UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
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EFEITOS DAS DIETAS RICAS EM ÔMEGA-3 OU ÔMEGA-6 NA EXPRESSÃO GÊNICA DE NEOPLASIAS MAMÁRIAS DE RATAS SPRAGUE-DAWLEY SOB O TRATAMENTO COM TAMOXIFENO

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Tese apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista – UNESP para obtenção do título de Doutor em Patologia.

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"Ás vezes vejo o homem velho | O rosto enrugado é o seu troféu
Tem na lembrança tanto amor, que é o seu andor
Ás vezes vejo ele levantar, andar tão solto só pra ensinar seu passo.
Eu passo sempre quando ele sorri"
Cascadura

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"I get by with a little help from my friends"
The Beatles

"Quando eu disse ao caroço de laranja que dentro dele dormia um laranjal inteirinho, ele me olhou estupidamente incrédulo."

Hermógenes

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Resumo

Estudos prévios mostraram que uma dieta rica em óleo de peixe aumentou a eficácia quimioprotetora do tamoxifeno contra a carcinogênese mamária induzida pela N-metil-N-nitrosuréia (MNU) em ratas Sprague-Dawley. O presente trabalho evidenciou que o tratamento com tamoxifeno modifica a expressão gênica de tumores mamários dependendo to tipo de gordura administrado na dieta dos animais. Ratas Sprague-Dawley iniciadas com MNU e tratadas com tamoxifeno receberam uma dieta rica em óleo de milho ou óleo de peixe. Após oito semanas, tumores do mesmo tipo histológico (cribriformes) foram coletados, e análise do RNA mensageiro (mRNA) foi executada através de microarray de expressão gênica e seguinte validação dos resultados por PCR em tempo real. A maior expressão do mRNA dos genes SerpinB10, Wisp2 e Apod em tumores de animais tratados com óleo de peixe é indicativo de que esses tumores eram altamente diferenciados. A redução da expressão de mRNA dos genes H19 e Igf2 nos grupos tratados com tamoxifeno, e de Thrsp e Wnt5b no grupo tratado com óleo de peixe e tamoxifeno pode estar relacionado à redução do crescimento tumoral e baixa capacidade metastática. A expressão aumentada do nível de transcrito Irf7 nos animais tratados com óleo de peixe sugere melhora na resposta imune anti-tumoral (padrão Th1), enquanto o aumento nos transcritos dos genes Fcer1a, Hdc, Ms4a2, Slp1, Mcpt1 and Mcpt2 nos animais tratados com óleo de peixe e tamoxifeno podem indicar uma mudança no padrão de resposta imune para Th2. O padrão Th2 representa um potencial mecanismo de escape da resposta imune adquirido pelas células tumorais dos animais tratados com óleo de peixe e tamoxifeno. Esses resultados sugerem que, apesar do tamoxifeno modular a expressão gênica levando à redução do crescimento tumoral, modulações de genes adicionais são influenciadas pela dieta rica em óleo de peixe, em parte, aumentando o efeito quimiopreventivo do tamoxifeno contra carcinogênese mamária induzida por MNU.

Abstract

Studies have shown that a fish oil-rich diet increased the chemopreventive efficacy of tamoxifen (Tam) against N-methyl-N-nitrosourea (MNU)-induced rat mammary carcinogenesis. Herein, we evidence that tamoxifen treatment modifies gene expression of mammary tumors depending upon the type of dietary fat fed to the animals. Rats initiated with MNU and treated with Tam were fed a diet rich in corn oil (CO) or fish oil (FO). After 8 weeks, tumors of the same histological type (cribriform) were collected and comprehensive analysis of messenger RNA expression was performed. The mRNA expression of genes such as SerpinB10, Wisp2 and Apod in tumors from FO-treated rats is indicative of highly differentiated tumors. Decreased expression of H19 and Igf2 mRNA in Tam-treated groups, and Thrsp and Wnt5b mRNA in FO+Tam group may be related to tumor growth impairment and lower metastatic capacity. Increased Irf7 transcript levels in FO-treated animals suggests an improved immune response against tumors (Th1 pattern) whereas decreased mRNA of Fcer1a, Hdc, Ms4a2, Slp1, Mcpt1 and Mcpt2 may indicate a shift of the immune response towards Th2 pattern. The Th2 pattern of gene expression represents a potential mechanism of escape from the immune response caused by FO+Tam treatment. These data show that, although tamoxifen modulates the expression of genes leading to tumor growth impairment, further modulations of genes are influenced by FO altering Tam-modulated expression of genes in a manner that may, in part, account for its enhancing chemopreventive effect against MNU-induced mammary carcinogenesis.

Capítulo 1

Revisão da Literatura

1. Câncer: evidências epidemiológicas

O câncer é uma doença crônico-degenerativa responsável por aproximadamente 12% das causas de morte no mundo. Atualmente, o câncer é a segunda doença de maior mortalidade no mundo, perdendo apenas para doenças coronarianas [1]. Porém a Organização Mundial da Saúde (*World Health Organization,* WHO) estima que em poucos anos o número de mortes por câncer será mais elevado do que em doença coronariana isquêmica [2] (Figura 1).

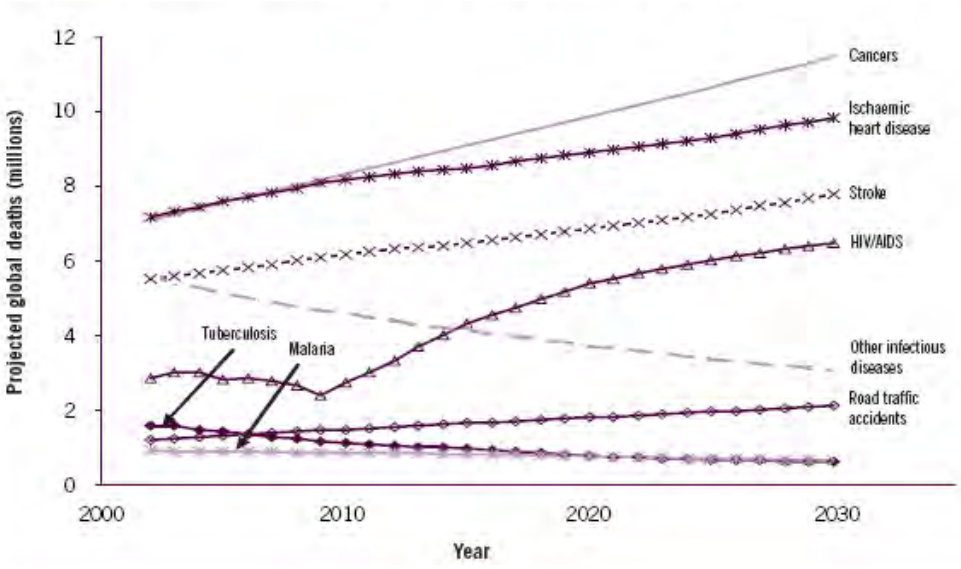

Figura 1 – Estimativa de taxas de mortalidade entre 2002 e 2030. Fonte: Mathers et al., 2006 [2].

A alta taxa de morbidade/mortalidade do câncer se deve a inúmeros fatores. Dentre eles, incluem-se a mudança de estilo de vida decorrente do processo crescente na industrialização e a redução nas taxas de mortalidade, em geral, com aumento da expectativa de vida e da prevalência de doenças crônico-degenerativas, como o câncer, doenças cardiovasculares, diabetes tipo 2, doença de Alzheimer e outros agravos relacionados ao envelhecimento e à obesidade [1, 3, 4]. Além disso, o diagnóstico tardio e as dificuldades no tratamento do câncer auxiliam no aumento das taxas de mortalidade relacionadas a essa doença [5, 6]. Em geral, em estágios avançados de desenvolvimento tumoral, as células transformadas possuem uma série de alterações gênicas que as tornam altamente invasivas e resistentes a medicamentos antitumorais. Essas características associadas aos mecanismos intrínsecos das células neoplásicas, como mecanismos de escape da vigilância imunológica -

perda da expressão de moléculas de complexo principal de histocompatibilidade, baixa expressão de antígenos tumorais, produção de citocinas inibitórias, dentre outros - impossibilitam o sucesso do tratamento em alguns casos [7].

Mundialmente, foram estimadas para 2010 uma incidência maior de novos casos de câncer de próstata, pulmão, cólon/reto e bexiga urinária em homens e câncer de mama, pulmão, cólon/reto e colo do útero, em mulheres [8] (Figura 2).

Males			Females		
Prostate	217,730	28%	Breast	207,090	28%
Lung & bronchus	116,750	15%	Lung & bronchus	105,770	14%
Colon & rectum	72,090	9%	Colon & rectum	70,480	10%
Urinary bladder	52,760	7%	Uterine corpus	43,470	6%
Melanoma of the skin	38,870	5%	Thyroid	33,930	5%
Non-Hodgkin lymphoma	35,380	4%	Non-Hodgkin lymphoma	30,160	4%
Kidney & renal pelvis	35,370	4%	Melanoma of the skin	29,260	4%
Oral cavity & pharynx	25,420	3%	Kidney & renal pelvis	22,870	3%
Leukemia	24,690	3%	Ovary	21,880	3%
Pancreas	21,370	3%	Pancreas	21,770	3%
All Sites	**789,620**	**100%**	**All Sites**	**739,940**	**100%**

Figura 2 – Estimativa de novos casos de câncer em homens e mulheres em 2010. Fonte: Jemal et al., 2010 [8].

Similarmente, estudos epidemiológicos e clínicos na população brasileira indicam as neoplasias de próstata, pulmão, cólon/reto, além de estômago, esôfago, como as mais prevalentes nos homens brasileiros, enquanto nas mulheres as neoplasias de mama, colo uterino, pulmão e cólon/reto [1]. Quanto à mortalidade, neoplasias de pulmão, próstata, estômago, esôfago, boca e faringe são responsáveis pelo maior índice em homens, e neoplasias de mama, pulmão, colo de útero, estômago, cólon e reto são responsáveis pelo maior índice em mulheres [9]. Estima-se que em 2010 foram registrados mais de 480 mil novos casos de câncer no Brasil, sendo que os principais órgãos afetados foram mama em mulheres e próstata em homens [10] (Figura 3).

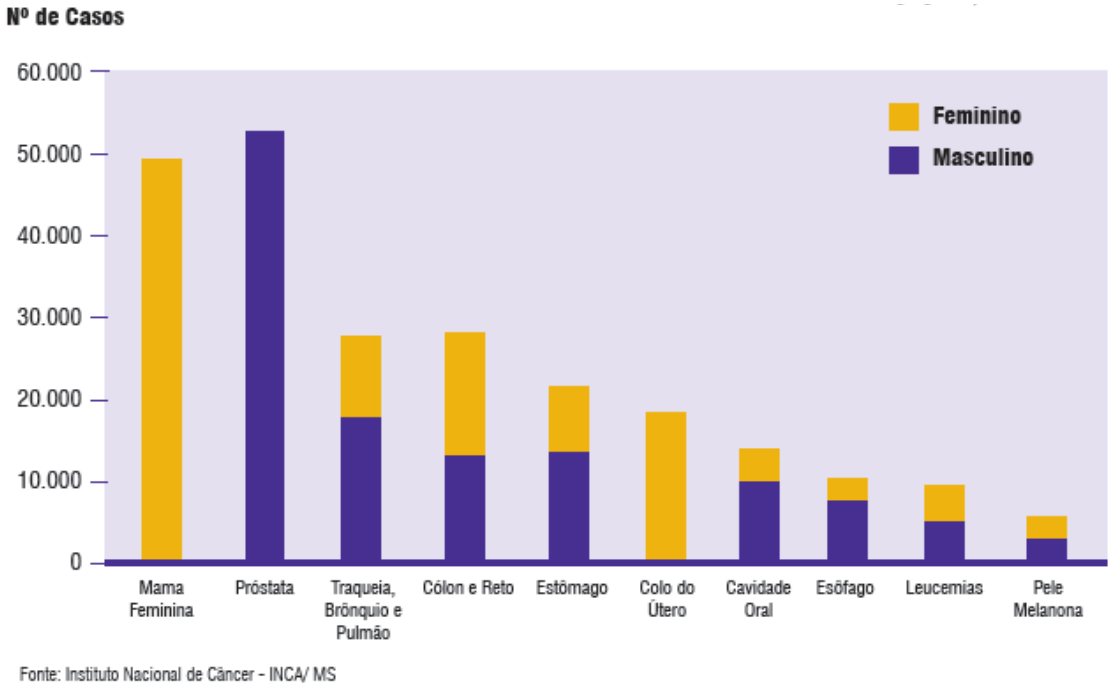

Figura 3 – Tipos de câncer mais incidentes estimados para 2010 na população brasileira. Fonte: INCA, 2009 [(10)].

Esses dados populacionais impulsionam os pesquisadores a tentarem entender melhor a evolução dessa doença para descobrir suas causas e/ou relacioná-la com fatores de risco e a procurarem novos métodos de prevenção e tratamento. Para estudos de métodos de prevenção e tratamento do câncer são utilizados modelos experimentais *in vitro*, utilizando-se de linhagens tumorais humanas, ou modelos experimentais *in vivo* em roedores através da inoculação de células tumorais ou da administração de agentes químicos carcinogênicos para indução de tumores (carcinogênese química).

2. Carcinogênese química: aspectos gerais

Os agentes químicos começaram a ser reconhecidos como cancerígenos para o homem no século XVIII, observando-se os altos índices de câncer de pele de escroto em limpadores de chaminé e de carcinogênese nasal pelo uso de rapé. Com isso, no século XX, desenvolveram-se modelos animais de carcinogênese química para o estudo da biologia tumoral e detecção de substâncias com potencial cancerígeno [11, 12].

Os cancerígenos químicos podem atuar nas diferentes etapas da carcinogênese. Os agentes iniciadores podem ser enquadrados em duas grandes categorias: 1) compostos de ação direta, que não exigem biotransformação para exercer sua carcinogenicidade e 2) compostos de ação indireta, ou pré-cancerígenos, que exigem conversão metabólica para produzir cancerígenos finais com caráter nucleofílico, como por exemplo, agentes alquilantes que induzem mutações no DNA ou a produção de radicais de oxigênio e nitrogênio, que são capazes de levar a lesões oxidativas e transformar as células-alvo [13]. As células iniciadas, com alterações permanentes em seu DNA, passam a se proliferar em ritmo diferente das outras células normais, adquirem novas mutações e respondem de maneira mais efetiva aos compostos promotores, ou não genotóxicos [13].

De forma geral, os modelos *in vivo* de carcinogênese química experimental requerem longo tempo de acompanhamento dos animais para observação de neoplasias, tornando-os dispendiosos e de execução laboriosa. Por esta razão, existe grande interesse na padronização de ensaios mais rápidos, que permitam detectar alterações que precedam o aparecimento das neoplasias [14]. Essas alterações, designadas biomarcadores, estão associadas a estágios iniciais do processo de carcinogênese e podem ser identificadas em nível molecular, celular ou morfológico [15-17]. Como exemplo, podem ser citadas as alterações estruturais precoces no cólon, mama e bexiga de roedores. Na mama podem ser encontradas as displasias e hiperplasias epiteliais [18]; na bexiga, as hiperplasias simples e as hiperplasias papiliferas/nodulares [19, 20] e na mucosa do cólon, as criptas aberrantes, que podem ser identificadas como únicas ou na forma de focos de criptas aberrantes [16].

O processo de carcinogênese química ocorre através de múltiplas etapas denominadas de iniciação, promoção e progressão [21-23]. A iniciação e promoção estão bem estabelecidas experimentalmente. Entende-se por iniciação a interação de um agente químico ou físico com o DNA da célula-alvo. É um processo irreversível, porque altera permanentemente a estrutura do DNA, através de ligações covalentes, distorção ou quebra da molécula em fita simples ou dupla [21-23]. A promoção acontece quando tal alteração é expressa, ocorrendo o aparecimento

das lesões pré-neoplásicas [21-23], que apresentam importantes modificações em níveis moleculares e morfológicos, incluindo genes que controlam a proliferação celular, diferenciação e apoptose [24, 25]. Quando as lesões adquirem características de malignidade tem-se a fase de progressão [21, 22], na qual são observadas alterações no genoma, relacionadas à proliferação celular/apoptose, invasividade, capacidade de produzir metástases e determinadas alterações bioquímicas, fornecendo substrato biológico para a manifestação clínica do câncer [11, 12]. O processo de metástase em um tecido tumoral depende de uma série de características adquiridas pelas células malignas no decorrer do processo de transformação celular. Essas alterações caracterizam tumores malignos capazes de invadir e se disseminar para tecidos distantes do próprio hospedeiro. Algumas alterações bem descritas em tumores malignos são: (a) afrouxamento das junções intercelulares das células tumorais umas das outras e do tecido adjacente, (b) degradação da matriz extracelular e da membrana basal, (c) fixação a componentes ancoradouros de locomoção da matriz (fibronectina, laminina e colágeno) e (d) migração e colonização de tecidos distantes [12]. Assim, na metástase, as células malignas se espalham por diversos tecidos e órgãos do paciente ou animal. Nesses novos locais, essas células utilizam nutrientes, espaço e desequilibram o microambiente quimio-eletrostático das células normais, podendo resultar em caquexia, falência múltipla de órgãos e morte do paciente ou animal.

A etapa de metástase é dificultada em indivíduos imunocompetentes. Os leucócitos são capazes de eliminar a maior parte das células malignas presentes tanto no interior do sistema vascular como nos tecidos primários e secundários. Apenas uma em cada 10.000 células que atravessaram o endotélio é capaz de sobreviver aos múltiplos ataques do sistema imunológico e colonizar tecidos distantes [26]. As células malignas que sobreviveram aos sucessivos ataques dos leucócitos no tecido primário e no interior do sistema vascular interagem com células endoteliais de tecidos distantes, extravasam (diapedese), migram entre as fibras do estroma e se fixam na nova localidade. No tecido receptor (secundário), as células transformadas que resistirem aos novos ataques do sistema imunológico se estabelecem e iniciam o desenvolvimento de um clone tumoral [26].

Além disso, as células neoplásicas metastáticas formam blocos trombóticos no sistema vascular e implantes monoclonais em múltiplos tecidos do hospedeiro. Em ambos os casos o tecido de origem pode ser reconhecido de acordo com as características morfológicas das células, dependendo do grau de diferenciação em que estão. A diferenciação celular é um processo complexo e de múltiplas etapas no qual a célula adquire funcionalidades e especializações para a formação de um determinado tecido do organismo. O passo inicial da

diferenciação celular é a instalação de um programa genético específico ao tecido em questão, que inclui o silenciamento de alguns genes e ativação de outros de acordo com a futura função da célula no tecido [27]. No decorrer das etapas de diferenciação, as funções biológicas das células são reguladas de acordo com o tipo celular, resultando em células que podem ser distinguidas das demais células de funções diferentes no mesmo tecido. A diferenciação terminal é a última etapa, na qual as células assumem uma função definida no tecido. Assim, nas células malignas, os controles do ciclo celular e diferenciação estão em desequilíbrio, resultando em células com elevada capacidade de multiplicação e baixa diferenciação [12]. Apesar do alto grau de anaplasia (indiferenciação celular) dos enxertos metastáticos, muitas vezes algumas características herdadas do tecido de origem permanecem nas células malignas enxertadas.

Por razões práticas, a maioria dos estudos experimentais de carcinogênese mamária é conduzida em roedores devido à baixa frequência de tumores espontâneos observados nesses animais em estudos de longa duração [28,29]. Tumores experimentais, induzidos pela administração de cancerígenos químicos, constituem ferramentas úteis para o entendimento das múltiplas etapas da carcinogênese mamária [29,30], bem como testes de potencial cancerígeno de agentes químicos ambientais [31-33] e estudos preventivos e terapêuticos da carcinogênese mamária [34, 35]. As neoplasias mamárias quimicamente induzidas são, em geral, carcinomas hormônio-dependentes. A incidência, multiplicidade e tipos de tumores mamários são influenciados pela idade, tempo de exposição ao cancerígeno, história reprodutiva, desregulação endócrina, dieta e outros fatores que alteram o desenvolvimento e o grau de diferenciação da glândula mamária [35]. As substâncias químicas mais utilizadas nos modelos experimentais de indução da carcinogênese mamária em fêmeas de ratos e camundongos são a 7,12-dimetilbenz(a)antraceno (DMBA) e a N-metil-N-nitrosoureia (MNU). A exposição de ratas jovens a esses cancerígenos antes da primeira prenhez aumenta a suscetibilidade das glândulas mamárias à iniciação do câncer [36].

3. Resposta imunológica anti-tumoral

Na tentativa de evitar multiplicação de células iniciadas - o que proporcionaria o subsequente crescimento tumoral - e de evitar a formação de metástases - o que levaria à subsequente instalação de células tumorais em sítios secundários - o sistema imunológico adota mecanismos de resposta anti-tumorais [26]. Basicamente, uma resposta anti-tumoral efetiva é baseada na resposta imediata de células Natural Killer (NK) e na resposta de células T auxiliares (T helper - Th) perfil Th1. Dependendo do estímulo dado a elas, através de células apresentadoras de antígeno (antigen presenting cells - APC, como macrófagos e celulas dendríticas) [37], as células Th indiferenciadas podem se diferenciar em Th1 ou Th2 [38]. As APCs apresentam epítopos específicos da célula tumoral através do complexo principal de histocompatibilidade (major histocompatibility complex – MHC) de classe I ou II. Em um padrão de resposta Th1, APCs apresentam os epítopos para células Th através de MHC II, enquanto apresentam epítopos para células T citotóxicas (Tc) através do MHC I [37]. Após a apresentação, Th e Tc se diferenciam e liberam citocinas (por exemplo, interferon-γ, IL2, IL15) [37, 39], completando, assim, sua diferenciação e expansão. Finalmente, as Tc estão aptas a agir contra as células tumorais (Figura 4). Portanto, no padrão de resposta imunológica tipo Th1, as células efetoras basicamente são as Tc.

Porém, se a célula Th indiferenciada receber estímulos para se diferenciar em Th2, o padrão de resposta é distinto e as células efetoras, geralmente, são macrófagos, mastócitos e linfócitos B. Em geral, essas linhagens celulares são pouco efetivas na resposta contra células tumorais [38, 40, 41]. Além disso, o aumento do padrão de resposta Th1 pode levar à diminuição do padrão Th2 e vice-e-versa [39] (Figura 4).

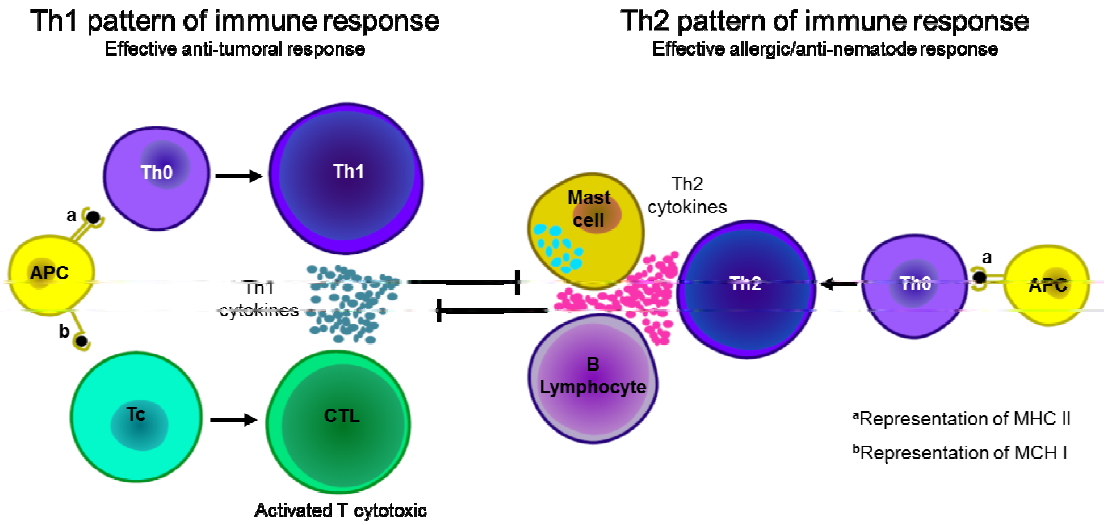

Figura 4 – Representação esquemática das respostas imunológicas padrão Th1 e Th2.

Existem vários artigos que descrevem a polarização da resposta imune para o padrão Th2 relacionada com a redução da resposta imune anti-tumoral e o consequente desenvolvimento tumoral. De fato, essa polarização pode ser utilizada pela célula tumoral como um mecanismo de escape da resposta imune anti-tumoral [38, 40, 41].

4. Câncer de mama

O câncer de mama é a neoplasia mais frequente em mulheres e sua etiologia não é totalmente conhecida [5,42]. A distribuição de regiões do planeta com maior ou menor incidência de câncer de mama não é uniforme, provavelmente devido a diferentes exposições aos diversos agentes ambientais e variáveis, desiguais estilos de vida [5] (Figura 5).

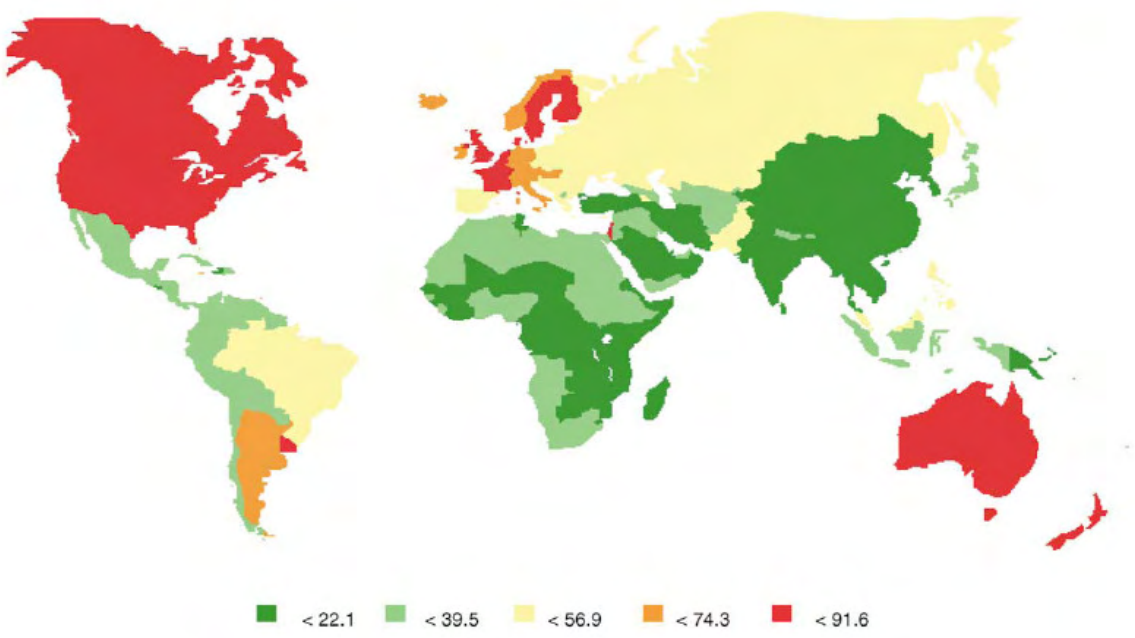

Figura 5 – Incidência mundial de câncer de mama/100.000 mulheres no ano de 2000. Fonte: Parkin et al., 2001 [5].

Estudos sugerem que a dieta, em especial o tipo e a quantidade de gordura ingerida, pode estar relacionada aos diferentes índices de incidência e mortalidade por câncer de mama [5].

No Brasil, as estimativas de incidência de câncer para 2008 e 2009 são de 466.730 novos casos. O número de novos casos de câncer de mama femininos esperados para o Brasil em 2008/2009 foi de 49.400, ou seja, há um risco estimado de 51 novos casos/100 mil mulheres (www.inca.gov.br). Assim como ocorrem disparidades de incidência ao redor do mundo, as várias regiões brasileiras também apresentam diferentes taxas de incidência, sendo sudeste e sul as regiões mais incidentes [10] (Figura 6).

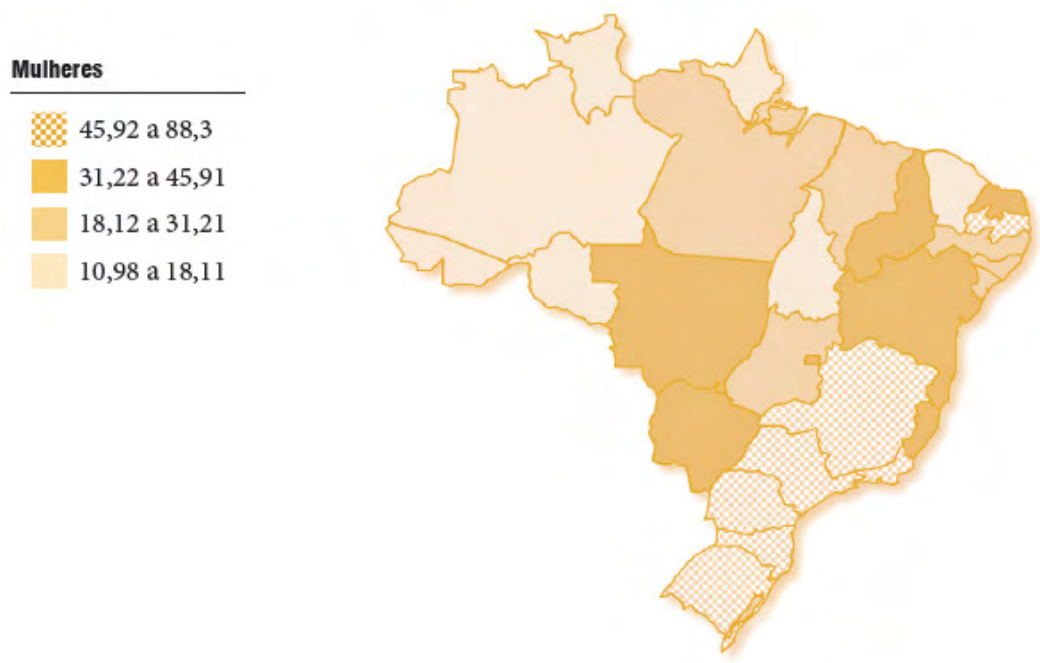

Figura 6 – Incidência brasileira de câncer de mama/100.000 mulheres no ano de 2010. Fonte: INCA, 2009 [10].

Por ser uma doença altamente heterogênea, o câncer de mama é representado por neoplasias que possuem história natural diversa, histologia complexa e resposta variável às terapias convencionais (quimioterapia, braquioterapia e radioterapia) [42]. Suas taxas de mortalidade e morbidade vêm aumentando gradualmente na maioria das "sociedades industrializadas" nas últimas décadas [6, 43, 44].

Pelo pouco conhecimento de sua causa e das razões desse aumento, uma prevenção efetiva ainda é difícil de ser traçada, porém alguns fatores de risco são bem estabelecidos, tais como a exposição à radiação [45,46], ao tabaco [47,48], a cancerígenos químicos, poluentes ambientais, praguicidas, drogas e luz ultravioleta [49]. Estima-se que 50% dos casos de câncer de mama possam ser atribuídos a fatores de risco que incluem idade, estilo de vida e história familiar e reprodutiva [50, 51].

Como medida preventiva, o Instituto Nacional do Câncer recomenda o rastreamento anual para todas as mulheres a partir de 40 anos de idade. A partir dos 50 anos é recomendado o rastreamento mamográfico no máximo a cada 2 anos. Mulheres em grupo de risco (com histórico familiar de câncer de mama em parentes de primeiro grau) devem iniciar os exames anuais a partir dos 35 anos de idade [52]. Quanto ao tratamento, a cirurgia é recomendada, podendo ser conservadora ou radical (dependendo do estadiamento clínico e do tipo histológico). Em alguns casos, a radioterapia é aconselhada para destruir as células

remanescentes após a cirurgia ou para reduzir o tamanho do tumor antes da cirurgia [52]. Dependendo do risco de recorrência, terapias adjuvantes e/ou neoadjuvantes são indicadas [52]. As terapias (neo)adjuvantes tem se mostrado benéficas no tratamento de câncer mamário com receptor de estrógeno positivo (ER+). Entre as terapias (neo)adjuvantes, a supressão ovariana, os inibidores de aromatase e a administração de tamoxifeno e seus derivados estão sendo propostas [53]. Inicialmente aprovado em 1977 pela FDA (EUA) para tratamento de câncer mamário metastático, o tamoxifeno (1-[4-(2-dimethylamino-ethoxy)phenyl]-1,2–diphenylbut-1(Z)-eno) (Figura 7) tem sido utilizado como tratamento para os estágios de câncer mamário com ER+ (54, 55).

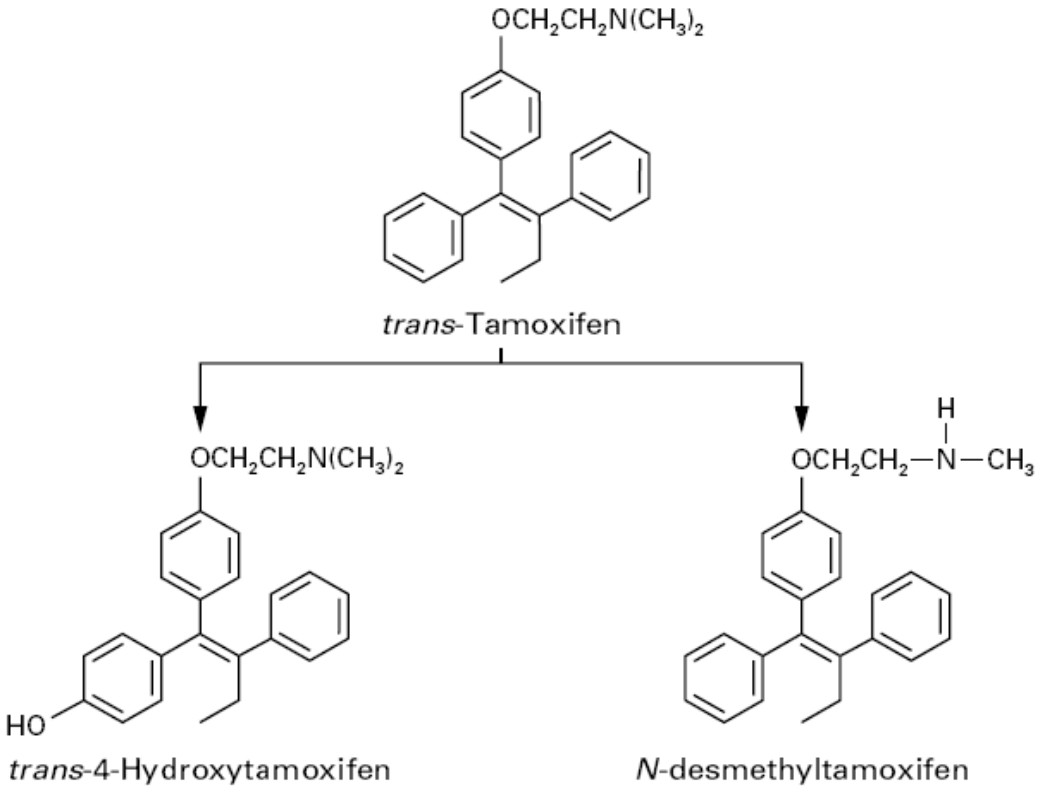

Figura 7 – Estrutura química do tamoxifeno e de seus principais metabólitos em humanos. Fonte: Osborne et al., 1998 [54].

O tratamento com tamoxifeno aumenta o tempo de sobrevivência das pacientes e o tempo de latência das neoplasias mamárias independente da idade, da positividade em linfonodos e estado menopausal [54,55]. Tamoxifeno também tem sido utilizado como um agente quimiopreventivo em mulheres com alto risco de desenvolvimento de câncer mamário [56,57]. O tamoxifeno é uma droga antagonista de estrógenos da família dos Moduladores

Seletivos dos Receptores de Estrógenos (SERMs). Estudos epidemiológicos demonstram que, quando o tratamento oral com tamoxifeno é aplicado a mulheres com câncer de mama ER-positivo por 5 anos após a ressecção cirúrgica do tumor, as chances de recorrência tumoral anual caem pela metade e as taxas de mortalidade pós-cirúrgica são reduzidas a um terço [58].

Os SERMs, como o tamoxifeno, se ligam com alta afinidade ao domínio de ligação do receptor de estrógeno (ERα). Essa ligação impede a ativação do receptor de estrógeno por bloquear a sua interação com os estrógenos. Esse efeito antiestrogênico é conhecido como antagonização dos estrógenos por competição pelo ER-α [53, 55].

Os efeitos biológicos do 17β-estradiol (E_2) são mediados pelos receptores nucleares ERα e ERβ. A ligação de E_2 ao ERα causa a ativação do receptor por fosforilação, dimerização com outro receptor, e ligação com regiões promotoras de genes estrógeno-responsivos, que são subsequentemente transcritos. Esses genes estão envolvidos em vias de promoção da proliferação celular, inibição da apoptose, estimulação da metástase e angiogênese. Co-reguladores são essenciais para essa transcrição e podem agir na promoção (co-ativadores) ou inibição (co-repressores) da mesma [53-55].

O ERα também pode interagir com outros fatores de transcrição (c-Jun ou c-Fos) e outros promotores (AP-1 e SP-1), modulando a transcrição gênica independentemente de interagir diretamente com o DNA. Adicionalmente, E_2 também pode se ligar ao ERα localizado no citoplasma e ativar receptores de fatores de crescimento (epidermal growth factor receptor - EGFR, IGF1R e HER-2) [53].

Os tumores ER-positivos são dependentes do estímulo estrogênico para proliferação de suas células. Por esse motivo, a presença do tamoxifeno bloqueando o ER impede esse estímulo de proliferação. Além desses importantes efeitos anti-proliferativos, o tamoxifeno demonstra efeitos complementares pró-apoptóticos. A combinação de efeitos anti-proliferativos e pró-apoptóticos resulta em uma regressão drástica no número de células nos tumores estrógeno-dependentes, justificando o uso dessa droga em pacientes com câncer de mama [53].

No entanto, o tratamento com tamoxifeno está intimamente associado a graves efeitos colaterais, como um aumento significativo no risco de câncer de endométrio e de fígado [59-61]. O aumento de incidência desses cânceres deve ser em decorrência dos danos genotóxicos/mutagênicos induzidos pelo tamoxifeno nesses tecidos. Adutos de DNA foram detectados principalmente no fígado de ratos tratados com tamoxifeno e iniciados para carcinogênese hepática [59]. Os adutos de DNA não reparados com eficácia levam a mutações no material genético da célula, direcionando à transformação neoplásica celular nesse tecido.

A extensão do dano de DNA depende do tempo de exposição e da dose de tamoxifeno aplicados [61]. Dentre outros efeitos colaterais importantes descritos, estão presentes: ondas de calor, sangramento, corrimento vaginal ou prurido vulvar, anorexia, náusea, vômitos, hipercalcemia, trombocitopenia, leucopenia, neutropenia, aumento do risco de ocorrência de eventos tromboembólicos, esteatose hepática, colestase e hepatite [62]. Além disso, a fosforilação do receptor de estrógeno e de seus co-reguladores (*molecular cross-talk*) podem levar à resistência a esse tratamento [53]. Portanto, seria interessante a administração de substâncias que interajam com o tamoxifeno, amenizando seus efeitos deletérios, prevenindo o aparecimento da resistência ao tamoxifeno ou possibilitando a administração de doses menores dessa droga, mantendo-se ou até aumentando seus efeitos benéficos.

5. Quimioprevenção do câncer: ácidos graxos ômega 3 e ômega 6

A quimioprevenção do câncer pode ser definida como a prevenção, inibição ou reversão do processo de carcinogênese pela administração de substâncias químicas naturais ou sintéticas [63, 64]. As substâncias quimiopreventivas podem ser classificadas em três categorias, de acordo com seus efeitos nas diferentes fases da carcinogênese: 1) inibidor da formação do cancerígeno; 2) bloqueador do metabólito ativo e, 3) supressor do desenvolvimento tumoral [65]. Entretanto, é difícil a classificação precisa dos agentes quimioprotetores, visto que os mecanismos de ação da maioria dos compostos são desconhecidos e muitos deles atuam sobre o processo de carcinogênese através de diferentes modos ou mecanismos de ação [63].

De fato, existem diferenças nas incidências de câncer quando são consideradas diferentes regiões do planeta e muitas destas estão diretamente relacionadas aos hábitos de vida, exposição ocupacional, condições sócio-econômicas e de alimentação [66-68]. Pela sua natureza de múltiplos estágios, há a possibilidade de ações quimiopreventivas com compostos que atuam em mecanismos alvo-específicos, envolvidos na iniciação, promoção ou progressão do câncer [64, 69]. Atualmente, o foco da quimioprevenção vem ganhando espaço na literatura científica em virtude do grande número de trabalhos científicos publicados acerca de diferentes produtos ou moléculas naturais em diferentes ensaios *in vitro* e *in vivo* [16, 69-71].

Existem vários estudos relacionando o consumo de frutas, cereais, vegetais e chás (fibras, retinóides, fenóis, inibidores de proteases, indoles, isocianatos, polifenóis, entre outros) [71-74] e micronutrientes [70,75,76] e a quimioprevenção do câncer. Os resultados desses estudos estimulam pesquisas futuras com o objetivo de identificar quais compostos de nossa alimentação são responsáveis por tais reduções [64, 77].

Inicialmente a maioria dos estudos epidemiológicos relacionando dieta e câncer foi realizada para identificar componentes da dieta associados à alta incidência de certos tipos de câncer, como a de colorretal e mama em países desenvolvidos e a incidência de câncer de estômago e esôfago em alguns países em desenvolvimento [77]. Esses resultados sugeriram que a intervenção no processo de carcinogênese, seja bloqueando a ação dos fatores causais, seja atuando na identificação de fatores moduladores, é de fundamental importância na estratégia preventiva contra esta doença [77].

Na década de 70, foram publicados artigos reportando baixas incidências de doenças coronárias e câncer em esquimós residentes em *Greenland*, mesmo eles tendo uma dieta rica em gorduras. Estudando-se a composição dessa dieta, uma das causas dessa baixa incidência foi considerada a grande quantidade de peixes ingerida [78]. Hoje em dia, câncer, doenças

cardiovasculares, obesidade e diabetes são responsáveis por mais de 80% da mortalidade relacionada a doenças nos Estados Unidos, e os lipídios (tanto a quantidade ingerida quanto o tipo de lipídios da dieta) parecem exercer papéis críticos em todas essas doenças [79-82]. De fato, o total de gordura ingerida e a proporção de ácidos graxos ômega-6:ômega-3 tem aumentado significantemente na dieta Ocidental desde a Revolução Industrial [78, 83, 84]. Relatórios do *US Department of Health & Human Services* [85-88] e trabalhos de revisão [89-93] relacionam o efeito preventivo de uma dieta rica em ômega-3 em doenças cardiovasculares, câncer e síndrome metabólica em humanos.

Ácidos graxos ômega-3 e ômega-6, também conhecidos como ácidos graxos essenciais, podem ser sintetizados *de novo* ou obtidos através da dieta. No entanto, os mamíferos não possuem desaturases necessárias para a síntese de n-3 e n-6 PUFAs (*n-3/n-6 PolyUnsatured Fatty Acids*, ômega-3 e ômega-6); portanto, nos mamíferos, esses ácidos graxos essenciais devem ser obtidos através da dieta [79, 94]. Ácidos graxos ômega-6 são representados pelo ácido linoléico (LA) e ácidos graxos ômega-3 são representados pelo ácido alfa-linoléico (LNA). LA, sintetizado por plantas terrestres, é abundante em sementes e na maioria dos óleos vegetais consumidos pelos seres humanos (óleos de milho, oliva, etc). LNA é encontrado no cloroplasto de vegetais folhosos (espinafre, brócolis, mostarda, etc), soja, castanhas e canola. Ambos ácidos graxos são metabolizados em ácidos graxos de cadeia longa de 20 e 22 átomos de carbono. LA é transformado em ácido aracdônico (AA) e LNA em ácido eicosapentanóico (EPA) e ácido docosahexanóico (DHA) (Figura 8) [78, 95]. Porém, a maioria dos vegetais que é rica em LNA também é rica em LA. Portanto, a maioria dos vegetais que produz ômega-3 também produz grandes quantidades de ômega-6 [79].

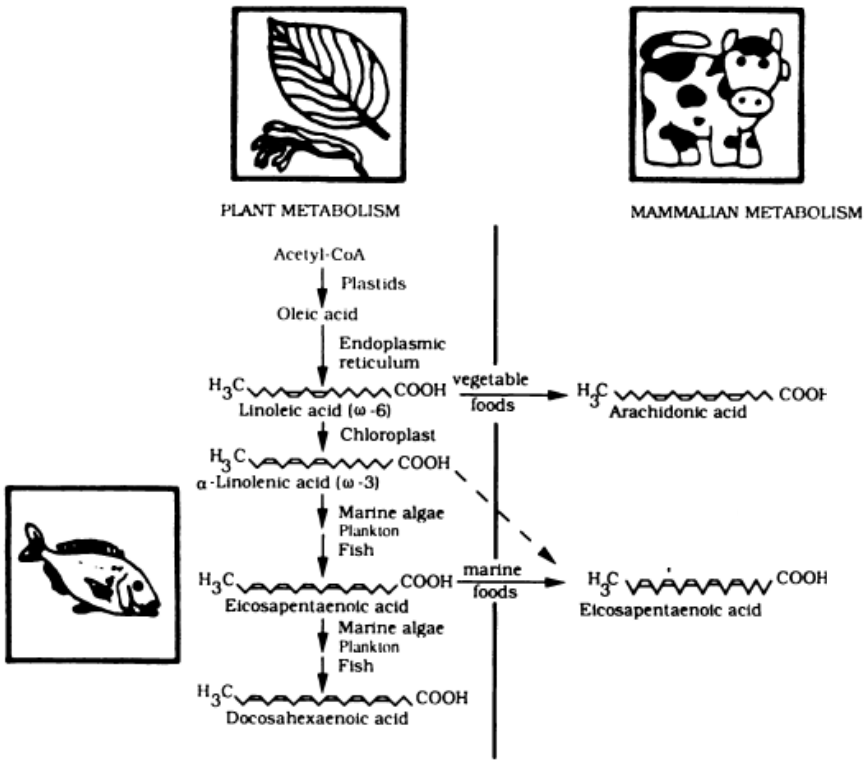

Figura 8 – Origem dos PUFAs ômega-3 e ômega-6. Modificado de Simopoulos et al., 1991 [78].

Adicionalmente, concentrações altas de ácidos graxos de cadeia longa n-3 são encontrados primariamente em alguns peixes de águas profundas na forma de EPA e DHA. Os peixes adquirem esses ácidos através da ingestão de fitoplâncton e zooplâncton [80]. A quantidade desses ácidos graxos varia de acordo com a espécie de peixe e a localização geográfica [81]. Em geral, cavalinha, atum e salmão possuem as maiores concentrações de EPA e DHA e baixas quantidades de ácidos graxos n-6 [81].

Uma vez ingeridos, os ácidos graxos ômega-3 e ômega-6 competem pelas mesmas enzimas de elongação (desaturases) (Figura 9); portanto, uma dieta altamente rica em ômega-3 pode reduzir os níveis de ômega-6 no organismo e vice-e-versa [94-96]. Em geral, a população ocidental ingere 20 vezes mais ácidos graxos ômega-6 do que realmente necessitam [95].

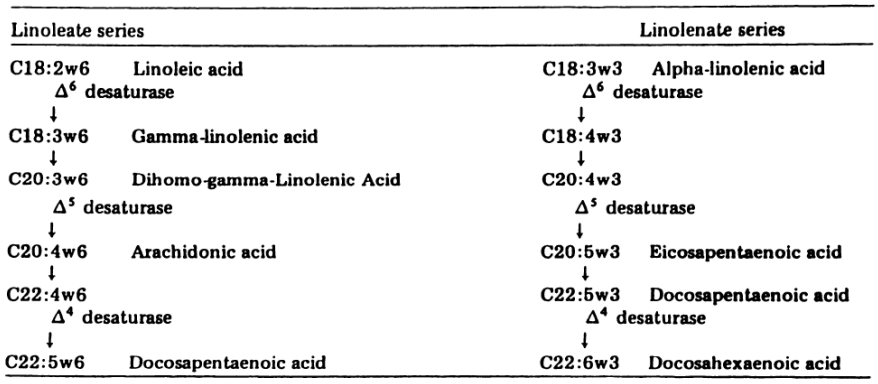

Figura 9 – Desaturação e elongação dos ácidos graxos ômega 6 e ômega 3. Fonte: Simopoulos et al., 1991 [78].

Interações complexas e substituições entre ômega-3 e ômega-6 ocorrem no plasma e nos lipídeos celulares, o que pode ocorrer através de diferentes perfis de dieta. Os passos iniciais de ativação celular, como a geração de mensageiros secundários intracelulares, podem ser ativados por esses ácidos graxos [78]. Adicionalmente, estudos demonstram que esses ácidos graxos podem induzir diferentes funções da membrana, bem como modificações na transcrição gênica [97].

Estudos mostram que ácidos graxos ômega-3 são essenciais para o crescimento e desenvolvimento normais e podem ter um papel importante na prevenção de hipertensão, artrite, câncer e doenças auto-imunes e inflamatórias [78, 95, 98]. De fato, o benefício de uma dieta rica em ômega-3 tem sido enfaticamente descrita em doenças coronarianas, acidente vascular encefálico e doenças com componentes inflamatórios (diabetes tipo 2, degeneração macular, asma, entre outras) [99] por mecanismos de proteção que incluem inibição de agregação plaquetária, diminuição da viscosidade sanguínea e supressão da formação de leucotrienos (mediadores lipídicos para agregação de macrófagos e neutrófilos) [100].

Com isso, um novo *approach* para o uso de ácidos graxos como tratamento adjuvante de doenças vem sendo explorado; ações sinergísticas entre ômega-3/droga, e possíveis reduções da toxicidade de algumas drogas devido à administração de ômega-3 podem ocorrer [78]. O tratamento de roedores com ômega-3 aumentou a eficiência de algumas drogas utilizadas no tratamento de câncer, tais como doxorrubicina, mitomicina C e potencializou os efeitos do tamoxifeno em xenografos estrógeno-dependentes. Esses estudos apontam para um efeito significativo do tipo de ácido graxo na dieta como tratamento adjuvante à quimioterapia convencional [93, 101, 102].

Vários estudos de carcinogênese em animais evidenciam que a alta ingestão de ômega-6 estimula o desenvolvimento de câncer mamário, principalmente na fase de promoção [82, 103, 104]. Outros trabalhos relacionam ácidos graxos ômega-3 com a quimioprevenção de câncer mamário em roedores [93, 101, 102]. Uma explicação plausível para os efeitos quimioprotetores do ômega-3 na carcinogênese de mama é a ação antiinflamatória que esse ácido graxo e seus metabólitos podem exercer [98].

Adicionalmente, Manni e colaboradores (2010) [105] mostraram que uma dieta rica em óleo de peixe (ômega-3) aumentou a eficiência do tratamento com tamoxifeno, reduzindo a incidência de neoplasias mamárias em 47%. Estudos observacionais em humanos mostram a correlação inversa do consumo de ômega 3 ou seus metabólitos e o risco de câncer colo-retal e prostático [106, 107] e o risco de câncer colo-retal, prostático e mamário em estudos experimentais [79]. Ácidos graxos ômega-6 parecem ter efeitos contrários aos do ômega-3. Em geral, produtos derivados de ômega-6 possuem efeitos pró-inflamatórios, enquanto os derivados de ômega-3 são anti-inflamatórios. Eles também parecem possuir efeitos opostos no crescimento de células neoplásicas, invasão e angiogênese [79]. Apesar de alguns resultados apontarem para a modulação do controle da expressão gênica por esses ácidos graxos [108], a interação gene-dieta na saúde ainda não foi totalmente esclarecida [79, 109].

Considerando as modificações fenotípicas em neoplasias mamárias de ratas tratadas ou não com tamoxifeno e dietas ricas em ômega-3 ou ômega-6 [105], nós elaboramos a hipótese de que o tratamento com tamoxifeno poderia modificar a expressão gênica das células do tecido mamário tumoral, dependendo da dieta fornecida aos animais. Portanto, nós utilizamos uma dieta rica em óleo de milho como a principal fonte de ômega-6 e uma dieta rica em óleo de peixe como principal fonte de ômega-3 e analisamos o perfil de expressão gênica nos tumores induzidos por MNU.

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Objetivos

Os objetivos do presente estudo foram avaliar os efeitos das dietas ricas em ômega 3 ou ômega 6 no transcriptoma de câncer mamário de ratas Sprague-Dawley sob o tratamento ou não com tamoxifeno.

Capítulo 2 - Editorial

Omega-3 fatty acids: a potential booster for tamoxifen therapy? [£]

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Introduction

The treatment of breast cancer patients with non-steroidal antiestrogen tamoxifen (Tam) increases the survival of patients, irrespective of the nodal and menopausal status or age [1]. Tam is a drug of the Selective Estrogen Receptor Modulators (SERMs) family, which has high affinity with the estrogen receptor α (ERα). The binding Tam-ERα avoids the receptor activation by blocking its interaction with the estrogens [1, 2]. The activation of ER increases the binding in promoters of estrogen-responsive genes, stimulating their transcription. In tumors, these genes are involved in the cellular proliferation, inhibition of apoptosis and stimulus of metastasis and angiogenesis [2]. Moreover, ERα is able to interact with other transcription factors and promoters, and modulate the gene transcription with no direct interaction with the DNA. Furthermore, estradiol can interact with ERα in the cytoplasm and activate growth factors [2]. Thus, the blocking of the ER by tamoxifen decreases the proliferating stimulus, which may decelerate the tumor growth. Further, Tam has shown important pro-apoptotic complementary effects. The combination of anti-proliferative and pro-apoptotic effects of this drug results in a drastic regression of the number of neoplastic cells in the patient [2]. Epidemiological studies have shown that a five-year oral treatment with Tam after ER+ breast cancer resection reduces the tumor recurrence in half, and the mortality rates post-surgery are decreased to one-third [1].

However, Tam presents several side effects, such as a significant increase in the risk of developing endometrial cancer [1, 3]. Additionally, “molecular cross-talk,” hypersensitivity of ER to low levels of estrogens, and increased expression of co-activators are among the numerous factors that contribute to Tam resistance in tumors, decreasing tumor response to Tam [3]. Therefore, it would be of great interest if the administration of substances capable of reducing Tam’s deleterious effects, could increase its beneficial effects or reverse tumor resistance to the drug.

Reports show a low incidence of breast cancer in individuals who ingest large quantities of various types of fish [4]. High concentrations of polyunsaturated fatty acids (PUFAs) omega 3 are found in cold water fish, primarily as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These compounds are formed by desaturation of alpha-linoleic acid (ALA) [5]. Indeed, complex interactions and substitution of omega-6 by omega-3 fatty acids happen in the plasma and cellular lipids, and this process is influenced by the diet. The intracellular second messenger generation and transcription of genes can be activated by these fatty acids [4]. So, a new approach for the use of fatty acids in the adjuvant treatment has been

explored; synergistic effects of omega-3 and drug, and/or possible reductions of drug toxicity can happen [4].

Omega 3 fatty acids and breast cancer

There is *in vitro* and *in vivo* evidence that the administration of omega-3 or its metabolites is able to reduce cellular proliferation and increase apoptosis. The treatment of MCF-7 or MDA-MB-231 cells with DHA or EPA reduced cellular proliferation [6-8] and inhibited DNA synthesis [6, 8]. Additionally, treatment with the omega-3 metabolites arrested the cells in G2 phase, [7] which may be explained by the down-regulation of CDK1-cyclin B1 complex, an indispensable mitosis initiator [7]. Similarly, breast cancer cells injected into *nude* mice fed with fish of flaxseed oils (rich in omega-3) formed smaller tumors, with lower cell proliferation [6, 9]. The animals also presented lower expression of HER2 and EGFR, and reduced levels of pMAPK [9]. Interestingly, female Sprague-Dawley rats that received a low-fat n-3 PUFA diet during prepuberty and were DMBA-initiated had lower mammary tumor incidence than the control, with lower PCNA protein expression in the tumors [10]. On one hand, slowing down cellular proliferation will reduce tumor growth; on the other, the stimulation of apoptosis will reduce tumor volume, by inducing programmed cell death of the tumor cells. Studies have shown that lipid peroxidation in the tumor may be responsible for the increase in the apoptotic rate [11]. Several studies show that cells and animals treated with omega-3 or its metabolites have increased apoptotic rate, [6-8] with increments of reactive oxygen species formation [6] and lipid peroxidation [6, 10]. In fact, Akt directly promotes cell survival by phosphorylating and inactivating components of apoptotic machinery. Akt also can activate transcription factors such as NFκB. This transcription factor is able to protect cells from apoptosis by activating anti-apoptotic genes [12]. One of the mechanisms of action attributed to the apoptosis augment in DHA/EPA-treated MDA-MB-231 cells was impairment of Akt phosphorylation and NFκB activity [8]. In line with this, *in vivo* studies showed increased apoptotic index of MCF-7 cells injected in flaxseed oil-fed *nude* mice. This reduction was probably due to the downregulation of tyrosine kinase receptors such as EGFR and HER2, and subsequent downregulation of Akt [9].

Besides the variety of mechanisms that omega-3 PUFAs may be involved with in and of themselves, there are articles that correlate the administration of tamoxifen and omega-3, leading to improved treatment. The treatment of rodents with omega-3 fatty acids potentiate the effects of tamoxifen in estrogen-dependent xenographs [13]. Moreover, Manni et al.

(2010) [14] showed that the administration of a fish oil-rich diet combined with tamoxifen inhibited mammary tumor incidence and multiplicity [14]. DeGraffenried et al. (2003) [15] showed that EPA was effective in the inhibition of Akt; further, EPA-treated MCF-7 cells had a more sensitive response to the effects of tamoxifen, which could be a potential treatment against tamoxifen resistance, once the activation of Akt is one of the factors that lead to tamoxifen resistance.

Ovariectomized Balb/C mice treated with raloxifene analogs and fed with fish oil showed a reduction in the production of the inflammatory cytokines IL-1b and IL-6 [16]. In fact, during the inflammatory response there are released pro-proliferative cytokines that can stimulate the tumor growth. Inflammatory response is part of the innate immune response. Innate immune cells present in the tumors may have a dual effect: although tumor-associated macrophages may kill tumor cells, they are able to produce angiogenic and lymphangiogenic growth factors, cytokines and proteases that can potentiate the neoplastic progression and blunt anti-tumor response of T cytotoxic cells [17]. By analyzing a cDNA microarray of tumors of corn oil-fed (an omega-6-rich oil) + tamoxifen-treated rats we found that these animals had increased expression of genes related to the increment of the inflammatory response, that was not found in fish oil+TAM-treated animals [18]. Additionally, the treatment of Sprague-Dawley rats with fish oil increased the modulation of genes that indicate a better immune response against tumors (polarized Th1 immune response) [19].

The incorporation of omega-3 PUFAs increases the fluidity of the membrane, which may allow greater influx, and increased uptake or permeability of chemotherapeutical drugs, resulting in enhanced drug concentrations inside the cell [20]. This may increase the effectiveness of the breast cancer treatment. In addition, omega-3 has been reported to reduce the toxic side effects of chemotherapy, such as diarrhea associated with pathological changes in the intestines, and hematological changes. As an added benefit, these fatty acids may interfere with wasting syndrome, also known as cachexia, which is often accompanied by weakness, anemia and suppression of immune functions [20].

In summary, there are several pathways by which omega-3 fatty acids can act in anti-cancer response. The down-regulation of the CDK1-cyclin B1 complex, resulting in a lengthening of G2-phase, and the down-regulation of MAPK pathway may be responsible for slowed breast cancer growth. The increased lipid peroxidation with accumulation of ROS, and the down-regulation of Akt may be the responsible for an increased apoptotic index. The improvement of the immune response against tumors, and the lack of inflammatory cytokines in tumors of animals fed omega-3 and treated with tamoxifen, contribute in the impairment of

the tumor growth. The decrease of wasting syndrome in patients with advanced cancer may contribute to their overall health. Finally, the increased membrane fluidity may improve the internalization of the chemotherapeutic drugs. Therefore, the combination of these mechanisms represents a potential boost to tamoxifen therapy.

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Capítulo 3

Fish oil alters tamoxifen-modulated expression of mRNAs that encode genes related to differentiation, proliferation, metastasis, and immune response in rat mammary tumors£

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Running title: Diet and tamoxifen in breast cancer treatment

£ Submitted to Cancer Prevention Research

Abstract

We have previously shown that a fish oil-rich diet increased the chemopreventive efficacy of tamoxifen (Tam) against *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary carcinogenesis. Herein, we evidence that tamoxifen treatment modifies gene expression of mammary tumors depending upon the type of dietary fat fed to the animals. Rats initiated with MNU and treated with Tam were fed a diet rich in corn oil (CO) or fish oil (FO). After 8 weeks, tumors of the same histological type (cribriform) were collected and comprehensive analysis of messenger RNA expression was performed. The mRNA expression of genes such as SerpinB10, Wisp2 and Apod in tumors from FO-treated rats is indicative of highly differentiated tumors. Decreased expression of H19 and Igf2 mRNA in Tam-treated groups, and Thrsp and Wnt5b mRNA in FO+Tam group may be related to tumor growth impairment and lower metastatic capacity. Increased Irf7 transcript levels in FO-treated animals suggests an improved immune response against tumors (Th1 pattern) whereas decreased mRNA of Fcer1a, Hdc, Ms4a2, Slp1, Mcpt1 and Mcpt2 may indicate a shift of the immune response towards Th2 pattern. The Th2 pattern of gene expression represents a potential mechanism of escape from the immune response caused by FO+Tam treatment. These data show that, although tamoxifen modulates the expression of genes leading to tumor growth impairment, further modulations of genes are influenced by FO altering Tam-modulated expression of genes in a manner that may, in part, account for its enhancing chemopreventive effect against MNU-induced mammary carcinogenesis.

Keywords: Adjuvant treatment, Breast cancer, cDNA microarray, Diet, Fatty acids, Tamoxifen.

Introduction

Breast cancer is the most frequently diagnosed type of cancer and is the second cause of death in women in the United States and in Brazil (1). Based on current rates, it is estimated that more than 200,000 women were diagnosed with breast cancer in 2010 (1). Adjuvant and neoadjuvant endocrine therapies have shown benefits in estrogen receptor-positive breast cancer treatments. Adjuvant therapies proposed for the treatment of this malignancy include estrogen receptor antagonists, aromatase inhibitors and ovarian suppression (2). Treatment with the non-steroidal antiestrogen tamoxifen (Tam) increases the recurrence-free and overall survival of patients with localized disease, irrespective of the nodal status, menopausal status or age (3). Tamoxifen has also been shown to be an effective chemopreventive agent (4). However, side effects of Tam include a significant increase in the risk of developing endometrial cancer, depending on the dose and length of treatment (5). In order to improve the chemopreventive action of Tam, our laboratories have tested the combination of this drug with a fish oil rich diet in a model of MNU-induced mammary carcinogenesis (6). Omega-3 fatty acids, which are abundantly present in fish oil, have been shown to increase the cytotoxic action of drugs such as doxorubicin and mitomycin-C in breast cancer cells (7, 8). In addition, the consumption of an omega-3 fatty acid rich protects against breast cancer in most preclinical studies (9). We have reported that the chemopreventive effect of tamoxifen against MNU-induced mammary carcinogenesis was superior in FO fed rats compared to rats fed with CO (6). In order to understand the molecular mechanisms underlying the effects of FO alone and in combination with Tam on mammary carcinogenesis, we performed a transcriptomic analysis of tumors arising in rats fed a FO vs. CO rich diet in the presence and in the absence of concomitant Tam treatment.

Material and Methods

The mammary tumors used in this experiment were obtained from a previous chemoprevention study (6). Briefly, twenty one day-old rats received a single i.p. injection of 50 mg MNU/kg body weight and were distributed into four different groups: Group 1 (CO group) received a diet with 20% of CO; Group 2 (CO+Tam group) received a diet with 20% of CO, and 100 μg/Kg s.c. 5 days/week of tamoxifen; Group 3 (FO group) received a diet with 17% of FO and 3% of CO; Group 4 (FO+Tam group) received 17% of FO and 3% of CO and tamoxifen. After the eight weeks of treatment, the rats were euthanized by CO_2 asphyxiation, and the palpable tumors were dissected and cut in half. One half was frozen in liquid nitrogen and kept at -80ºC for molecular biology analysis and the other was fixed in 10% neutral buffered formalin, processed and paraffin embedded. Six micrometers sections were processed for routine H&E staining and tumor diagnosis. Only cribriform tumors were selected for RNA isolation, microarray and real time PCR analysis to exclude possible genomic expression heterogeneity among different tumor types.

Gene expression array

Total RNA was isolated from cribriform tumors using RNeasy Mini Kit (Qiagen Inc., Valencia, CA) following manufacture's protocol. After DNase I treatment, RNA concentration was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was assessed using capillary electrophoresis using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Two hundred ng of total RNA from three cribriform tumors per group were processed for microarray hybridization using Quick Amp Labeling Kit-one color (Agilent Technologies, Palo Alto, CA) following the manufacturer's protocol. Labeled cRNAs were hybridized to microarray slides (Whole rat genome-4X44K oligo microarrays-G4112F, Agilent Technologies) at 65°C for 17 hours. Slides were then washed and scanned following Agilent's recommendations. The digitalized images were decoded and submitted to quality control tests through Feature Extractor v9.5.3.1 using the default protocol GE1-v5_95_Feb07 (Agilent Technologies, Palo Alto, CA).

Microarray data analyses

Preprocessing and differential expression analyses of microarray data were performed using LIMMA (Linear Methods for Microarray data Analysis) package (10) in the R/Bioconductor platform. Mean signal intensities were background-corrected using the normexp method, and quantile normalization was performed. Prior to differential expression analysis, a non-specific filter was applied, in which probes without a valid Entrez ID annotation were excluded. In addition, probes with signal close to the background noise level for all 4 groups under consideration were excluded. About 11,000 probes remained for further analysis.

Empirical Bayes moderated t-statistics was used to assess significance among treatments (CO vs. CO+Tam; FO vs. FO+Tam and CO vs. FO comparisons). Genes with fold-change of at least 3.0 at $p < 0.01$ were considered differentially expressed and used for bioinformatics analyses. Gene Ontology (GO) (11) enrichment analysis was performed using GeneSpring v.11.0 (Agilent Technologies, Palo Alto, CA) and biological processes with p value < 0.05 were considered enriched.

Validation by real time PCR

In order to validate the expression of selected genes found in the microarray analysis (Supplemental Table 1), real time PCR was performed on the same RNA samples used for microarray, as previously described by our group (12). Total RNA was reverse transcribed using the ABI High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Standard curves were made using serial dilutions from pooled cDNA samples. Real time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol and run on the ABI Prism 7300 Sequence Detection System. A TaqMan® assay was performed for RT1-Aw2 mRNA, following the manufacturer's instructions (Applied Biosystems).

Quantification was performed for each gene and normalized to a housekeeping gene (18S ribosomal RNA). Normalized expression values were log2 transformed, analysis of variance (ANOVA), and Tukey's post hoc test were performed. Statistical analysis was performed in the JMP 7 software (SAS Institute, Cary NC). Differences were considered statistically significant with a P value less than 0.05.

Results

The effects of our interventions on tumorigenesis and tumor multiplicity have been described in our previous report (6). We report here the transcriptomic changes occurring in the tumors which developed despite our interventions. The dietary influence on tamoxifen effects was assessed by comparing the CO vs. CO+Tam and FO vs. FO+Tam groups. The comparison of CO vs. FO was performed to evaluate the effects of the diet *per se*. We obtained 12 arrays in which, after quality control analysis, normalization and preprocessing of the microarray data, 11,079 out of 44,000 probes remained for our analysis. All the microarray data was deposited in the Gene Expression Omnibus (GEO) public repository under the series record GSE27465. We used enriched Gene Ontology analysis and analysis of the relation of each differentially expressed gene with cancer-related processes. After analyses, a set of genes of interest was chosen for further real time PCR validation.

Number of differentially expressed genes

Thirty two genes were differentially expressed in the comparison CO vs. CO+Tam (Supplemental Table 2), and 58 genes were differentially expressed between FO vs. FO+Tam (Supplemental Table 3). In the comparison CO vs. FO, 19 differentially expressed genes were found (Supplemental Table 4). An indirect analysis of the data was performed in order to assess the effects of tamoxifen independently of diet. By studying the commonality between the effects of tamoxifen in a CO-rich diet (CO vs. CO+Tam comparison) and the effects of tamoxifen in FO-rich diet (FO vs. FO+Tam comparison), three genes, H19, Igf2 and Ccl5, were found that were differentially expressed by tamoxifen in either CO- or FO-rich diets.

Genes directly related to the breast cancer profile

Differences in the expression of genes were found in the comparisons FO vs. FO+Tam and CO vs. CO+Tam (H19, Igf2, Sncg, Thrsp, Wnt5b, Lcn2, Aqp1, Mme (CD10), Pdgfra, Lrrn1, Fmod, Chl1, Aspn, Spock2, Ptms and LOC298116). These genes play an active role in breast cancer progression, stage, prognosis, metastasis and disease-free survival. Regarding the effects of the diet *per se* in the gene expression of the tumor cells, genes that are typically expressed in tumors with a more differentiated phenotype were noticed in the FO-treated group than in the CO-treated group (SerpinB10, Apod and Wisp2).

We have already reported that the administration of tamoxifen to CO- or FO-fed rats reduces the incidence of the mammary tumors (6). In order to test the transcriptomic changes that were associated with these effects, eight of the differentially expressed genes directly related to the breast cancer profile were selected for validation by real time PCR: H19, Igf2, Serpinb10, Wisp2, Apod, Sncg, Thrsp and Wnt5b. H19 and Igf2 genes were selected in order to evaluate the effects of tamoxifen independently of the diet. Specifically, Thrsp, Sncg and Wnt5b mRNA were examined as genes responsible for tumor growth impairment, whereas SerpinB10, Wisp2 and Apod mRNAs as markers of differentiation in tumors.

Figure 1 shows the side-by-side comparison of $\log_2$ values of selected genes in both microarray and real time PCR analysis. H19 and Igf2 mRNA were decreased in the FO+Tam group. Moreover, Igf2 mRNA was decreased in CO+Tam group. H19 transcript levels were decreased in the CO+Tam group although this transcript reached statistically significant only in the microarray quantification. Fish oil treatment decreased mRNA for SerpinB10; although there was an increase in the expression of Apod and Wisp2 mRNA, this was not statistically significant (p=0.058 and p=0.13, respectively; figure 1) in the real time PCR quantification. Finally, FO+Tam treatment reduced Sncg mRNA expression. The decrease in Wnt5b and Thrsp transcripts was statistically significant only in the microarray.

Genes related to immune response

Enrichment GO analysis showed that fish oil treatment modified several genes related to immune response (Table 1). Based on the GO classification and on previous reports, we chose two of the genes differentially expressed by FO to be validated by real time PCR (RT1-Aw2 and Irf7), considering their relation to the cellular immune response. Real time PCR data confirmed increased mRNA for the genes Irf7 in the FO-fed animals in comparison to the CO-fed animals (fold change 4.99, $P<0.05$). The differential expression of RT1-Aw2 was not confirmed in the comparison CO vs. FO.

Similarly, enriched GO analysis showed that the transcripts differentially expressed in the tamoxifen-treated group that received fish oil diet (FO+Tam) were related to immune response (Table 2). Considering the interaction and the function of these genes in the immune response, seven mRNA (Fcer1a, Hdc, Klra22, Mcpt1, Mcpt2, Ms4a2 and Slpi) were selected to be quantified by real time PCR (Figure 2). Hdc and Slpi mRNA levels were increased whereas alterations in mRNA for Fcer1a (P=0.20), Klra22 (P=0.17), Ms4a2 (P=0.07), Mcpt1 and Mcpt2 (p=0.13) were not statistically significant (Figure 2) when assessed by real time

PCR. Although real time PCR did not confirm increased mRNA of RT1-Aw2 gene in CO vs. FO (primarily chosen for validation in this comparison), it was found to be 3.33 times increased in FO+Tam compared to FO group ($P<0.05$). In the comparison CO vs. CO+Tam, there were three differentially regulated genes related to immune response (RT1-Bb, Pls and Il33), and they were confirmed by real time PCR (Figure 2).

Discussion

In the present study, it was observed that FO diet alone and in combination with Tam treatment is responsible for changes in the transcriptomic profile of mammary tumors that may influence the behavior of this malignancy. The changes observed are mainly in transcripts which encode for proteins related to differentiation and progression of tumors, and immune response.

The FO diet dysregulated genes related to tumor differentiation, such as SerpinB10, Apod and Wisp2. Wisp2 expression gradually decreases as breast cancer progresses from a well-differentiated to a poorly differentiated stage (13). This is confirmed by experiments that show high expression of Wisp2 in less aggressive human breast cancer cells (MCF7 and ZR-75-1), minimal expression in moderately aggressive breast cancer cells (SKBR-3) and no expression in highly aggressive breast cancer cells (MDA-MB-231) (14). Based on these results, it is possible that increased of Wisp2 expression in the present study indicates that tumors in FO-treated group are more differentiated than those observed in the CO-treated group. Likewise, breast tumors require a certain degree of differentiation to produce the Apod protein (15). As shown herein, this mRNA is increased in tumors of animals treated with FO. In addition, there is a correlation of low Apod levels and a shorter relapse-free survival and poor survival of patients with breast cancer (15). Furthermore, the decrease in mRNA for SerpinB10 in the FO group may predict either a more differentiated mammary tumor, a reduction in the cellular proliferation and/or an increase of apoptosis. Decrease of SerpinB10 mRNA levels was observed in NRP-152 and NRP 154 prostatic epithelial cell lines after induction of differentiation (16). An up-regulation of this gene was observed in HL-60 cancer cells compared to normal myeloid cells (17).

Tumors which arose from Tam-treated rats had decreased expression of H19 and Igf2 mRNA independently of the type of dietary fat fed to the animals (either FO or CO). *In vitro* studies correlated increased H19 with a more malignant cell phenotype, as assessed by colony formation capacity in soft-agar and enhanced adhesiveness in type I collagen (18). Adrianssens et al. (19) showed that estradiol stimulated and tamoxifen decreased H19 gene expression in MCF-7 cells. Similarly, Igf2 is well-established as a growth factor both *in vitro* and *in vivo* (20). Reinforcing this concept, cells with disrupted Igf2 function, when injected in transgenic mice, showed reduction in tumor cell growth, reduced malignancy and a significant number of apoptotic bodies (21). These studies suggest that estrogen may affect the expression of Igf2 and its components. By using mRNA *in situ* hybridization, Manni et al.

(22) found that the expression of Igf2 mRNA is under positive endocrine regulation, since its levels decreased in regressing tumors following ovariectomy, and the normal expression levels were re-established after estradiol repletion. In line with the results presented herein, in a T61 human breast cancer xenograft model, treatment of cells with tamoxifen produced a ten-fold reduction in the baseline level of Igf2 mRNA (23).

In addition to the changes that Tam caused irrespective of diet, the combination of FO and tamoxifen treatment increased the regulation of genes that may lead to a better prognosis of mammary cancer. Sncg is undetectable in normal breast tissue and in most of the benign lesions, whereas this gene is expressed in breast cancer with a positive correlation with stage, poorer prognosis, metastasis, and negative correlation with disease-free survival and overall survival (24). Over-expression of Sncg increases anchorage-dependent cell growth (25), motility and invasiveness of MDA-MB 435 cells, and metastatic potential *in vivo* (26). Furthermore, over-expression of Sncg in MCF-7 cells increases cell proliferation after estradiol administration, probably due to the increase of ER-α ligand-dependent transcriptional activity (25). It is plausible that the decreased of Sncg mRNA by tamoxifen in presence of FO diet may be impairing tumor growth and/or reducing its metastatic potential. Equally important is the decrease of Thrsp mRNA. This gene is increased in breast carcinoma cells, and is associated with a poor prognosis, high grade tumors, reduced disease-free survival and higher metastatic potential (27). Increased expression of Thrsp is related to an accelerated growth of breast cancer cells in culture, whereas the transfection of antisense RNA augmented apoptosis (28). Taken together, these results show that Tam administered to FO-fed rats decreased expression of Sncg and Thrsp, which may account for the enhanced chemopreventive efficacy of this combination regimen described previously (6).

Based on the gene expression patterns, FO treatment may improve the immune response against tumors, as summarized in Figure 3. Basically, an effective anti-tumoral response is based on T helper 1 (Th1) pattern response. Undifferentiated T helper cells are able to differentiate either to Th1 or Th2 (29), depending on the stimulus, and on the antigen presentation by antigen-presenting cells (APCs) (30). APCs present tumor cell-specific antigens (epitopes) through both MHC (major histocompatibility complex) class I and II. APCs present the epitopes to Th cells through MHC II, whereas APCs present the epitopes to T cytotoxic cells (Tc) through MHC I (30). After presentation, both Th and Tc are able to differentiate and release lymphokines (such as interferon-γ, IL2, IL15) (30, 31), completing their differentiation and expansion. Finally, Tc cells are able to be effective against tumor cells. In this context, FO treatment increased Irf7 mRNA expression. Irf7 increases antitumor

activities of macrophages (32), which are antigen-presenting cells, and to function as a possible tumor suppressor gene (33). The proteins of the RT1 locus are part of the MHC I (34). The supposition that a FO diet may improve the immune response against tumors is supported by improved immune response against tumors after n-3 PUFAs administration to animals, as reported in the literature (35, 36).

The FO given in combination with tamoxifen increased mRNA of RT1-Aw2, which may confirm the MHC I increment caused by a FO-rich diet (34). Surprisingly, FO+Tam strongly increased the expression of several mRNAs that may be related to the Th2 pattern of immune response. Generally, the augmentation of a Th2 response down-regulates the Th1 response (31). It is believed that a polarized immune response towards the Th2 pattern is related to a reduced cellular immunity against cancer (29, 37, 38). In fact, the increased transcript levels of Fcer1a, Ms4a2, Hdc, Slpi, Mcpt1 and Mcpt2 genes by the FO+Tam treatment suggests an increment of this pattern of response (Figure 3).

Fcer1a and Ms4a2 are subunits of Fc receptor located on mast cells (39, 40). The up-regulation of these genes triggers the IgE-mediated immune response. As a consequence, T-cell releases mediators that initiate the inflammatory response and allergic reaction (39, 40). Histamine is one of the mediators produced by mast cells and basophils, through the action of histidine decarboxylase (Hdc) (39, 41). Some studies show an increase in Hdc activity in breast cancer, in comparison to normal tissue. Moreover, histamine promotes migration of breast cancer cells, which may have an important role in invasion and metastasis (41). Additionally, histamine shifts pattern of immune response from Th1 to Th2 (38). The increase of Fcer1a, Ms4a2 and Hdc mRNAs in the FO+Tam group may in turn increase histamine release and the Th2 response pattern in the tumor microenvironment.

The increases in mRNA of Mcpt1 and Mcpt2 genes in the FO+Tam group is of interest, because these enzymes are released during nematode infection (42, 43), and Mcpt1 expression is up-regulated in response to maturation of mast cells, and consequent Th2 response (42, 44). Finally, Slpi mRNA is increased in FO+Tam group. This gene is commonly increased in gastric, ovarian and lung carcinomas; its serum level is positively correlated with tumor stage, and it may promote tumor formation and development through the prevention of the formation of endostatin, an anti-angiogenic factor (45-47). These data led us to hypothesize that mammary tumor cells in FO+Tam-treated group escaped from the immune response against tumors through up-regulation of Th2 pattern of immune response in the tumor microenvironment.

In conclusion, treatment of female rats with FO resulted in differential expression of several genes that promote more differentiated tumors and a more efficient immune response against tumors, when compared to tumors of CO fed counterparts. In addition, cribriform tumors of FO-fed animals that received Tam showed decreased mRNA for genes directly related to tumor growth and metastasis; thus, Tam treatment was more efficient in a fish oil than in a corn oil background. However, FO+Tam treatment increased transcripts for proteins responsible for the Th2 immune response pattern, which may be a mechanism of escape of tumors from the immune response. More studies are warranted in order to better describe the beneficial effects of tamoxifen when it is administered to rats fed with different diets, and to further explain the possible mechanism of escape of these tumors from the immune response.

Acknowledgements

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Tables and figures

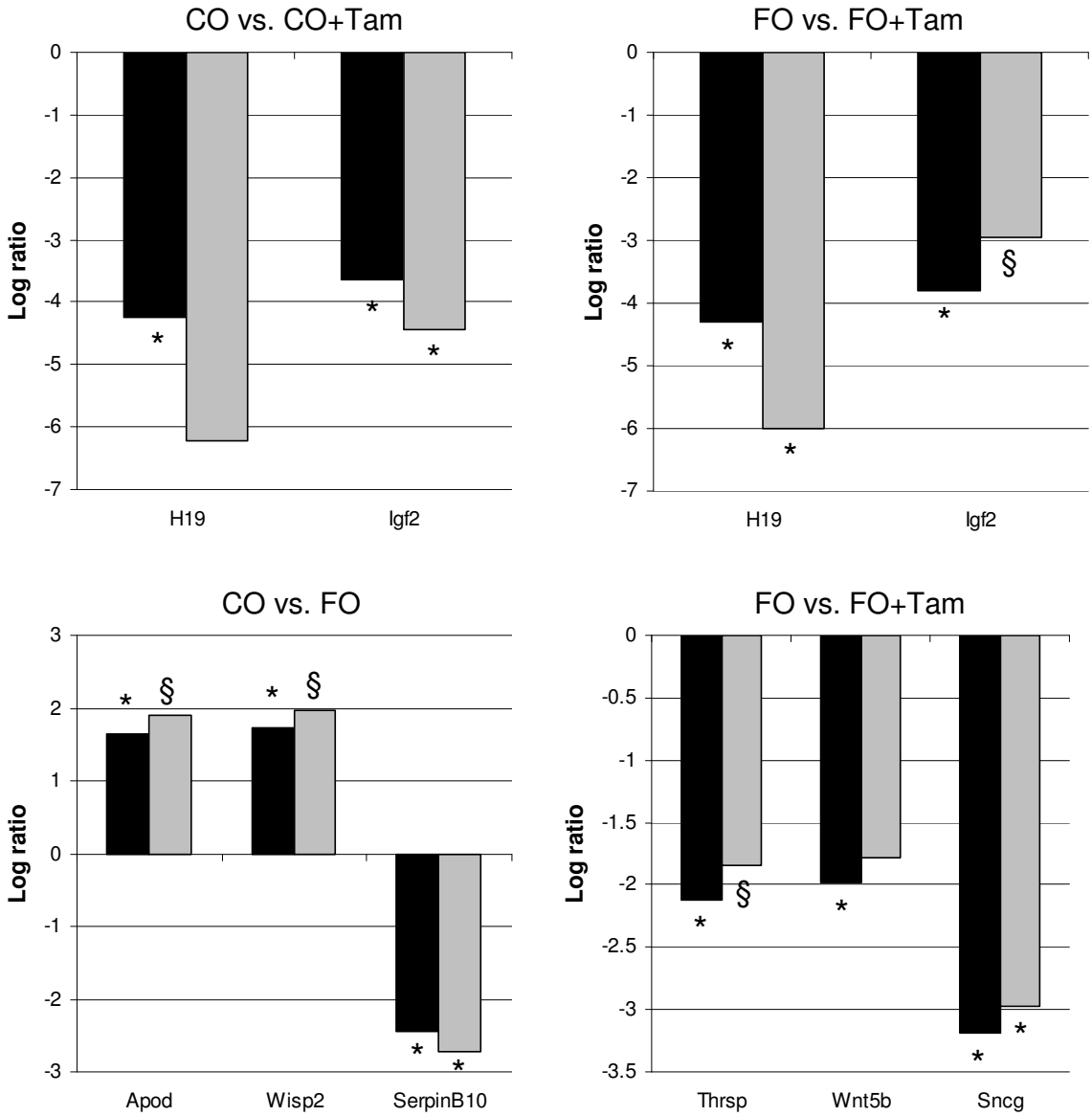

Figure 1 – Side-by-side log_2 values of mRNA expression in microarray (Black bars) and real time PCR (grey bars) of genes directly related to the tumor profile. * $p<0.05$; § $0.05<p<0.20$, with fold change > 3.0 ($log_2>1.58$).

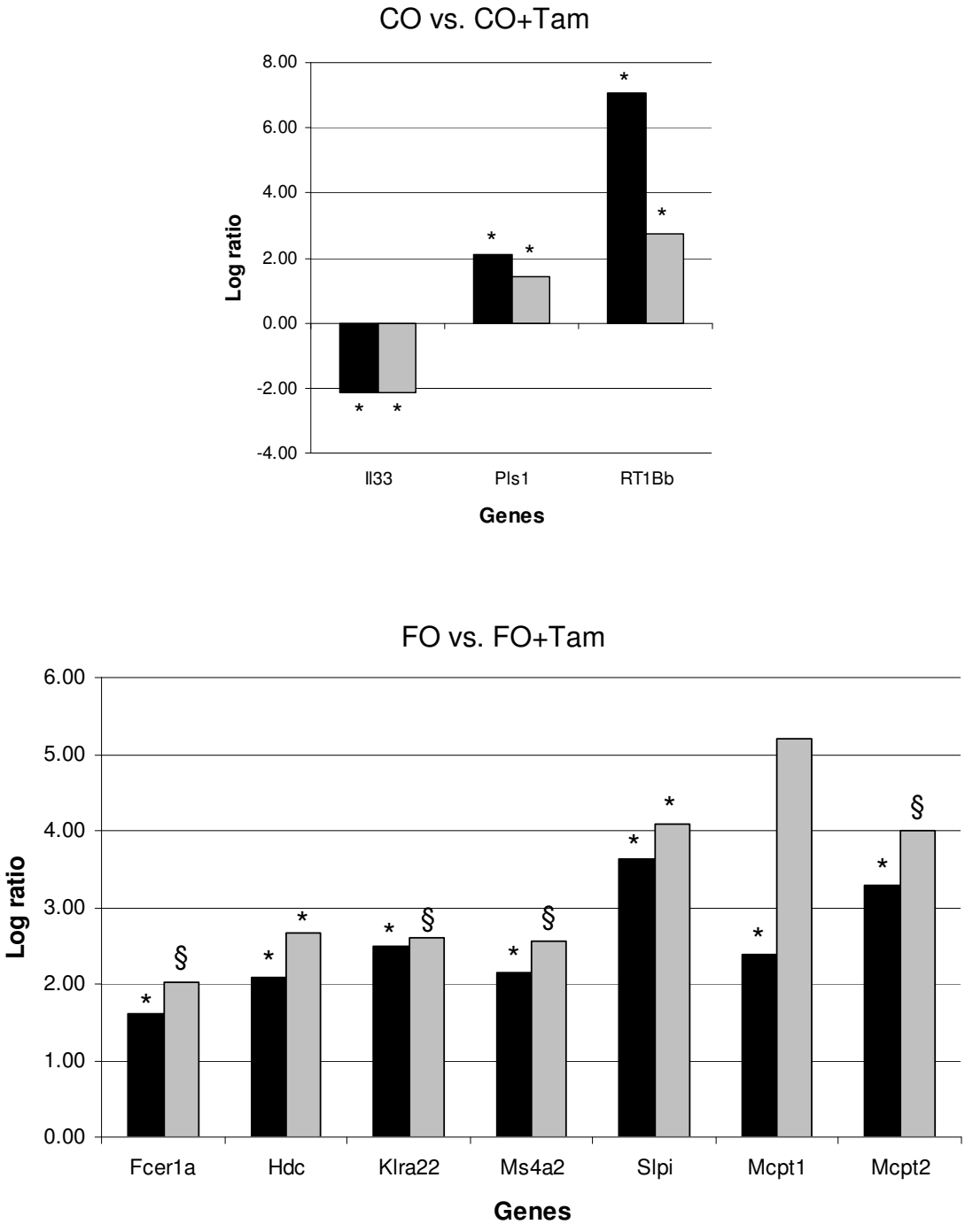

Figure 2 – Side-by-side $\log_2$ values of mRNA expression in microarray (Black bars) and real time PCR (grey bars) of genes related to the immune response. * $p<0.05$; § $0.05<p<0.20$, with fold change > 3.0 ($\log_2>1.58$).

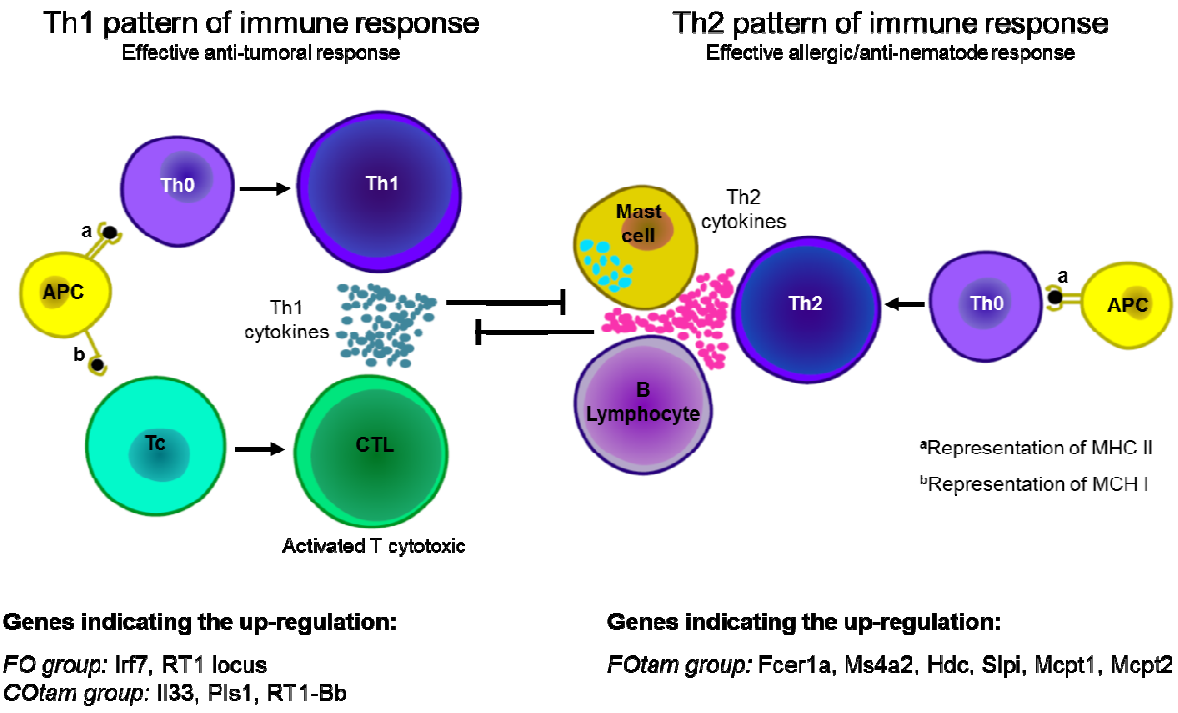

Figure 3 - Diagram depicting the role of the immune response against tumors. Genes indicated in the figure were differentially expressed in the microarray results.

Table 1 – Enrichment of GO terms ($p<0.05$) by differentially expressed genes between CO vs. FO treatments.

GO:0006955 - Immune Response ($p=3.66x10^{-4}$)		
GO:0002376 - Immune System Process (p=0.003)		
Probe name	*Symbol*	*Gene name*
A_43_P15750	Cfd	complement factor D (adipsin)
A_44_P1039994	Irf7	interferon regulatory factor 7
A_44_P214846	Mx2	myxovirus (influenza virus) resistance 2
A_44_P196401	RT1-Aw2	RT1 class Ib, locus Aw2
A_44_P274061	RT1-CE3	RT1 class I, CE3
A_44_P867246	RT1-CE16	RT1 class I, CE16
A_44_P422630	Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1
GO:0045087 - Innate Immune Response (p=0.01)		
Probe name	*Symbol*	*Gene name*
A_43_P15750	Cfd	complement factor D (adipsin)
A_44_P214846	Mx2	myxovirus (influenza virus) resistance 2
A_44_P422630	Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1
GO:0019882 - Antigen Processing and Presentation (p=0.02)		
Probe name	*Symbol*	*Gene name*
A_44_P196401	RT1-Aw2	RT1 class Ib, locus Aw2
A_44_P274061	RT1-CE3	RT1 class I, CE3
A_44_P867246	RT1-CE16	RT1 class I, CE16
GO:0045861 - Negative Regulation of Proteolysis (p=0.03)		
GO:0009895 - Negative Regulation of Catabolic Process (p=0.04)		
Probe name	*Symbol*	*Gene name*
A_44_P1004757	Serpinb10	serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 10
A_44_P422630	Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1

Table 2 – Enrichment of GO terms ($p<0.05$) by differentially expressed genes between FO vs. FO+Tam treatments.

GO:0002526 - Acute Inflammatory Response ($p=1.38x10^{-4}$)

Probe name	*Symbol*	*Gene name*
A_43_P21634	C4b	complement component 4B (Childo blood group)
A_44_P419064	Cfb	complement factor B
A_44_P468468	Lbp	lipopolysaccharide binding protein
A_42_P661600	Reg3a	regenerating islet-derived 3 alpha
A_44_P271658	Reg3b	regenerating islet-derived 3 beta
A_44_P524718	Serpina1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1

GO:0006954 - Inflammatory Response ($p=1.72x10^{-4}$)
GO:0006952 - Defense Response (p=0.004)
GO:0009611 - Response to Wounding (p=0.006)

Probe name	*Symbol*	*Gene name*
A_44_P1052607	Aox1	aldehyde oxidase 1
A_43_P21634	C4b	complement component 4B (Childo blood group)
A_44_P304323	Ccl5	chemokine (C-C motif) ligand 5
A_44_P419064	Cfb	complement factor B
A_44_P468468	Lbp	lipopolysaccharide binding protein
A_43_P15496	Ms4a2	membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for; beta polypeptide)
A_42_P661600	Reg3a	regenerating islet-derived 3 alpha
A_44_P271658	Reg3b	regenerating islet-derived 3 beta
A_44_P524718	Serpina1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1

GO:0009605 - Response to External Stimulus (p=0.002)

Probe name	*Symbol*	*Gene name*
A_44_P393531	Adipoq	adiponectin, C1Q and collagen domain containing
A_44_P1017367	Alb	albumin
A_44_P1052607	Aox1	aldehyde oxidase 1
A_43_P21634	C4b	complement component 4B (Childo blood group)
A_44_P304323	Ccl5	chemokine (C-C motif) ligand 5
A_44_P419064	Cfb	complement factor B
A_44_P428326	Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)
A_44_P468468	Lbp	lipopolysaccharide binding protein
A_43_P15496	Ms4a2	membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for; beta polypeptide)
A_42_P661600	Reg3a	regenerating islet-derived 3 alpha
A_44_P271658	Reg3b	regenerating islet-derived 3 beta
A_44_P524718	Serpina1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1

GO:0006953 - Acute-Phase Response (p=0.004)

Probe name	*Symbol*	*Gene name*
A_44_P468468	Lbp	lipopolysaccharide binding protein
A_42_P661600	Reg3a	regenerating islet-derived 3 alpha
A_44_P271658	Reg3b	regenerating islet-derived 3 beta
A_44_P524718	Serpina1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1

GO:0043306 - Positive Regulation of Mast Cell Degranulation (p=0.017)
GO:0033005 - PositiveRregulation of Mast Cell Activation (p=0.017)
GO:0043302 - Positive Regulation of Leukocyte Degranulation (p=0.017)
GO:0043304 - Regulation of Mast Cell Degranulation (p=0.027)

GO:0033003 - Regulation of Mast Cell Activation (p=0.027)
GO:0043300 - Regulation of Leukocyte Degranulation (p=0.04)

Probe name	*Symbol*	*Gene name*
A_44_P461402	Fcer1a	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide
A_43_P15496	Ms4a2	membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for; beta polypeptide)

GO:0048583 - Regulation of Response to Stimulus (p=0.024)

Probe name	*Symbol*	*Gene name*
A_43_P21634	C4b	complement component 4B (Childo blood group)
A_44_P419064	Cfb	complement factor B
A_43_P12786	Fabp4	fatty acid binding protein 4, adipocyte
A_44_P461402	Fcer1a	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide
A_43_P15496	Ms4a2	membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for; beta polypeptide)
A_43_P20937	Tmprss6	transmembrane protease, serine 6

GO:0006950 - Response to Stress (p=0.027)

Probe name	*Symbol*	*Gene name*
A_44_P393531	Adipoq	adiponectin, C1Q and collagen domain containing
A_44_P1017367	Alb	albumin
A_44_P1052607	Aox1	aldehyde oxidase 1
A_43_P21634	C4b	complement component 4B (Childo blood group)
A_44_P244851	Car3	carbonic anhydrase 3
A_44_P304323	Ccl5	chemokine (C-C motif) ligand 5
A_44_P419064	Cfb	complement factor B
A_44_P461402	Fcer1a	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide
A_44_P468468	Lbp	lipopolysaccharide binding protein
A_43_P15496	Ms4a2	membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for; beta polypeptide)
A_42_P661600	Reg3a	regenerating islet-derived 3 alpha
A_44_P271658	Reg3b	regenerating islet-derived 3 beta
A_44_P524718	Serpina1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1

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Supplemental table 1 – Primers used in the real time PCR

Symbol	RefSeq	Gene name	Forward primer	Reverse primer
H19	NR_027324	H19 fetal liver mRNA	AGCAAGGAGGCTGCAGTGGG	AGACCCAGGGACTGAGCGGT
Igf2	NM_031511	insulin-like growth factor 2	CCTTCGCCCGCTGTTCGGTT	CCCATTGGTACCTGGAAGCCGG
Serpinb10	NM_153733	serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 10	AGGAGCTGCAAACCATAGGCGT	TCTCCAGCTGTTTCAGGCCTTCC
Apod	NM_012777	apolipoprotein D	TGCGTCCTGACGGAACCCTG	TGCCGGTGGCATCAACGAGA
Wisp2	NM_031590	WNT1 inducible signaling pathway protein 2	CGGCGCAAGGACACCAACTT	TGGCTATGCCCAGCCCACAG
Sncg	NM_031688	synuclein, gamma (breast cancer-specific protein 1)	GAGGAGGGCGAAGAGGCCAA	TGTGCCAGCTTGGGACAGGG
Thrsp	NM_012703	thyroid hormone responsive	ACGGGGCAGGTCCTGTAGGT	GCCGCCTTTGCATCCACCTG
Wnt5b	NM_001100489	wingless-type MMTV integration site family, member 5B	CGTGGGAGCTCCGCTTTGGA	GCCAGCGACCACCAGGAGTT
Fcer1a	NM_012724	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	TGGGTCATTGTGAGTGCCACCATT	GCAGCCACTCTTGCATCACGT
Hdc	NM_017016	histidine decarboxylase	CTGCACTGGGTTCTGGGTCAAGG	TGGCGACACCAGAGTTCGCAT
Klra22	NM_173291	killer cell lectin-like receptor subfamily A, member 22	GCTGGAAACAGAGTCTGCTGGC	ACTGCAACCGATACTAGCCGAAAGA
Mcpt1	NM_017145	mast cell protease 1	GCGTGGGCCTTTCCACAAGGT	GGCCCACCAGAGTCTCCAGTGTA
Mcpt2	NM_172044	mast cell protease 2	GCGTGGGCAGTCCCACAACTT	CGCCAGAGTCTCCCATGAATGC
Ms4a2	NM_012845	membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for; beta polypeptide)	GCCTTTTGCAGTGCCGTGCT	AGACGGTCATCGGGGACCTTACT
Il33	NM_001014166	interleukin 33	GCACAATCAGGAGACGGTGTGGA	AGGCCTGATCTGGCGGAGAGA
Pls1	NM_001108178	plastin 1 (I isoform)	TGCACCGTGGTCAACATCGGT	AGCAGCGCGATCAAGGCTTCAT
RT1-Bb	NM_001004084	RT1 class II, locus Bb	GGTGTGAGTCCTGGTGACTGCC	CGAAATCCCTTGGGGAGTCTCTGC
Irf7	NM_001033691	interferon regulatory factor 7	ACCCAGTTCTGATGACTGAGCGC	AGCACTGTGCGGCCCTTGTA

Supplemental table 2 – mRNA expression changes induced by CO+Tam treatment.

Probe name	Symbol	Gene name	Fold change
			Decreased expression
A_44_P342289	H19	H19 fetal liver mRNA	19.04
A_44_P1037953	Igf2	insulin-like growth factor 2	12.50
A_44_P247286	Cxcl14	chemokine (C-X-C motif) ligand 14	5.19
A_44_P247281	Cxcl14	chemokine (C-X-C motif) ligand 14	4.65
A_44_P283385	Gip	gastric inhibitory polypeptide	4.36
A_44_P1029131	Il33	interleukin 33	4.35
A_44_P170502	Mup5	major urinary protein 5	4.32
A_44_P310596	Il33	interleukin 33	3.94
A_44_P503562	Gip	gastric inhibitory polypeptide	3.86
A_44_P1029697	Chl1	cell adhesion molecule with homology to L1CAM	3.65
A_44_P211878	Ndufa11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11	3.40
A_43_P12707	Ptms	parathymosin	3.28
A_44_P296478	Hba-a2	hemoglobin alpha, adult chain 2	3.21
A_44_P281402	Arsi	arylsulfatase family, member I	3.18
A_44_P365516	Tsc22d1	TSC22 domain family, member 1	3.08
A_44_P414630	LOC298116	alpha-2u-globulin (L type)	3.02
			Increased expression
A_44_P587679	Ldb3	LIM domain binding 3	3.01
A_44_P318861	RGD1564142	similar to chromosome 10 open reading frame 64	3.07
A_42_P604331	Spock2	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2	3.10
A_43_P10690	LOC690045	similar to immunoreceptor Ly49si1	3.10
A_44_P304323	Ccl5	chemokine (C-C motif) ligand 5	3.10
A_44_P393696	LOC308990	hypothetical protein LOC308990	3.18
A_43_P15590	Fmod	fibromodulin	3.21
A_44_P684180	Lrrn1	leucine rich repeat neuronal 1	3.23
A_42_P537501	Dpp4	dipeptidylpeptidase 4	3.30
A_44_P253073	Aspn	asporin	3.36
A_42_P457003	Pdgfra	platelet derived growth factor receptor, alpha polypeptide	3.51
A_43_P11484	Mme	membrane metallo endopeptidase	3.53
A_44_P869362	LOC688150	similar to isthmin 1	3.56
A_43_P14915	C6	complement component 6	3.83
A_43_P22744	Adcy9	adenylate cyclase 9	3.84
A_44_P545553	Dgkb	diacylglycerol kinase, beta	3.91
A_42_P782235	Rnase2	ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin)	4.08
A_44_P958139	Pls1	plastin 1 (I isoform)	4.26
A_44_P552452	RT1-Bb	RT1 class II, locus Bb	132.28

Supplemental table 3 – mRNA expression changes induced by FO+Tam treatment.

Probe name	Symbol	Gene name	Fold change
			Decreased expression
A_44_P342289	H19	H19 fetal liver mRNA	19.63
A_44_P1037953	Igf2	insulin-like growth factor 2	13.85
A_42_P570313	Ces3	carboxylesterase 3	11.73
A_44_P393531	Adipoq	adiponectin, C1Q and collagen domain containing	10.34
A_44_P384017	Sncg	synuclein, gamma (breast cancer-specific protein 1)	9.12
A_43_P12786	Fabp4	fatty acid binding protein 4, adipocyte	8.08
A_44_P517258	Pck1	phosphoenolpyruvate carboxykinase 1 (soluble)	7.75
A_44_P244851	Car3	carbonic anhydrase 3	7.33
A_43_P20937	Tmprss6	transmembrane protease, serine 6	5.71
A_42_P633019	Grifin	galectin-related inter-fiber protein	4.75
A_44_P1017367	Alb	albumin	4.70
A_44_P1042754	Slc7a10	solute carrier family 7, (neutral amino acid transporter, y+ system) member 10	4.69
A_44_P271658	Reg3b	regenerating islet-derived 3 beta	4.69
A_44_P468468	Lbp	lipopolysaccharide binding protein	4.47
A_44_P1052607	Aox1	aldehyde oxidase 1	4.35
A_43_P11520	Thrsp	thyroid hormone responsive	4.34
A_42_P615191	Csn1s1	casein alpha s1	4.31
A_44_P391057	Angptl4	angiopoietin-like 4	4.22
A_44_P714007	Cidec	cell death-inducing DFFA-like effector c	4.09
A_44_P299571	Olah	oleoyl-ACP hydrolase	3.98
A_44_P945616	Wnt5b	wingless-type MMTV integration site family, member 5B	3.95
A_43_P21634	C4b	complement component 4B (Childo blood group)	3.87
A_44_P414625	Pck1	phosphoenolpyruvate carboxykinase 1 (soluble)	3.84
A_44_P419064	Cfb	complement factor B	3.82
A_42_P624773	Cpz	carboxypeptidase Z	3.63
A_42_P638620	Lcn2	lipocalin 2	3.46
A_44_P1042756	Slc7a10	solute carrier family 7, (neutral amino acid transporter, y+ system) member 10	3.44
A_42_P661600	Reg3a	regenerating islet-derived 3 alpha	3.43
A_43_P14597	Sgcg	sarcoglycan, gamma (dystrophin-associated glycoprotein)	3.18
A_42_P751720	LOC683720	similar to keratin 6L	3.17
A_44_P121063	Aqp7	aquaporin 7	3.14
A_42_P525170	Abcd2	ATP-binding cassette, sub-family D (ALD), member 2	3.13
A_44_P428326	Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	3.03
A_44_P299247	Aqp1	aquaporin 1	3.01
			Increased expression
A_44_P389132	Rgs18	regulator of G-protein signaling 18	3.04
A_44_P461402	Fcer1a	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	3.05
A_44_P360539	Ly49i2	Ly49 inhibitory receptor 2	3.06
A_44_P1057055	Ccl12	chemokine (C-C motif) ligand 12	3.07
A_42_P500080	Nkg7	natural killer cell group 7 sequence	3.07

A_43_P11543	Klrd1	killer cell lectin-like receptor, subfamily D, member 1	3.26
A_42_P466283	Gzmk	granzyme K	3.28
A_44_P1029706	Cma1	chymase 1, mast cell	3.31
A_44_P536089	Klre1	killer cell lectin-like receptor, family E, member 1	3.32
A_44_P363009	Scin	scinderin	3.42
A_44_P420503	Slpil2	antileukoproteinase-like 2	3.50
A_44_P1029714	Cma1	chymase 1, mast cell	3.68
A_44_P524718	Serpina1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	3.73
A_44_P454157	Mcpt8	mast cell protease 8	3.76
A_44_P304323	Ccl5	chemokine (C-C motif) ligand 5	4.04
A_44_P868963	Tnfrsf14	tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)	4.09
A_44_P288401	RGD1559887	similar to Transducin-like enhancer protein 4 (Groucho-related protein 4) (Grg-4)	4.17
A_42_P573296	Hdc	histidine decarboxylase	4.25
A_43_P15496	Ms4a2	membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for; beta polypeptide)	4.43
A_42_P641517	Mcpt1	mast cell protease 1	5.22
A_44_P527205	Rnase1l2	ribonuclease, RNase A family, 1-like 2 (pancreatic)	5.45
A_44_P266817	Klra22	killer cell lectin-like receptor subfamily A, member 22	5.65
A_44_P238556	Mcpt2	mast cell protease 2	9.78
A_44_P372254	Slpi	secretory leukocyte peptidase inhibitor	11.82
A_43_P19944	Upk3a	uroplakin 3A	11.96
A_44_P372261	Slpi	secretory leukocyte peptidase inhibitor	12.34
A_44_P110120	RGD1563818	similar to secretory leukocyte protease inhibitor	12.88
A_44_P636717	LOC685106	similar to ribosomal protein L6	26.76

Supplemental table 4 – mRNA expression changes comparing CO vs. FO treatments.

Probe name	Symbol	Gene name	Fold change
			Decreased expression
A_44_P236820	LOC685608	hypothetical protein LOC685608	25.18
A_42_P515028	Alpk2	alpha-kinase 2	6.65
A_44_P1004757	Serpinb10	serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 10	5.44
			Increased expression
A_43_P11558	Apod	apolipoprotein D	3.13
A_44_P714007	Cidec	cell death-inducing DFFA-like effector c	3.14
A_44_P867246	RT1-CE16	RT1 class I, CE16	3.18
A_44_P422630	Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1	3.18
A_44_P199187	MGC108823	similar to interferon-inducible GTPase	3.19
A_42_P629321	Wisp2	WNT1 inducible signaling pathway protein 2	3.32
A_44_P274061	RT1-CE3	RT1 class I, CE3	3.36
A_43_P15750	Cfd	complement factor D (adipsin)	3.67
A_44_P1052607	Aox1	aldehyde oxidase 1	3.72
A_44_P1039994	Irf7	interferon regulatory factor 7	4.15
A_44_P214846	Mx2	myxovirus (influenza virus) resistance 2	5.74
A_44_P517258	Pck1	phosphoenolpyruvate carboxykinase 1 (soluble)	5.79
A_44_P200539	Cbll1	Cas-Br-M (murine) ecotropic retroviral transforming sequence-like 1	5.85
A_44_P244851	Car3	carbonic anhydrase 3	6.56
A_44_P196401	RT1-Aw2	RT1 class Ib, locus Aw2	7.54
A_43_P12786	Fabp4	fatty acid binding protein 4, adipocyte	7.60

Conclusões

O tratamento de ratas Sprague-Dawley com óleo de peixe alterou o padrão transcriptômico de vários genes, promovendo tumores mais diferenciados e resposta imune anti-tumoral mais eficiente em comparação com os grupos tratados com óleo de milho. Adicionalmente, tumores cribriformes de animais que receberam óleo de peixe e tamoxifeno apresentaram uma redução nos níveis de mRNA de genes diretamente relacionados com crescimento tumoral e metástase, o que pode explicar a redução da incidência tumoral nesse grupo. Em conclusão, o tratamento com tamoxifeno foi mais eficiente em ratas que receberam óleo de peixe do que em ratas que receberam óleo de milho.

Anexos

Andrea Manni M.D. - IACUC Approval of Protocol # 2008-034, ("Combination of low-dose antiestrogens with a diet rich in omega-3 fatty acids for safe and effective breast cancer prevention")

From: Carol Kelly
To: Manni M.D., Andrea
Date: 4/22/2010 1:45 PM
Subject: IACUC Approval of Protocol # 2008-034, ("Combination of low-dose antiestrogens with a diet rich in omega-3 fatty acids for safe and effective breast cancer prevention")
CC: Doster, Lisa

Dear Dr. Manni:

Your protocol # 2008-034, entitled "Combination of low-dose antiestrogens with a diet rich in omega-3 fatty acids for safe and effective breast cancer prevention" has been approved by the IACUC on 4/20/10.

This protocol will expire on 4/19/11.

Carol Kelly
Research Compliance Coordinator
Penn State College of Medicine
Department of Comparative Medicine, H054
P.O. Box 850
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717-531-0003 X285895

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R. Margaux Anel-Tiangco, M.D.
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Carina Signori, D.O.
Gerti Tashko, M.D.

December 14, 2010

Lucas Bidinotto
Fox Chase Cancer Center

RE: Lucas Tadeu Bidinotto

To Whom It May Concern:

The project, "Transcriptomic changes on mammary tumors treated with Tamoxifen in omega-3 or omega-6 fed Sprague-Dawley rats" is part of a general project entitled, "Combination of low-dose antiestrogens with a diet rich in omega-3 fatty acids for safe and effective breast cancer prevention" which was approved by the IACUC (#2008-034). Mr. Lucas Tadeu Bidinotto is one of the researchers involved in this project.

Sincerely,

Andrea Manni, M.D.
Professor of Medicine

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Faculdade de Medicina de Botucatu

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CEEA Ética
Comissão de Ética em Experimentação Animal

Botucatu, 24 de fevereiro de 2011. Of. 004/11-CEEA

Ilustríssimo Senhor
Prof. Dr. Luís Fernando Barbisan
Departamento de Morfologia do
Instituto de Biociências de Botucatu

Prezado Prof. Luis Fernando.

De ordem da Senhora Presidente desta CEEA, informo que com referência a sua solicitação de homologção do Projeto de Pesquisa **"Efeitos das dietas ricas em Omega-3 ou Omega 6 na expressão gênica de neoplasias mamárias de ratas Sprague-Dawley sob tratamento com tamoxifeno"** recebemos a informação abaixo transcrita da Seção de Pós Graduação da Faculdade de Medicina.

- Após consulta à Seção de Pós Graduação fomos informados que nos experimentos realizado em outro País com animais, uma vez tendo sido aprovado na Instituição de origem, não há necessidade de obtenção de novo documento homologando a aprovação.

Atenciosamente,

Alberto Santos Capelluppi
Secretário da CEEA