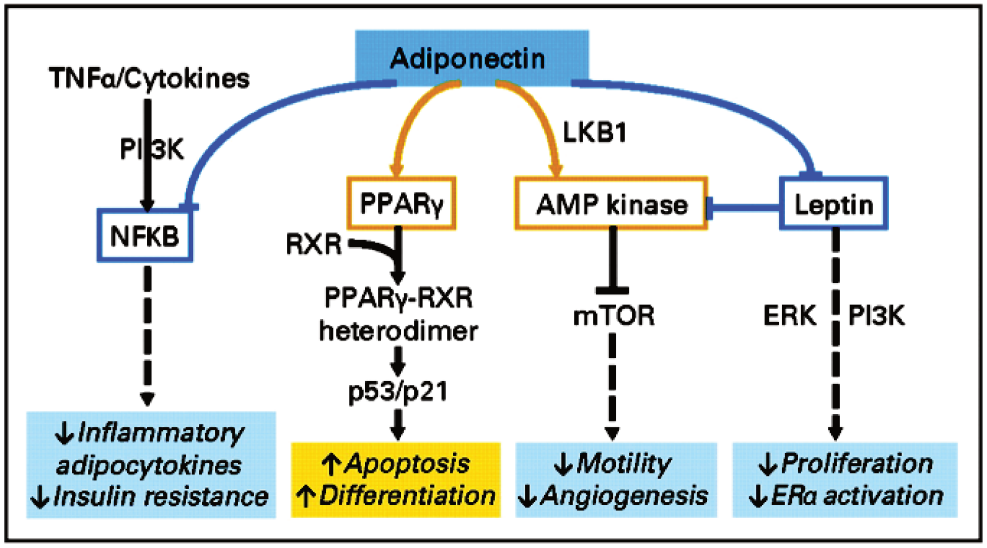

Figura 1

Ativação: azul; Bloqueio: amarelo
ERK: *extracellular signal-related kinase*
mTOR: *mammalian target of rapamycin*
NFKB: *nuclear factor-kB*
PI3K: *phosphatidylinositol-3-kinase*
PPARγ: *peroxisome-proliferator-activated receptor γ*
RXR: *retinoid X receptor*
REα: *receptor de estrógeno α*
TNFα: *tumor necrosis factor α*

7.5. Anexo 5 – Carta da Comissão de Pesquisa – DTG/CAISM

UNICAMP

Comissão de Pesquisa do DTG / CAISM

Campinas, 7 de junho de 2010.

Protocolo nº: 024/2011

O protocolo de pesquisa *"O papel da Soroamilóide A (SAA), da Adiponectina, dos receptores de Adiponectina tipos 1 e 2 (AdipoR1/R2) e da Fatty Acid Binding Protein (FABP) no tecido mamário normal, nas lesões precursoras e no carcinoma invasor da mama"*, do pesquisador Rodrigo Pinto Guimenez, orientado pela Profa. Dra. Maria Salete Costa Gurgel, foi aprovado pela Comissão de Pesquisa do DTG/CAISM em 7/06/2011.

Atenciosamente,

PROF. DR. JOSÉ GUILHERME CECATTI
Presidente da Comissão de Pesquisa do DTG/CAISM

Rua Alexander Flemming, n.º101 – Cidade Universitária Zeferino Vaz – Campinas-SP
Fone: (19) 3521-9400
comissaopesquisa@caism.unicamp.br

7.6. Anexo 6 – Parecer doCEP – FCM/UNICAMP

UNICAMP

COMITÊ DE ÉTICA EM PESQUISA

www.fcm.unicamp.br/fcm/pesquisa

CEP, 23/08/11
(Grupo III)

PARECER CEP: N° 824/2011 (Este n° deve ser citado nas correspondências referente a este projeto).
CAAE: 0740.0.146.000-11

I - IDENTIFICAÇÃO:

PROJETO: **"O PAPEL DA SOROAMILÓIDE A (SAA), DA ADIPONECTINA, DOS RECEPTORES DE ADIPONECTINA TIPOS 1 E 2 (ADIPOR1/R2) E DA FATTY ACID BINDING PROTEIN (FABP) NO TECIDO MAMÁRIO NORMAL, NAS LESÕES PRECURSORAS E NO CARCINOMA INVASOR DA MAMA".**
PESQUISADOR RESPONSÁVEL: Rodrigo Pinto Gimenez
INSTITUIÇÃO: CAISM/UNICAMP
APRESENTAÇÃO AO CEP: 11/08/2011
APRESENTAR RELATÓRIO EM: 23/08/12 (O formulário encontra-se no *site* acima).

II – OBJETIVOS.

Comparar o padrão de expressão da Soroamilóide A (SAA), da Adiponectina, dos receptores de Adiponectina tipos 1 e 2 (Adipo R1/R2) e daFatty Acid Binding Protein (FABP) no tecido mamário normal, nas lesões precursoras e no carcinoma invasor da mama e correlaciona-lo com parâmetros antropométricos, reprodutivos, clínicos e histológicos.

III – SUMÁRIO.

Serão incluídos os blocos de parafina de 450 mulheres com carcinoma invasor da mama e de 450 mulheres com biópsias negativas para câncer de mama, tratadas na Divisão de Oncologia Ginecológica e Patologia Mamária do CAISM/UNICAMP de janeiro de 2004 a dezembro de 2007 e preparadas lâminas de Tissue Microarray (TMA). A expressão de SAA, Adiponectina, AdipoR1/R2 e FABP será realizada por imunohistoquímica e avaliada no tecido tumoral, na gordura peritumoral e na gordura mamária normal nos casos de câncer e no tecido epitelial e gordura mamários nos casos benignos. Os dados serão avaliados descritivamente através do cálculo de frequências absolutas (n) e relativas (%) para variáveis categóricas e através de média, mediana e desvio-padrão para as variáveis contínuas.

IV - COMENTÁRIOS DOS RELATORES.

O projeto apresenta-se bem redigido, com metodologia adequada. Os critérios de inclusão, exclusão e descontinuação dos sujeitos estão bem definidos; cálculo do tamanho amostral e planejamento de análise estatística adequados. Os aspectos éticos estão bem discutidos no projeto e solicita-se dispensa do Termo de Consentimento Livre e Esclarecixo por se tratar de estudo retrospectivo que avalia material de biópsia conservado em blocos de parafina. O orçamento é detalhado e o estudo já tem financiamento aprovado pela Fapesp.

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 VIII Reunião Ordinária do CEP/FCM, em 23 de agosto de 2011.

Prof. Dr. Carlos Eduardo Steiner
PRESIDENTE do COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP